Mechanism and Selectivity of the Effects of Halothane on Gap Junction Channel Function

Ding Sheng He, Janis M. Burt

Abstract—Volatile anesthetics alter tissue excitability by decreasing the extent of gap junction–mediated cell-cell coupling and by altering the activity of the channels that underlie the action potential. In the present study, we demonstrate, using dual whole-cell voltage-clamp techniques, that coexpression of connexin (Cx) 40 and Cx43 rendered cells more sensitive to uncoupling by halothane than cells that express only Cx40 or only Cx43. The halothane-induced reduction in junctional conductance was caused by decreased channel mean open time and increased channel mean closed time. The magnitude of the effect of halothane on channel open time was least for Cx40-like channels and greatest for heteromeric channels. Thus, the data indicate that halothane gates gap junction channels to the closed state in a dose-dependent and connexin-specific manner. One consequence of the selectivity of halothane is that the profile of single-channel events observed in the presence of halothane may not be quantitatively representative of the population of channels contributing to macroscopic conductance in cells that express more than one connexin. In addition, in tissues that express multiple connexins, such as heart and blood vessels, the capacity of the gap junctions to transmit electrical and chemical signals in the presence of halothane could vary according to the pattern of connexin expression. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2000;86:e104-e109.)

Key Words: gap junctions • connexins • volatile anesthetics • vascular smooth muscle • arrhythmia
decrease in channel mean open time for a nearly 100-fold decrease in channel $P_o$. The effects of halothane on the various channels expressed in A7r5 cells was, however, not uniform. Many of the heteromeric channels were more sensitive to these gating effects than homomeric/homotypic Cx43 or Cx40 channels. Cx40 channels were the least sensitive to halothane. These differences in sensitivity are discussed with respect to their possible implications for the effects of halothane on heart rhythmicity.

Materials and Methods

Cells and Culture Conditions

A7r5 cells, a rat embryonic aortic smooth muscle cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, Va). These cells coexpress Cx40 and Cx43, which form heteromeric as well as heterotypic channels. Homomeric/homotypic Cx43 and Cx40 channels were studied in Rat-1 cells and N2A cells stably transfected with Cx40, respectively. All cells were cultured as described previously.2

Electrophysiology

Confluent A7r5, Rat-1, or N2A-Cx40 cells were trypsinized (0.25% trypsin in Ca$^{2+}$-Mg$^{2+}$-free PBS) and replated at low density on glass coverslips. Thirty minutes to 5 hours after plating, coverslips with attached cells were mounted in an experimental chamber and the cells bathed at room temperature in a solution containing (in mmol/L) NaCl 142.5, KCl 4, MgCl$_2$ 1, Na$_2$HPO$_4$ 0.9, dextrose 5, sodium pyruvate 2, HEPES 10, CsCl 15, tetraethylammonium chloride 10, and BaCl$_2$. Patch-type microelectrodes (5 to 10 M$\Omega$) were filled with a pipette solution containing (in mmol/L) CsCl 67.8, potassium glutamate 67.8, tetraethylammonium chloride 10, CaCl$_2$ 0.5, MgCl$_2$ 3, dextrose 5, HEPES 10, EGTA 10, Na$_2$ATP 5, and sodium creatine phosphate 6.7. Using dual whole-cell voltage-clamp techniques as previously described, $g_j$ was monitored until stable, and halothane (1, 2, or 4 mmol/L) was then suffused over the cells.9 This protocol ensured that observed changes in $g_j$ (and single-channel events; see below) were induced by halothane, rather than a function of changing series resistance or run-down. The range of 1 to 2 mmol/L halothane corresponds to the 1% to 3% minimum alveolar concentration used clinically.10

Single-channel data were obtained from poorly coupled cells (control conditions, no halothane) as well as from well-coupled cells treated with halothane to reduce $g_j$ and reveal single-channel events. No obvious changes in event-amplitude histograms occurred as a function of whole-cell clamp duration in any of the three cell types (data not shown), which further supports the absence of run-down in these cells. The A7r5 and Rat-1 cells assemble junctions very rapidly at 37°C. To obtain poorly coupled pairs, 30 minutes after plating (and incubation at 37°C), the cells were placed in a room-temperature incubator. Single-channel events were observed immediately on removal from the incubator and several hours later were not different. Regardless of incubation conditions before the actual experiment, all electrophysiological experiments for all cell types were conducted at room temperature. Channel events were typically obtained with a transjunctional driving force of 40 mV. Data were monitored continuously by chart recorder and acquired in digitized format for subsequent analysis. Effective rise time of the equipment was 6.6 ms. Mean open and closed times were determined according to Ramanan et al.14 Open times for channels of specific amplitudes were obtained from cell pairs with only one channel of specific amplitude active and were measured by one of the investigators from the chart records. Precision of these measurements was ±3.125 pS for event amplitude and ±25 ms for event duration.

Results

Halothane reversibly reduces $g_j$ between cells.9,15 We examined the dose-dependent effects of halothane on $g_j$ in cells that coexpress Cx40 and Cx43 (A7r5 cells) and compared the results to those obtained from cells that express only Cx43 (Rat-1 cells) or only Cx40 (N2A-Cx40 cells). Figure 1 shows the steady-state effects of halothane on $g_j$ for these three cell types. The initial $g_j$ for each cell type was not different (Table 1). The data demonstrate that A7r5 cells were the most sensitive and N2A-Cx40 cells the least sensitive to uncoupling by halothane.

The time required to reach steady-state $g_j$ in each cell type decreased as the halothane concentration increased. These differences in time course are evident in Figure 2, which illustrates normalized $g_j$ as a function of time (first 150 seconds) and halothane concentration (1, 2, and 4 mmol/L), as well as in time to steady state and 50% uncoupling, as presented in Table 1. These data provide further evidence supporting high sensitivity of A7r5 cells to uncoupling by halothane.

To determine how halothane affected channel gating, we measured open and closed times in the presence and absence of halothane in A7r5 cells. Single-channel records obtained from A7r5 cells in the absence of halothane and during the early recovery period after uncoupling by halothane application.  

**TABLE 1. Time Required for 2 and 4 mmol/L Halothane to Reduce $g_j$ to Steady State ($t_{ss}$) or by 50% ($t_{50}$) in A7r5, Rat-1, and N2A-Cx40 Cells**

<table>
<thead>
<tr>
<th>Cell Type (n)</th>
<th>Cx</th>
<th>Halothane, mmol/L</th>
<th>$g_j$, nS</th>
<th>$t_{ss}$, seconds</th>
<th>$t_{50}$, seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7r5 (4)</td>
<td>40/43</td>
<td>2</td>
<td>15±5</td>
<td>133±35</td>
<td>60±9</td>
</tr>
<tr>
<td>Rat-1 (7)</td>
<td>43</td>
<td>2</td>
<td>22±3</td>
<td>194±34</td>
<td>104±21†</td>
</tr>
<tr>
<td>N2A-Cx40 (4)</td>
<td>40</td>
<td>2</td>
<td>16±8</td>
<td>449±125</td>
<td>146±10†</td>
</tr>
<tr>
<td>A7r5 (4)</td>
<td>40/43</td>
<td>4</td>
<td>8±1.5</td>
<td>58±19</td>
<td>42±5‡</td>
</tr>
<tr>
<td>Rat-1 (3)</td>
<td>43</td>
<td>4</td>
<td>21±6</td>
<td>55±14</td>
<td>38±12‡</td>
</tr>
<tr>
<td>N2A-Cx40 (5)</td>
<td>40</td>
<td>4</td>
<td>11±6</td>
<td>206±47</td>
<td>98±22</td>
</tr>
</tbody>
</table>

*Data are mean±SEM.
†Significantly different from A7r5 (2 mmol/L, data).
‡Significantly different from N2A-Cx40 (4 mmol/L, data).
tion were acquired. In the absence of halothane (Figure 3A), channels remained open for long periods of time (>1 second; Table 2) whereas during recovery from halothane-induced uncoupling (Figure 3B), channel open times were quite brief (<0.2 seconds). Analysis of Po in multiple single-channel records comparable to those shown in Figure 3, with the use of software developed by Ramanan et al., which makes no distinctions between channels with different amplitudes, revealed that halothane induced an approximate 10-fold decrease in mean open time and 20-fold increase in mean closed time (Table 2). The net effect of these changes was a 95% reduction in Po.

In A7r5 cells, Cx40 and Cx43 form functional Cx40-Cx43 heteromeric channels, some of which can be recognized by event amplitudes not observed in cells that express only Cx43 or only Cx40. To determine whether these heteromeric channels were more sensitive to the effects of halothane than homomeric/homotypic Cx43 or Cx40 channels, we compared single-channel event amplitudes observed in the presence versus absence of halothane exposure. In the absence of halothane, a diversity of channel amplitudes was observed in the A7r5 cells (Figure 3A). In the all-points histogram (displayed on the right), two features characteristic of a mixed-channel population were evident: the peak-to-peak separations did not correspond to actual event amplitudes, and the peaks were broad. In the presence of halothane (Figure 3B), this diversity of channel amplitudes was absent, and the all-points histogram revealed narrow peaks whose separations corresponded to the observed event amplitudes. The event amplitude observed most often in the early period of recovery from halothane was 140 pS, which is the amplitude most commonly observed for homomeric/homotypic Cx40 channels.

To determine whether this altered amplitude-frequency histogram might reflect nonuniform effects of halothane on the Po of the various channel types evident in the histogram, we measured the open times of these channels in the presence versus absence of halothane. Figure 5 shows that open time was significantly decreased by halothane for events of nearly all amplitudes, providing qualitative confirmation of the result derived from use of the analysis program of Ramanan et al. However, events of different amplitudes were not affected to the same extent (Figure 6A). Interestingly, open times for channels whose amplitudes corresponded to those observed for homomeric/homotypic Cx40 (165 pS) and Cx43 (90 pS) were somewhat longer in the presence versus absence of halothane. However, these channel events were sufficiently infrequent (Figure 4) that their contribution to cumulative open time was quite small (Figure 6B). The prominence of the 140-pS event in the halothane cumulative

**Table 2. Biophysical Parameters of Gap Junction Channels in A7r5 Cells Under the Control and Halothane Treatment Conditions at a Transjunctional Voltage of 40 mV**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean Open Time, ms</th>
<th>Mean Closed Time, ms</th>
<th>P0</th>
<th>P0</th>
<th>P0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6, 886)</td>
<td>1287±1632</td>
<td>875±80</td>
<td>0.57±0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Halothane (4, 390)</td>
<td>132±99†</td>
<td>17 095±15 145†</td>
<td>0.032±0.039†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data are mean±SD.
†Significantly different from control.

Figure 2. Halothane reduced gj in a dose- and time-dependent fashion in A7r5 (A), Rat-1 (B), and N2A-Cx40 (C) cells. M, A, and A indicate 1, 2, and 4 mmol/L halothane, respectively. The gj at each time point was normalized to the initial value for each cell pair, and mean±SEM of all cell pairs at a given dose was plotted relative to time. Sample sizes are reported in Table 1.

Figure 3. Channel events observed in A7r5 cells in the absence of halothane (A) and during early recovery from halothane-induced uncoupling (B). A diversity of event amplitudes is evident under control conditions, and channel open times are long. Peak-to-peak distances in the all-points histogram are variable. B-1, Channel activity early in recovery is characterized by long closed times and very short open times. As recovery progresses, open time increases and closed time decreases. B-2, Expanded section of B-1, shows that 140-pS events predominate during early recovery. Peak-to-peak distances in the all-points histogram are homogeneous. In both cases, transjuncational voltage was 40 mV with cell 1 at 0 mV and cell 2 at 40 mV; current from cell 1 is displayed. The asterisk marks zero junctional current.
open time plot (Figure 6B, black bars) is consistent with the insensitivity of N2A-Cx40 cells to uncoupling by halothane, as illustrated in Figures 1 and 2, and consequent early recovery of this channel type during halothane washout. Many, but certainly not all, of the channels whose open durations were most severely affected by halothane (Figure 6A) correspond to channel amplitudes not typical of the homomeric/homotypic setting. These events accounted for a large percentage of the cumulative open time under control conditions but significantly less so in the presence of halothane (Figure 6B).

Discussion

Halothane, 2-bromo-2-chloro-1,1,1-trifluoroethane, is a volatile anesthetic agent that reversibly uncouples many cell types.\(^9\,13\,15\) In the present study, we demonstrated that coexpression of Cx40 and Cx43 rendered cells more sensitive to uncoupling by halothane than cells that express only one of these connexins. Uncoupling was the result of a significant decrease in \(P_o\) caused by increased channel mean closed time and decreased mean open time. These effects on channel open time were not uniform for all channel amplitudes. Channel amplitudes common to the homomeric/homotypic setting (165 and 90 pS) were least affected. Indeed, at halothane concentrations where open times of heteromeric channels were greatly reduced, the open times of these Cx40- and Cx43-like channels were increased. However, the contribution to cumulative open time was small due to low frequency. The contribution to cumulative open time of the remaining channels, which are likely heteromeric in composition, was substantial, and the open times of many of these channel types were severely decreased by halothane. These data indicate that the dose-dependent differences in effect of halothane on \(g_j\) reflected the connexin composition of the channels comprising the junction, with some heteromeric channels displaying a high degree of sensitivity. The data suggest that for cells expressing connexins capable of forming functional heteromers, the diversity of channel amplitudes observed in the presence of halothane may quantitatively underestimate the diversity of channel types present in the
macroscopic junction. Possible implications of these data for conduction of impulses in the healthy and diseased heart are discussed.

Our data indicated that many of the heteromeric Cx40-Cx43 channels in A7r5 cells were more sensitive to halothane-induced closure than homomeric/homotypic Cx43 or Cx40 channels. Four lines of evidence support this conclusion. First, the dose dependence of uncoupling indicated greater sensitivity to halothane in the A7r5 versus Rat-1 (homomeric/homotypic Cx43) and N2A-Cx40 cells (homomeric/homotypic Cx40). Second, the diversity of channel amplitudes observed in A7r5 cells in the presence versus absence of halothane was reduced. Third, the open times of many channels whose amplitudes are not typical of homomeric/homotypic Cx40 or Cx43 channels were reduced to a far greater extent than the open times of channels common to this setting. Finally, the channels most severely affected by halothane contribute ~50% of cumulative open time in the absence of halothane. Reversibility of the effect of halothane, the absence of change in macroscopic gj under control conditions, and the absence of differences in single-channel amplitude histograms as a function of whole-cell clamp duration rule out any role of run-down in the effects of halothane reported in the present study. Thus, our results strongly suggest that many heteromeric Cx40-Cx43 channels are more sensitive to halothane-induced closure than homomeric/homotypic Cx43 or Cx40 channels.

Other investigators have examined chemical-dependent gating behavior of heteromeric channels and hemichannels. Bevans et al17,18 reported that reconstituted heteromeric Cx32-Cx26 hemichannels are more sensitive to gating by aminosulfonates and cyclic nucleotides than homomeric Cx32 hemichannels. Lee and Rhee19 found that Cx32-Cx26—coexpressing cells are more sensitive to pH-dependent uncoupling than cells that express only Cx26. Preliminary data recently presented by Gu et al20 suggest that heteromeric Cx40-Cx43 channels exhibited greater pH sensitivity than homomeric Cx40 or Cx43 channels. Thus, the available literature on heteromeric channels suggests that the chemical-dependent gating behavior of heteromeric channels (at least by pH and halothane) differs from that of either corresponding homomeric channel and is indeed not well predicted by the gating behavior of the corresponding homomeric channels. Interestingly, Wang and Peracchia21 found that the pH-dependent gating behavior of heteromeric channels composed of Cx32 and a C-terminal mutant form of Cx32 is dominated by the wild-type connexin. This observation might suggest that the alterations of chemical-dependent gating behavior observed for heteromeric channels composed of two connexin isoforms reflect subunit interactions in membrane spanning or extracellular domains.

Interestingly, heteromeric channels seem to be more resistant to voltage-dependent gating than their homomeric counterparts, which suggests that subunit interactions influence chemical- and voltage-dependent gating mechanisms very differently. Junctions composed of Cx37-Cx43 heteromeric channels2 displayed variable response to voltage, with the extremes of response outside the range predicted by either of the homomeric channels. Similarly, Cx40-Cx43 channels appear to be less sensitive to voltage-dependent gating than their homomeric/homotypic counterparts.2 The behavior of heteromeric hemichannels is also quite different from either homomeric hemichannels.22 These data suggest that heteromerization alters subunit interactions in a fashion that leads to enhanced Popen through a broader range of voltages than occurs in the homomeric channel and hemichannel.

In studies of the function of gap junctions, many investigators have used halothane (and other lipophilic agents) to reduce coupling between cells to reveal single-channel events.9,13,15 The population of channel amplitudes observed in the presence of halothane has been assumed to be representative of all the channels present in the gap junction. The data in the present study indicate that this assumption is not warranted and could be misleading. Indeed, Moore and Burt16 observed a preponderance of 70-, 108-, and 140-pS channels in A7r5 cells in the presence of halothane and concluded, on the basis of these and other results, that Cx40 and Cx43 did not form heteromeric or heterotypic channels. The present study indicates that the conditions of the study presented by Moore and Burt favored visualization of the homomeric/homotypic channel forms. Thus, quantitative evaluation of the diversity of channels formed in a setting where heteromerization is possible cannot be achieved in the presence of halothane.

Clinically, halothane is arrhythmogenic in some settings23,24 whereas in others it abolishes arrhythmias.25,26 This dual effect of halothane could reflect differences in connexin expression in the arrhythmogenic versus antiarrhythmic settings. Most cells of the heart normally express more than one type of connexin. However, spatial and quantitative changes in connexin expression occur as a normal part of cardiac development,27,28 as well as in aging,29 ischemia-induced remodeling,29,30 and arrhythmia-induced remodeling.31,32 For example, sustained atrial fibrillation induces a permanent increase in Cx43 expression in the atrium.31,32 Such an increase could predispose the remodeled heart to arrhythmias. Similarly, infarction causes a redistribution of Cx43 in the infarction border zone30,32 and an increased susceptibility to ventricular tachycardia. Expression of other connexins was not examined in these studies; however, a change in the expression of only one connexin in a cell that expresses multiple connexins would be expected to significantly shift the connexin composition of the channel population.5,21 Because halothane affects homomeric and heteromeric gap junction channels with different sensitivities, expression-dependent effects of halothane on cardiac rhythmicity would be expected. Interestingly, if heteromeric channels are selectively closed in the presence of halothane, then the remaining channels would be predominantly homomeric channels. Homomeric channels are far more likely in the Cx40 knockout mouse and in the heterozygous Cx43+/− mouse, and both animals display an increased susceptibility to arrhythmias.33,34

In summary, our data demonstrate that halothane reduces gap junction channel Popen in a dose-dependent and connexin-specific fashion. If comparable selectivity of effect were to occur in the heart, halothane could result in nonuniform
changes in the conductive pathway that would predispose the healthy heart to arrhythmias but, as a consequence of altered connexin expression, oppose arrhythmogenesis in the diseased heart.

Acknowledgments

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