Laboratory Investigation

Ca\textsuperscript{2+} channel modulation alters halothane-induced depression of ventricular myocytes

**Purpose:** This study examined the direct myocardial depressant effect of halothane and determined whether an L-type Ca\textsuperscript{2+} channel agonist and antagonists altered the myocardial depression induced by halothane in cultured rat ventricular myocytes.

**Methods:** Ventricular myocytes were obtained from neonatal rats by enzymatic digestion with collagenase and then cultured for 6 to 7 days. The myocytes were stabilized in a serum-free medium, and the spontaneous beating rate and amplitude were measured. To assess the halothane-induced conformational changes in L-type Ca\textsuperscript{2+} channel, receptor binding study was performed using a dihydropyridine derivative, \([\text{H}]\) PN 200-110, in cardiac membrane preparation.

**Results:** Halothane (1%, 2%, 3%, 4%) decreased the beating rate and amplitude in a concentration-dependent manner (P < 0.05). The myocardial depressant effects of halothane were potentiated by nifedipine or verapamil (P < 0.05). Bay K 8644, an L-type Ca\textsuperscript{2+} channel agonist, completely prevented the halothane-induced depression in amplitude (P < 0.05), but affected the beating rate less. Adding halothane (2%) decreased (P < 0.05) the maximum binding site density for \([\text{H}]\) PN 200-110 (from 198.6 ± 23.7 fmol/mg protein to 115.3 ± 21.6 fmol/mg protein) but did not affect binding affinity (from 0.461 ± 0.077 nM to 0.307 ± 0.055 nM).

**Conclusion:** The reduction of Ca\textsuperscript{2+} current via sarcolemmal L-type Ca\textsuperscript{2+} channel, probably due to conformational changes in dihydropyridine binding sites, plays an important role in halothane-induced myocardial depression in living heart cells.
HALOTHANE induces myocardial depression in vivo, as well as in vitro. However, the mechanism underlying this depressant effect is not fully understood. Altered intracellular Ca²⁺ availability is one potential explanation for halothane's negative inotropic effect. In cardiac myocytes, because Ca²⁺ influx via the sarcolemmal L-type Ca²⁺ channels triggers the Ca²⁺ release from the sarcoplasmic reticulum for contractile activation, halothane may inhibit the L-type Ca²⁺ channels and thus produce the observed myocardial depression. Therefore, modulating the sarcolemmal L-type Ca²⁺ channel may alter the effect of halothane on cardiac excitation-contraction coupling.

Potentiation of cardiodepressant effects has been reported between several Ca²⁺ channel antagonists and volatile anaesthetics. However, the effect of Ca²⁺ channel agonists on volatile anesthetic-induced myocardial depression is still somewhat controversial. Bay K 8644 (Bay K), an L-type Ca²⁺ channel agonist, that acts at the dihydropyridine site, improves myocardial contractility during halothane anaesthesia in chronically instrumented dogs. In human ventricular myocardiun, halothane 2% shifted the concentration-response curve to Bay K for isometric force to the right despite a lack of change in maximal inotropic effect. In contrast, Baum reported that Bay K did not affect the reduction in peak Ca²⁺ current occurring upon exposure to halothane 1% when the patch-clamp technique was used in single guinea pig ventricular myocytes. The direct effects of anaesthetics on myocardial contractility are difficult to assess in vivo because these effects result from various actions on different target tissues, including tissue of the central and autonomic nervous systems, vascular smooth muscle, and cardiac muscle. Moreover, most of these studies tested only one concentration of halothane. Therefore, further studies examining the direct effects of halothane on excitation-contraction coupling at the cellular level and its interaction with Ca²⁺ channel modulators at various concentrations are needed.

The direct effects of anaesthetics can be examined under physiological conditions in cultured ventricular myocytes because they beat spontaneously and haemodynamic, humoral, autonomic, and metabolic influences are excluded. Thus, in this study we sought to study the direct myocardial depressant effect of halothane in cultured rat ventricular myocytes to determine the interaction between halothane and the Ca²⁺ channel antagonists nifedipine and verapamil and an agonist (Bay K) in living cells and to confirm the association between conformational changes in the L-type Ca²⁺ channel receptor and halothane-induced myocardial depression by radiolabeled-ligand binding studies using [³H] PN 200-110, an L-type Ca²⁺ channel antagonist.

Materials and methods
All experiments were performed under the supervision of the Animal Care Committee of Sapporo Medical University. Primary cultured cardiac myocytes were prepared from ventricles of one-to-three-day-old Wistar rats by enzymatic digestion, as described previously. Neonatal rat hearts were removed and incubated at 37°C with collagenase (200 unit-mL⁻¹, Dainihon Seiyaku, Tokyo, Japan) - salt solution (Ca²⁺ and Mg²⁺ free). The isolated cardiac myocytes were seeded on 35-mm x 18-mm tissue culture dishes coated with collagen type I in 1.5 mL Dulbecco's modified Eagle's minimum essential medium (DMEM, Dainihon Seiyaku, Tokyo, Japan) - salt solution (Ca²⁺ and Mg²⁺ free). The isolated cardiac myocytes were seeded on 35-mm x 18-mm tissue culture dishes coated with collagen type I in 1.5 mL Dulbecco's modified Eagle's minimum essential medium (DMEM, Dainihon Seiyaku, Tokyo, Japan) - salt solution (Ca²⁺ and Mg²⁺ free). The isolated cardiac myocytes were seeded on 35-mm x 18-mm tissue culture dishes coated with collagen type I in 1.5 mL Dulbecco's modified Eagle's minimum essential medium (DMEM, Dainihon Seiyaku, Tokyo, Japan) - salt solution (Ca²⁺ and Mg²⁺ free).
colemmal L-type Ca²⁺ channel, we examined the concentration-response curve of halothane in the presence of the Ca²⁺ channel antagonists verapamil (10 nM) or nifedipine (10 nM) or the agonist Bay K (1 μM). Concentration studies were conducted in the same random fashion used previously. Five minutes before the administration of halothane, the myocytes were exposed to either vehicle, verapamil, nifedipine or Bay K. The myocytes served as their own controls: baseline data were collected at five minutes after vehicle, verapamil, nifedipine or Bay K, before halothane administration. Six culture dishes were analysed at each halothane concentration.

The halothane concentration in the media without cultured myocytes was measured using gas chromatography (GC-12A, Shimadzu, Kyoto, Japan) at seven minutes after the anaesthetic was introduced. The halothane concentrations (1%, 2%, 3%, and 4% at the vaporizer) in the culture dish were 0.30 ± 0.02, 0.57 ± 0.02, 0.89 ± 0.02, and 1.14 ± 0.03 mM (mean ± SEM; n = 5 at each concentration), respectively.

**Investigation of conformational changes**

Cultured cardiac membranes were prepared as described previously. Briefly, at day 7 of culture, the cardiac myocytes were rinsed with phosphate-buffered saline (PBS); then the cells were scraped from the dishes and lysed in ice-cold 50 mM Tris/HCl (pH 7.4) containing 10 μg·mL⁻¹ soybean trypsin inhibitor, 5 μg·mL⁻¹ of leupeptin, 200 μg·mL⁻¹ of bacitracin, 2 mM EDTA and 100 μM phenylmethylsulfonyl fluoride. The lysed cell fragments were centrifuged at 1000g for five minutes at 4°C. The pellet was resuspended and homogenized in ice-cold buffer using a Brinkmann Polytron homogenizer (Westbury, NY). Halothane, 2% (0.57 ± 0.02 mM), was administered by passing the carbogen (O₂, 95%; CO₂, 5%) through the vaporizer and directing the output through the membrane suspension. The suspended membranes were allowed to equilibrate for seven minutes; control membranes were equilibrated with the carbogen but without halothane and handled identically. Dihydropyridine binding sites were studied using [³H]PN200-110 (specific activity 83.3 Ci/mmol, NEN, Tokyo, Japan) as radiolabeled ligand (0.05-25 nM). Nonspecific binding of [³H]PN200-110 was determined in the presence of 10 μM nifedipine. The homogenate was incubated at 25°C for 90 min. These conditions allowed the receptor to equilibrate completely with the radioligand. The samples were filtered on a Brandel M-30 cell harvester through Whatman GF/B glass fiber filters with three 5-mL washes of 50 mM Tris/HCl. The filters were dried at 80°C for one hour, placed in 8 mL scintillation fluid (AQUASOL-2), and counted in a liquid scintillation counter (Beckmann LS-6000LL). The maximum number of binding sites (Bmax) and the affinity constant (Kd) were calculated by Scatchard-Rosenthal analysis. Protein contents were measured according to the method of Lowry et al. using bovine serum albumin as the standard.

**Statistical analysis**

The data for contractile variables were analysed as the percentage of the control values (0% halothane), and each dish served as its own control. The results are given as mean ± SEM. The dose-response curves were compared by one-way analysis of variance (ANOVA), followed by a Bonferroni/Dunn test. Comparisons between the halothane groups were made with two-way repeated measures ANOVA. When appropriate, statistical significance was estimated with an unpaired Student's t test. Values were considered significantly different at P < 0.05.

**Results**

After six to seven days of culture, the myocytes became a confluent monolayer and beat spontaneously at 131 ± 8 beats-min⁻¹. There were no differences in beating rate and amplitude among the groups at preand five minutes after drug administration (Table I).

Adding halothane to cultured rat ventricular myocytes reduced the beating rate and amplitude in a concentration-dependent manner (Figure 1); spontaneous beating returned to control levels after the washout period. Halothane caused concentration-dependent decreases in beating rate and amplitude (P < 0.05) (Figure 2). At halothane 1%, 2%, 3%, and 4%, amplitude decreased to 82% ± 3%, 65% ± 7%, 27% ± 6% and 3% ± 3% of control values, respectively, and the beating rate decreased to 87% ± 4%, 67% ± 4%, 33% ± 5% and 8% ± 4% of control values, respectively.

We further examined the effects of Ca²⁺ channel modulators on halothane-induced myocardial depression. Beating rate and amplitude were 96% ± 5% and 90% ± 5% of preadministration values at five minutes after nifedipine (halothane 0%); and 94% ± 3% and 100% ± 6% of preadministration values at five minutes after verapamil (halothane 0%) administration. When cultured cells were exposed to halothane 1-4% in the presence of either 10 nM nifedipine or verapamil, the halothane-induced depression of beating rate and amplitude was exacerbated (Figures 2A, 2B). Verapamil strongly potentiated the halothane-induced myocardial depression (P < 0.05) (beating rate and amplitude decreased to 26% and 32% of the halothane 1% -alone value, respectively) compared with nifedipine (beating rate and amplitude decreased to 91% and 87% of halothane 1%-alone value, respectively). When
TABLE I Beating rate and amplitude at pre- and 5 minutes after drug administration

<table>
<thead>
<tr>
<th></th>
<th>HAL + vehicle (n = 24)</th>
<th>HAL + Nif (n = 24)</th>
<th>HAL + Ver (n = 24)</th>
<th>HAL + Bay K (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-administration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beating rate (beat-min⁻¹)</td>
<td>131 ± 8</td>
<td>130 ± 9</td>
<td>131 ± 8</td>
<td>130 ± 7</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>21.5 ± 0.7</td>
<td>22.0 ± 0.8</td>
<td>21.7 ± 0.6</td>
<td>22.2 ± 0.8</td>
</tr>
<tr>
<td><strong>5 min after drugs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beating rate (beat-min⁻¹)</td>
<td>131 ± 8</td>
<td>125 ± 8</td>
<td>124 ± 7</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>21.5 ± 0.7</td>
<td>20.0 ± 0.8</td>
<td>21.5 ± 0.6</td>
<td>21.0 ± 0.8</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
HAL = halothane, Nif = nifedipine, Ver = verapamil, Bay K = Bay K 8644

TABLE II Effects of halothane on radioligand binding of [³H] PN 200-110 to L-type Ca²⁺ channels in membranes of cultured rat ventricular myocytes

<table>
<thead>
<tr>
<th>Binding Characteristic</th>
<th>Control (n = 6)</th>
<th>Halothane 2% (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmax (fmol·mg protein⁻¹)</td>
<td>198.6 ± 23.7</td>
<td>115.3 ± 21.6*</td>
</tr>
<tr>
<td>Kd (nM)</td>
<td>0.461 ± 0.077</td>
<td>0.307 ± 0.055</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, *P < 0.05 vs control.

cultured cells were exposed to halothane in the presence of 1 μM Bay K, the halothane-depressed amplitude was completely antagonized even at the highest halothane concentration (P < 0.05) (Figure 2A). The decrease in beating rate also was antagonized but to a lesser degree than occurred for amplitude (Figure 2B): the change in beating rate was statistically significant only at halothane 4%. The beating rate and amplitude were 110% ± 2% and 93% ± 3% of preadministration values at five minutes (halothane 0%) after Bay K administration, respectively.

To study whether the halothane-induced myocardial depression on cultured rat ventricular myocytes could be explained by an interaction with L-type Ca²⁺ channel binding sites, we investigated receptor binding using a dihydropyridine derivative, [³H] PN200-110, in the presence and absence of halothane 2%. Figure 3 shows a typical Scatchard plot, and Table II shows the summarized data for density (Bmax) of calcium antagonist binding sites and the affinity (Kd) of the channel for the calcium channel antagonist [³H] PN200-110 in cultured cardiac membranes. In the control group, the binding data revealed a Bmax of 198.6 ± 23.7 fmol·mg⁻¹ protein and a Kd of 0.461 ± 0.077 nM. In the halothane group, adding halothane 2% decreased Bmax by 42% (115.3 ± 21.6 fmol·mg⁻¹ protein) (P < 0.05), but did not affect Kd (0.307 ± 0.055 nM).

Discussion
We examined the direct myocardial depressant effect of halothane on the beating rate and amplitude in cultured rat ventricular myocytes. Halothane had direct negative chronotropic and inotropic actions at the cellular level. We also examined the effects of Ca²⁺ channel modulators on halothane-induced myocardial depression. The L-type Ca²⁺ channel blockers, nifedipine and verapamil, potentiated the negative chronotropic and inotropic effects induced by halothane. On the other hand, the L-type Ca²⁺ channel agonist, Bay K, prevented the halothane-induced reduction in amplitude even at the highest halothane concentration. Furthermore, halothane reduced the maximal binding of [³H] PN200-110 to the dihydropyridine binding sites of the Ca²⁺ channel without any significant change in binding affinity.

The effects of halothane on spontaneous beating in cultured rat myocytes have been confirmed by other investigations.14,15 Stung et al.15 compared the effects of halothane in rat heart cells cultured for various length of time. Although the concentration-dependent decreases in beating rate and strength that they found agree with our results, beating strength at halothane 1% was exceedingly depressed (-41%) in their cells cultured for 4 to 10 days compared with our results (-14%) at seven days. We reported previously that the beating rate increased as the culture period lengthened (one to eight days), and then gradually decreased after more than 10 days of culture.16 At seven days of culture, the myocytes became confluent and exhibited concomitant contractile activity with a constant rate.17 Therefore, we used ventricular myocardial cells cultured for seven days in this study. Stung et al. did not mention whether the basal beating rate changed or whether they removed the atrium. Such a difference in tissue preparation may explain why their findings differ from those in our present study.
The combination of Ca\textsuperscript{2+} antagonists and halothane produces pronounced myocardial depression in vivo\textsuperscript{7,18} and in vitro.\textsuperscript{5,6,8} However, experiments performed in animals and in humans could not eliminate the sympathetic nervous system stimulation occurring in response to decreased blood pressure caused by peripheral vasodilatation. Our present results clearly show that nifedipine and verapamil enhanced the depressive effect of halothane in ventricular cells. These findings agree with the additive direct negative inotropic effects of halothane and either nifedipine\textsuperscript{5} or verapamil\textsuperscript{8} found in studies of isolated hearts. Verapamil potentiated the halothane-induced myocardial depression more than nifedipine. This result agrees with findings observed in isolated guinea pig hearts.\textsuperscript{5} In our present results, halothane-induced myocardial depression was markedly potentiated in the presence of nifedipine (10 nM) or verapamil (10 nM) neither of which caused myocardial depression alone. In our preliminary experiments, higher doses (>100 nM) of these Ca\textsuperscript{2+} channel antagonists abolished the spontaneous beating. In addition, neonatal myocardium, unlike mature myocardium, relies predominantly on transsarcolemmal Ca\textsuperscript{2+} influx to support contraction than Ca\textsuperscript{2+} released from sarcoplasmic reticulum.\textsuperscript{19} Thus, the pre-existing reduction of Ca\textsuperscript{2+} influx by nifedipine or verapamil may cause a profound myocardial depression when halothane is applied.

The L-type Ca\textsuperscript{2+} channels are modulated by distinct chemical classes of Ca\textsuperscript{2+} antagonists, such as the dihydropyridines, phenylalkylamines, and benzothiazepines.\textsuperscript{20} The dihydropyridine receptor site of L-type Ca\textsuperscript{2+} channels has been proposed to localize to the extracellular surface of the lipid bilayer; in contrast, the verapamil binding domain (phenylalkylamine site) of L-type Ca\textsuperscript{2+} channel is proposed to be within the lipid bilayer.\textsuperscript{21} (Figure 4) These conformational differences may produce varying degrees of myocardial depression when volatile anaesthetics are given in the presence of Ca\textsuperscript{2+} antagonists. Because nifedipine and halothane are considered to exert similar effects at the sarcolemma,\textsuperscript{22} it is likely that halothane does not exert a greater effect on verapamil binding sites than on dihydropyridine binding sites.

Thar halothane inhibits Ca\textsuperscript{2+} antagonist binding to cardiac membranes has been observed in adult rats\textsuperscript{23} and in humans.\textsuperscript{9} To our knowledge, no study has evaluated the effect of halothane on Ca\textsuperscript{2+} antagonist binding to cardiac membranes in cultured rat ventricular myocytes. Caution is necessary, however, when membrane binding studies are used to explain physiological effects in intact tissues. In our present study, an approximately 40% decrease in B\textsuperscript{max} was observed in cultured rat ventricular myocytes. At the same concentration of halothane, a similar degree of myocardial depression was observed in cultured myocytes (Figure 2). Therefore, these results strongly suggest that conformational changes in the L-type Ca\textsuperscript{2+} channel is impor-
Kanaya et al.: Halothane and Calcium Channel Function

FIGURE 3 Typical Scatchard plot of the binding data when a dihydropyridine derivative, [3H] PN 200-110, was added to membranes of cultured rat ventricular myocytes. After exposure to 2% halothane (closed circle), the dihydropyridine binding site density decreased as compared with controls (open circle).

FIGURE 4 Schematic diagram of L- and T-type Ca\(^{2+}\) channels, and their antagonists and agonist. DHP = dihydropyridines (nifedipine, PN 200-110, Bay K 8644) binding site, VER = verapamil binding site.

It is not clear why Bay K completely prevented the decrease in beating amplitude but only partially prevented the decrease in beating rate. Malinconico et al.\(^{14}\) reported similarly that increased extracellular calcium concentrations ([Ca\(^{2+}\)]\(_o\)) reversed the halothane-depressed beating intensity of heart cells in culture; however, increased [Ca\(^{2+}\)]\(_o\) did not affect the halothane-depressed beating rate. Two different types of Ca\(^{2+}\) channels exist in the cardiac sarcolemma, the long-lasting (L-type) and transient (T-type).\(^{25}\) The L-type Ca\(^{2+}\) current is increased by dihydropyridine-derived Ca\(^{2+}\) channel agonists, such as Bay K, and decreased by Ca\(^{2+}\) channel antagonists, such as nifedipine. In contrast, the T-type Ca\(^{2+}\) current is insensitive to dihydropyridine Ca\(^{2+}\) channel agonists and antagonists. Although the physiological role of the T-type Ca\(^{2+}\) current in cardiac membranes is not well understood, blockade of T-type Ca\(^{2+}\) current may induce bradycardia by reducing the rate of depolarization in the later phase of pacemaker depolarization.\(^{25}\) Recently, Eskinder et al.\(^{26}\) reported that halothane depressed both L- and T-type Ca\(^{2+}\) channel currents to a similar extent in a study of single canine cardiac Purkinje cells using the patch-clamp technique. Although this effect of halothane on L- and T-type Ca\(^{2+}\) currents may explain our findings that Bay K pre-
vented depression of beating amplitude but only partially prevented depression in beating rate, further examination of halothane's effects on the physiological function of the T-type Ca$^{2+}$ channel will be required.

Extrapolating our data to the clinical situation must be considered with caution because of possible species differences, and differences between in vivo and in vitro conditions. Our study also has the limitations that cultured neonatal rat myocytes have some different patho-physiological properties compared with the adult rat heart, and there was no load on the cardiac myocytes. Despite these limitations, the model allows us to examine the direct effects of anaesthetics on myocardial contraction independence of haemodynamic, neural or locally-derived factors.

In conclusion, 1) the Ca$^{2+}$ channel antagonists, nifedipine and verapamil, potentiated the myocardial depression during exposure to clinical concentrations of halothane in cultured neonatal rat ventricular myocytes under physiologic conditions. 2) A calcium channel agonist, Bay K, completely prevented the depression in beating amplitude even at a halothane concentration of 4% but only partially prevented decreases in beating rate. 3) Halothane reduced the Bmax of a dihydropyridine derivative, $[^{3}H]$ PN200-110, in cultured ventricular myocyte membranes. 4) These results indicate that the reduction of Ca$^{2+}$ current via the sarcolemmal L-type Ca$^{2+}$ channel is probably caused by conformational changes in dihydropyridine binding sites, and is important in halothane-induced myocardial depression in living heart cells.

References


