Effects of Halothane on Whole-Cell Calcium Channel Currents in Cultured Lymnaea Neurones

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Abstract

1) The effects of four concentrations of halothane were studied on the macroscopic, high-voltage activated calcium channel currents of cultured neurones of the pedal I cluster of Lymnaea stagnalis, using the whole cell patch clamp technique.
2) Following application of increasing concentrations of halothane, in the clinical range, a rapid and reversible depression of both the peak and end-pulse Ca²⁺-channel currents was observed. These effects of halothane were clearly dose-dependent.
3) The rate of inactivation of the calcium current was significantly accelerated by halothane in a dose-dependent manner suggesting that halothane affects the channels both in the open and the closed state.
4) The observations of depression of chemical synaptic transmission, alteration of rate of firing, and alteration in the action potential amplitude, duration and after-hyperpolarization can all be partially explained on the basis of effects of halothane on calcium channels, but volatile anaesthetics appear to have multiple targets at the cellular and subcellular levels.

Keywords: Anaesthetics; Halothane; Calcium Currents; Calcium Channels; Cultured Neurones; Snail

Abbreviations

ABS: Antibiotic Saline; AKAP: A-Kinase Anchor Proteins; [Ca²⁺]: Intracellular Calcium Concentration; DM: Defined Medium; Ep: End-Pulse Current; GA: General Anaesthetic; Hp: Holding Potential; HEPES: 4-(2-Hydroxyethyl)Piperazine-1-Ethanesulfonic Acid; HVA: High Voltage Activated; I₉b: Barium Current; I₉c: Calcium Current; Iₚₚₚ: End-Pulse Current; Iₚₚₚ: Peak Current; I-V Curve: Current Voltage Curve; LVA: Low Voltage Activated; MAC: Minimum Alveolar Concentration; Msec: Millisecond; PKA: Protein Kinase A; PKC: Protein Kinase C; SD: Standard Deviation; SEM: Standard Error Of The Mean; Tp: Test Potential; Vc: Command Voltage; VDCC: Voltage Dependent Calcium Channel Or Alternatively; VGCC: Voltage-Gated Calcium Channel; V/V% Or Volume % - Volume Of Anaesthetic In Its Carrier Gas

Introduction

Within nervous systems, a major target site for the action of general anaesthetics is the cell membrane especially the membrane ion channels and receptors which determine neuronal excitability and promote the release of neurotransmitters. The least conflicting and the most promising target from amongst the voltage-gated ion channels for action of general anaesthetics seems to be the calcium channels [1], though potassium-selective channels have also been proposed to be the primary site for action of general anaesthetics [2,3] as have the GABAA receptors [4], whose activation normally results in hyperpolarisation due to an influx of Cl⁻, and the NMDA receptors [5], which normally gate Ca²⁺ and Na⁺ resulting in depolarization. However, there is no single target site that can explain the actions of anaesthetics.
so we have chosen to characterise both potassium (preliminary paper by Moghadam and Winlow, [6]) and high voltage activated [HVA] (L-type) calcium currents [7] in the highly accessible, identifiable, neurones of the pond snail, *Lymnaea stagnalis* (L).

L-type channels seem to be present in all excitable cells and many in excitable cells too. They were first described in peripheral neurones and cardiac cells [8]. In neurones and endocrine cells they are involved in exocytosis [8] while it has been postulated that those localised on the cell soma may be implicated in regulation of gene expression [9,10]. A reduction in amplitude of calcium channel current on exposure to volatile anaesthetics has been observed in rat dorsal root sensory neurones [11], recombinant cardiac L-type channels expressed in HEK 293 cells [12]; cultured neocortical astrocytes [13], CA1 neurones in hippocampal slices [14] clonal pituitary cells [1,15], isolated chromaffin cells [16,17,18], PC12 cells [19], Xenopus oocytes [20], human neuronal cell lines [21], guinea pig myocytes [22], etc. General anaesthetics have also been shown to exert marked effect on calcium channel currents in myocardial cells in rabbits [23] and dogs [24,25] in the clinically effective range.

In many of the reports using isolated neurones from various preparations it is difficult to demonstrate a direct correlation between reduction in calcium channel current and normal electrical and chemical activity in individual neurones in situ or in a network. It is equally important to correlate these findings with whole animal behaviour in the same preparation to deduce reasonable results about the mechanism of action of general anaesthetics. According to Gary Strichartz [26] any "inquiry regarding anesthesia should occur at many levels, behavioural, neurophysiological, biochemical and pharmacological, at the cellular domain and with an appreciation for diverse anesthetic actions on subcellular structures, including ion channels." Using the brain of *Lymnaea stagnalis* as a model for research on general anaesthetics, the behavioural [27], network [28] and cellular [3,29,30-38] actions of anaesthetics delivered at clinical concentrations [33,36,39] have been studied. *Lymnaea* and other gastropod molluscs have easily accessible, physically large, uniquely identifiable nerve cells [40,41,42], whose biophysical and pharmacological properties have been investigated extensively in many different laboratories. Behavioural studies on *Lymnaea* revealed that the ED$_{50}$ for halothane on the whole animal withdrawal reflex was 0.83% v/v, which is well within the clinical range [27]. Application of inhalation or systemic anaesthetics to the isolated brain of *Lymnaea* led to a gradual decline in discharge frequency and eventual quiescence or the occurrence of paroxysmal depolarizing shifts (PDS) during either spontaneous or evoked activity [43]. An alteration of the calcium-dependent components of the action potential, i.e., shortening or disappearance of pseudoplateau, change in after hyperpolarization amplitude and a change in amplitude of the action potential was also evident. In addition to reducing neuronal excitability in *Lymnaea stagnalis*, halothane also depressed chemical synaptic transmission [30,31,32]. High-voltage-activated (HVA) calcium channel currents (both N- and L-type) are thought to play a role in generation of action potentials and excitation-response coupling such as neurotransmitter secretion [8,44], but the precise actions of various types of calcium channels may vary from one type of neurone to another. Identified *Lymnaea* neurones in culture possess a high-voltage-activated (HVA) calcium current with L-type properties that can be studied in isolation under appropriate conditions [7]. We hypothesise that these observations may be explained if general anaesthetics reduce the entry of calcium via HVA calcium channels into neurones.

Here we show that halothane, at clinically relevant concentrations, reduces an isolated, high-voltage-activated, whole-cell calcium channel current [7] in a dose-dependent manner in cultured, identified *Lymnaea* neurones. Halothane was selected for this investigation as it is a commonly used inhalational anaesthetic. A preliminary report has been published elsewhere [45].

**Materials and Methods**

Whole cell patch clamp recordings were made according to the method of Hamill., et al. [46] from cultured, pedal I cluster neurones of the pulmonate mollusc *Lymnaea stagnalis* (L) as previously described [44]. To prevent neurite outgrowth neurones were cultured in defined medium (DM), which resulted in spherical cells. For comparative purposes, some neurones from the right parietal A group were also studied. Both types of neurones were usually derived from small snails in order to have cells in the range of 20 to 40 microns soma diameter. Calcium channel currents were recorded using 10 mM Ba$^{2+}$ as the charge carrier [7]. Barium currents (I$_{Ba}$), similar to those previously described [7,45], were seen with only an HVA barium current component. Concentrations of halothane selected were 0.5%,

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1.0%, 2.0% and 4.0%, v/v so as to cover the clinically useful spectrum (PM Hopkins, University of Leeds - personal communication). Halothane in air was prepared using an Ohmeda Fluotec 3 vaporiser. Anaesthetic solutions were prepared in glass bottles in which 300ml of saline was equilibrated with a known concentration of anaesthetic according to the methods of Girdlestone, et al [27]. The percentage concentration of the vaporised gas in the bottles was checked using a Datex “Normac” anaesthetic agent monitor (Datex Instrumentarium Corporation, Finland) and was delivered with the help of a 60 ml glass syringe connected to the culture dish via a small length of Teflon tubing. All experiments were performed at room temperature (21°C).

Results

Effect of halothane on the amplitude of the peak and the end-pulse current

Effect of 0.5% halothane: Peak current ($I_{peak}$) is the maximal current attained during a square voltage step and the end-pulse current ($I_{end}$) is the residual current just prior to the end of this command pulse. Data acquisition and analysis were as previously described [7]. The most striking feature of the action of halothane on the calcium channel currents was a reversible depression of $I_{peak}$. Figure 1a shows a family of calcium channel currents produced by depolarizing the cell from a constant holding potential ($h_p$) of -50 mV to test depolarizations between -30 mV to +30 mV, in increments of 10 mV (Figure 1d). Typical calcium channel currents that peaked with increasing speeds as voltage became more positive, and then inactivated to a non-zero steady-state by the end of pulse could be seen. The voltage clamp current traces at command voltages of -30, -20 and -10 mV showed very little, if any, inactivation [7] as illustrated in figure 1a. When the preparation was superfused with 0.5% halothane there was a depression of these calcium channel currents over the voltage range from -30 mV to +30 mV (Figure 1b). There was a mean decrease of 15% in $I_{peak}$ on application of 0.5% halothane which is significant ($p \leq 0.05, n = 9$). This effect was reversible when the preparation was washed with normal barium saline.

![Figure 1](image-url)

Figure 1: Reduction in the $I_{peak}$ by 0.5% halothane in an I-cluster cell from right pedal ganglion of Lymnaea. The neurone was voltage-clamped for 180 msec to voltages between -40 to +130 mV from an $hp$ of -50 mV. Current traces are shown first in the control Ba$^{2+}$saline (A), in 0.5% halothane (B) and after removal of halothane (C). The voltage protocol for current traces is shown in (D). Current-voltage relationship (E) of the peak Ba$^{2+}$ current recorded in the same cell using the same protocol. The maximum current in the I-V relation in control (open squares) was obtained with a clamp pulse to +10 mV and the current reversed at around +58 mV. The leakage current was subtracted from the records used for the I-V plot. In 2-3 minutes 0.5% halothane suppressed the current significantly without changing the configuration of the I-V curve (closed triangles). The effect of halothane was reversible after removal of halothane (open circles).

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**Effect of halothane on Ba\(^2+\) current-voltage relation:** Figure 1e shows the current-voltage relationship of the peak calcium channel currents in the absence and then in the presence of halothane and after wash out of halothane. Calcium channel currents were elicited with depolarizing voltage steps to -40 mV through to +130 mV from an hp of -50 mV. A clear though small effect of 0.5% halothane can be seen as a diminution of the I-V curve, without a shift of the curve along the voltage axis. Beyond the reversal potential of the calcium channel current, no appreciable difference was observed in the currents elicited in the control and in the presence of halothane indicating a minimal effect on the nonspecific outward currents [44]. Similar results were obtained in another nine experiments performed with this concentration of halothane. (Figure 1)

**Effect of increasing concentrations of halothane on calcium channel current:** As is apparent from Table 1 progressively higher concentrations of halothane caused a dose-dependent depression of the peak and the end-pulse calcium channel currents elicited at the peak of the I-V curves. The effect on end-pulse current was significantly greater as compared to the peak current at each concentration of halothane tested. The mean reduction of peak barium current (I\(_{ba}\)) by 1.0% and 2.0% halothane was 27.2% (n = 7) and 52% (n = 6) respectively. The effect of 1.0% halothane on peak current was significantly greater than that of 0.5% halothane (p < 0.005). Figure 2a shows the peak calcium channel current elicited by depolarization to +10 mV from a holding potential of -50 mV, first in the absence and then in the presence of 1.0% halothane. The post control current trace shows a near-complete recovery. There was an indication of an accelerated inactivation of Ba\(^2+\) currents as the current at the end of pulse was depressed to a greater extent as compared to the peak current. The effect of 1.0% & 2.0% halothane on I\(_{ba}\) was reversible and the maximal peak current amplitude was restored to within about 90% of control value (Figure 2b) in less than 5 minutes after beginning of the wash. 4.0% halothane abolished the Ba\(^2+\) current completely in some cases (mean reduction of peak Ca\(^2+\) channel current by about 89%, n = 6) and even an outward current could be seen on one or two occasions at the end of the depolarizing pulse of 180 msec (Figure 3a).

**Figure 2:** Reduction in the I\(_{ba}\) by 1.0% halothane in an I-cluster cell from right pedal ganglion of *Lymnaea*. The neurone was voltage-clamped for 180 msec to +10 mV from an hp of -50 mV. Current responses were obtained in the control Ba\(^2+\) saline, in 1.0% halothane and after removal of halothane (a). There was a clear depression of I\(_{ba}\) by halothane which was reversible. The rate of inactivation appears to be enhanced especially in the initial part of the relaxation phase of the current. These current traces were leak subtracted. (b) Current-voltage relationship of peak Ba\(^2+\) current recorded in the same cell held at -50 mV. 180 msec depolarizing pulses were applied from -40 to +130 mV in steps of 10 mV: in control (open squares), in 1.0% halothane (closed triangles), and after wash (open circles). In 2-3 minutes 1.0% halothane suppressed the current significantly without changing the configuration of the I-V curve. The effect of halothane was reversible after removal of halothane. There was no appreciable difference in the currents in three situations above the apparent reversal potential of the current.
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<table>
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<tr>
<th>Halothane %</th>
<th>% depression of $I_{\text{peak}}$</th>
<th>P</th>
<th>% depression of $I_{\text{end}}$</th>
<th>n</th>
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<td>*</td>
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<td>39 ± 2.4</td>
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<tr>
<td>4.0</td>
<td>89 ± 2.9</td>
<td>**</td>
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* $p <= 0.05; ** p <= 0.01; *** p <= 0.005; **** p <= 0.001; paired t-test

**Table 1:** Effect of four concentrations of halothane on peak and end-pulse calcium channel currents. Maximal calcium channel currents were elicited (the peak of I-V curves) and percentage depression of the peak and the end-pulse currents by different concentrations of halothane were calculated. Data is presented as mean ± S.E.M. The number of experiments are given under the column $n$. Paired t-tests were performed to test the significance of difference. The percentage depression of peak current is significantly different from that of end-pulse current at each concentration of halothane.

The I-V plots (Figure 3b) constructed from the peaks of $\text{Ba}^{2+}$ current traces in the absence and then in the presence of 4.0% halothane show an almost complete abolition of calcium channel current, the effect being more or less uniform with a marked depression of current over the whole voltage range. Halothane did not shift the peak of the current-voltage relation of $I_{\text{Ba}}$, and therefore did not show any voltage dependence in its effect. Another point to note is a more marked effect on the region beyond the reversal potential of the current. The current seems to be outward at the end of the pulse at positive voltages in the case of 4.0% halothane. The end-pulse current was similarly depressed by 4.0% halothane and this depression of end-pulse current was again significantly greater than the depression of peak current.

![Image](image_url)

**Figure 3:** 4.0% halothane almost blocked the calcium channel current. The neurone was voltage-clamped for 180 msec to +10 mV from a holding potential of -50 mV. Current responses were obtained in the control $\text{Ba}^{2+}$ saline, in 4.0% halothane and after wash out of halothane (a). There was a marked depression of $I_{\text{Ba}}$ by halothane which was reversible. The decay of $I_{\text{Ba}}$ was significantly increased by 4.0% halothane. (b) Current-voltage relationship of peak $\text{Ba}^{2+}$ current recorded in the same cell held at -50 mV. 180 msec depolarizing pulses were applied from -40 to +130 mV in steps of 10 mV: in control (open squares), in 4.0% halothane (closed triangles), and after wash (open circles). In 2-3 minutes 4.0% halothane suppressed the current significantly without changing the configuration of the I-V curve. The effect of halothane was reversible after removal of halothane. There was a clear depression of the outward current beyond the apparent reversal potential of $I_{\text{Ba}}$.

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Figure 4 bears out the enhanced effect of halothane on end-pulse current very clearly where maximal peak $I_{Ba}$ has been elicited by depolarization from a holding potential of -50 mV to +10 mV. The record under the effect of 1.0% (Figure 4a) and 2.0% (Figure 4b) halothane has been shown along with the normalized records obtained by multiplying every point with a constant factor (peak control/peak in halothane) in each case. The initial portion of the inactivation phase of the current is comparatively faster than the late one. When the peak current evoked under the effect of 4.0% halothane was normalized (Figure 4c), the same qualitative effect as seen with 1.0% and 2.0% halothane could be observed.

![Figure 4: Normalization of $I_{Ba}$ current traces in 1.0% and 2.0% halothane. (a) During whole-cell recording in barium saline the cell was held at an hp = -50 mV and a 180 msec long depolarizing voltage step was applied to a command voltage of +10 mV. This elicited the maximal peak current response which showed a rapid activation and a slow and incomplete inactivation of calcium channel current. When 1.0% halothane was added it depressed the $Ba^{2+}$ current. The $I_{Ba}$ trace under the effect of halothane was normalized (indicated by n) to its control by multiplying every point of this trace with a constant factor (peak control/peak halothane). 1% halothane enhanced the inactivation of $I_{Ba}$. (b) Same procedure for 2.0% halothane brought out a still greater enhancement of inactivation of $I_{Ba}$. Note that the initial part of relaxation phase of $I_{Ba}$ is very fast. (c) Normalized current trace for 4.0% halothane. From an hp of -50 mV a command voltage step of 180 msec duration was applied to potential of +10 mV and the response recorded in control and in 4.0% halothane.

A very clear dose dependent effect of halothane can be observed when normalized peak current (Figure 5a) and normalized end-pulse current (Figure 5b) are plotted as a function of voltage for all four concentrations of halothane applied to the same cell (Figures 5a to d). Four percent halothane caused a shift of reversal potential of barium current in the less depolarizing direction (Figures 5e, 5f) which could be due to some structural damage to the cell or some sort of change in the seal.

Figure 5: Comparison of effect of four concentrations of halothane on the same pedal I-cluster cell. Cell was held at hp of -50 mV and 180 msec long depolarizing pulses were applied in 10 mV increments. Families of I-V plots of relative peak current (A) and relative endpulse current (B) in control, 0.5%, 1.0%, 2.0% and 4.0% halothane, from the same cell. There is a very clear reduction of calcium channel current which is concentration-dependent. The peak of the IV curves are not shifted by halothane.

Figure 6: A) Concentration-dependent depression of peak $I_{Na}$ by halothane in right pedal I-cluster cells. The graph demonstrates an almost linear relationship between depression of peak maximal calcium channel current and the halothane concentration applied. Values are expressed as mean ± S.D. The numbers in parenthesis indicate the number of experiments on different cells. On three occasions, it was possible to get good results with all four concentrations of halothane from the same cell. B) Dose-response relationship showing the percentage depression of peak $I_{Na}$ by different concentrations of halothane in pooled data from 30 right parietal A group neurones. As in the previous figure, this graph also demonstrates an almost linear depression of peak current by halothane concentrations applied. The curve, however, illustrates a greater variability in response to halothane in various types of cells. Some of the cells exhibited a much greater sensitivity to applied halothane. In one extreme case 1.0% halothane was able to reversibly depress $I_{Na}$ by 59% and 2.0% halothane was able to reduce the calcium channel current by 80%. Values are expressed as mean ± S.D. The numbers in parenthesis indicate the number of experiments on different cells. On twelve occasions, it was possible to get good results with two or more concentrations of halothane from the same cell.
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Figure 6a demonstrates an almost linear relationship between the percentage of peak and end-pulse current depression and the halothane concentration applied. Values are expressed as mean ± S.D. When data were pooled together with those for right parietal A group cells, the concentration dependent effect was quite obvious (Figure 6b). Some of the cells especially from the right parietal A group showed a greater sensitivity to applied halothane (1 & 2%) and that led to a larger variability seen in this figure at 1.0% and 2.0% halothane. Thus, in addition to a differential effect of general anaesthetics on different ion channels [1] different cells could vary in their response to applied anaesthetics.

**Effect of halothane on percentage inactivation value:** Increasing concentrations of halothane not only depress the peak inward current, but also markedly reduce the end-pulse current. For instance, under control conditions the Ba\(^{2+}\) current decayed to a new steady state value after attaining its peak value during the course of a 180 msec pulse. This new value was usually greater than 65% of the peak current elicited at the peak of I-V curve. With 0.5% halothane, there was relatively greater inactivation of Ba\(^{2+}\) current. To bring out the effect on this parameter the % inactivation value was calculated. The % inactivation value increased significantly under the effect of halothane in comparison to control. Figure 7 shows histograms illustrating the mean ± S.E.M. of percentage inactivation value in control and in 0.5%, 1.0%, 2.0% and 4.0% halothane. There was a significant dose-dependent increase in the % inactivation value by halothane. The % inactivation value indicates that during a 180 msec pulse the total net influx of Ba\(^{2+}\) (or Ca\(^{2+}\)) would be much less under the influence of halothane as compared to control. This greater effect of halothane on the Ba\(^{2+}\) current at the end of the pulse may be because of a reduced ability of calcium channels to remain open for longer periods in response to a depolarizing step, i.e., an accelerated inactivation.

**Effect on time to peak and time to half maximal activation:** The time to peak of Ba\(^{2+}\) current was decreased by 2.0% halothane (25.70 ± 5.44 msec in control vs. 12.30 ± 1.83 in halothane, mean ± S.D.) as compared to control. The time to half maximal activation, which is a better measure of rate of activation, was not affected in a consistent manner. The same was true for 4.0% halothane. There was a reduction in the time to peak for Ba\(^{2+}\) current elicited from a holding potential of -50 mV to a test potential of +10 mV. The time to half maximal activation was reduced from 4.5 msec to 3.5 msec (n = 3), and time to peak was reduced from 14.5 msec to 7.5 msec. Thus, it seems as if halothane is quickening the activation process in addition to hastening the inactivation. This obviously requires a more detailed study of the activation and inactivation kinetics by looking at steady-state activation and inactivation curves using double-pulse protocols.
Time course of onset and recovery: Because of the lipid-soluble nature of halothane it was expected that the onset of action would be rapid. Indeed, this was observed when halothane was applied to these neurones in culture. Figure 8 shows the time course of onset of action and recovery to control levels after washing in case of 2.0% and 4.0% halothane. Peak currents were elicited by 180 msec depolarizations from a holding potential of -50 mV to the test potential of +10 mV. A voltage pulse was applied once every 30 seconds for the duration of the experiment, and the response was plotted as a function of time. As can be observed both the onset and recovery were quite rapid. The oscillations seen during the application of halothane are due to repeated applications of halothane. On removal of the drug by washing in the control saline an immediate recovery to within 80 % of control value was observed. A much more marked depression of I_{Ca} was observed when 4.0% halothane was applied to the same cell. After an immediate onset of action, which reached its peak in 1-2 minutes and persisted during the period of application of drug, an instantaneous partial recovery was observed when halothane was washed away. The current trace showed an almost complete abolition of calcium channel current which recovered to within 60 - 70% of control value on washing. It was not possible to obtain a complete recovery in the case of 4.0% halothane as mostly the cell seemed to become more fragile and flabby and almost invariably the seal was lost during the process of prolonged washing. The onset of the effect in the case of 0.5 % and 1.0 % halothane also occurred within 1 - 2 minutes and lasted as long as the drug was present in the bath.

Reproducibility of the actions of halothane: When the same concentration of halothane was applied repeatedly to a voltage clamped cell, while Ba^{2+} current I-V plots were obtained with standard protocols, the reduction of HVA current by 1.0% halothane was almost constant (Figure 9). In this particular experiment, there was a 28.09% reduction of peak maximal I_{Ba} by 1% halothane. Then the cell was washed for 5 minutes to let it recover to almost 90% of the control value when 2.0% halothane was superfused. This led to a 48.97% reduction of I_{Ba}. After the cell, had recovered from the effect of 2.0% halothane it was again exposed to 1.0% halothane to see if there was any difference in the response to 1.0% halothane compared to the first application. As is evident from figure 9, the response was quite

Figure 8: Time course of action of 2.0% and 4.0% halothane. A pedal I-cluster cell was held at potential of -50 mV and 180 msec command steps to +10 mV were applied every 30 seconds to elicit peak I_{Ba}. After attaining a steady-state, 2.0% halothane was superfused for 6 minutes. A rapid decline in the peak I_{Ba} was observed within seconds and within 2-3 minutes a relatively steady response was achieved. When the cell was washed with normal Ba^{2+} saline there was quick recovery to within 80 % of the control in 2-3 minutes. 4.0% halothane induced a rapid decline in the amplitude of I_{Ba} within 2-3 minutes and there was a rapid but incomplete recovery within 2-3 minutes on removal of the drug.

similar to the one obtained in the first instance. Experiments performed with 0.5% and 2.0% halothane in the same way produced similar results, at least over a time period of 15 minutes. Thus, the effects of 0.5 to 2.0% halothane were quite reproducible in a preparation and the higher concentration of halothane did not leave a residual effect on Ca\(^{2+}\) currents of neurones. However, it was observed on a few occasions that if a neurone was exposed to halothane for longer periods of time (30 - 60 minutes), Ca\(^{2+}\) currents showed a progressive decline in amplitude and the cell made only a partial recovery on removal of halothane. It is possible that continued exposure to halothane led to irreversible changes in the intracellular factors controlling the Ca\(^{2+}\) channel function.

**Figure 9:** Comparison of the effect of halothane applied at different times but same concentration to show the reproducibility of effect. The cell was held at a holding potential of -50 mV and 180 msec command steps were applied every 10 seconds to potentials between -40 mV to +90 mV. Peak currents were plotted as a function of voltage in control situation (open squares) after normalizing the data. 1.0% halothane was then superfused for 3 minutes and same protocol repeated (open triangles). 2.0% halothane was then superfused and data collected for that concentration of halothane (data not shown). After washing the preparation for 5 minutes to get a near-complete recovery of calcium channel currents, 1.0% halothane was applied again. A second set of observations was obtained with the same protocol as before and data plotted on the same graph (open circles) after normalization. The fractional decrease in \(I_{Ca}\) is the same in two situations.

**Discussion**

The principal finding of the present study was the concentration-dependent decrease of the conductance of calcium channels and an acceleration of the inactivation of calcium channel currents by clinically relevant doses of halothane. These effects became apparent within a few seconds, were usually maximal within 3 - 5 minutes, and were partially reversible within 3 - 5 minutes. The halothane-induced reduction in calcium channel currents probably contributes to the halothane-dependent decrease in action potential height and duration, and depression of synaptic transmission in *Lymnaea* neurones. However, because of their lipid solubility anaesthetics can have numerous sites of action both at the cell membrane and at intracellular sites.

Possible mechanisms of inhibition of the HVA calcium current: The inactivation of the Ca\(^{2+}\) channel current in neurones involves at least two processes \[47\]: a voltage-dependent process \[48\] and a Ca\(^{2+}\)-dependent process \[49,50\]. Anaesthetics may affect both of this process and understanding their effects on HVA calcium currents is of the utmost importance since they are implicated in traumatic brain injury \[51\] because calcium ions are major regulators of cellular functions and toxic calcium overload can lead to cell death in vulnerable populations of neurones \[52,53,54\]. The normal compensatory mechanisms to prevent calcium overload involve both voltage- and calcium-dependent inactivation of HVA calcium channels \[55,56,57\] and may be disturbed by both volatile \[58,59\] and intravenous \[60\] anaesthetics, which can raise the intracellular calcium concentration.

Role of phosphorylation: The run-down of HVA Ca\(^{2+}\) channel current and its recovery on addition of ATP, cAMP and PKA etc. is suggestive of a role of phosphorylation in the proper functioning of HVA calcium channels \[61-64\]. Most of the inactivation is calcium-dependent \[65\] and has been shown to be regulated by phosphorylation and inactivation during a depolarizing pulse results from dephosphorylation of the channel, and recovery from inactivation after the pulse is a consequence of rephosphorylation \[66\]. Agents that decrease the level of phosphorylation can simultaneously decrease the amplitude and increase the rate of inactivation of the Ba\(^{2+}\) current, whereas inhibiting the dephosphorylation of the channels can simultaneously increase the amplitude and decrease the rate of inactivation of Ca\(^{2+}\) current \[67\].

Inactivation of calcium current is an important mechanism in limiting the influx of Ca\(^{2+}\) ions through the voltage-activated Ca\(^{2+}\) channels and, Rankovic, et al. \[57\] have demonstrated that A-kinase anchor proteins (AKAP) target PKA to L-type channels allowing their phosphorylation leading to modulation of calcium dependent inactivation in rat thalamocortical relay neurones. The inactivation time course has been suggested to be a function of the rise of intracellular free Ca\(^{2+}\). Ca\(^{2+}\) channel current inactivation was evident as a decay from the peak of the inward current during prolonged depolarizing steps. Thus, the data presented here may be partly explained by a phosphorylation-dependent modulation of the kinetics of inactivation of the channels or a closely associated regulatory protein, or to the inactivation of cAMP-induced phosphorylation of Ca\(^{2+}\) channels, following a decrease of cAMP content of the cell by halothane \[68,69\]. This is supported further by the fact that the inactivation of Ca\(^{2+}\) current is enhanced by halothane in a dose-dependent manner. However, inhibition of L-type currents by halothane and isoflurane has been found to be independent of protein kinase C (PKC) inhibition in isolated aortic myocytes \[70\], but this does not rule out "the possibility that volatile anaesthetics may stimulate atypical PKCs and thereby inhibit calcium channels" \[70\] as previously implied \[71\].

Role of intracellular calcium: Since in our experiments halothane modulated the Ca\(^{2+}\)-dependent phase of inactivation the question arose whether Ca\(^{2+}\) was involved in the action of halothane on the inactivation process. This possibility was eliminated by using Ba\(^{2+}\) as the charge carrier and studying Ba\(^{2+}\) current inactivation. Barium currents through Ca\(^{2+}\) channels are larger in amplitude and their relaxation from the peak is slow as compared to Ca\(^{2+}\) currents due to removal of Ca\(^{2+}\)-dependent inactivation. The halothane produced inactivation had similar time-courses in Ba\(^{2+}\) as well as in Ca\(^{2+}\). Thus, a Ca\(^{2+}\)-specific influence on the inactivating action of halothane appeared unlikely, but it was possible that changes of intracellular free divalent cations are a prerequisite for its action. However, in our experiments the changes in internal free Ca\(^{2+}\) (or Ba\(^{2+}\)) were buffered with 10 mM EGTA., but Inhibition of Ca\(^{2+}\) current by halothane reported by other investigators might well be a reflection of halothane-induced elevation in [Ca\(^{2+}\)]i \[58,60,72\] which mediates calcium-dependent inactivation \[14\]. If halothane increased inactivation of the HVA current by affecting, at least in part, calcium-dependent inactivation, then the effect of halothane on Ba\(^{2+}\) currents might differ from those on calcium currents. In our previous paper \[7\] we substituted Ca\(^{2+}\) (4 mM) for Ba\(^{2+}\) in the recording medium for some experiments; this concentration of Ca\(^{2+}\) produced peak currents of slightly less than half the magnitude of those obtained in 10 mM Ba\(^{2+}\), but the rate of decay of the calcium currents was very similar to that of barium currents when calcium channel currents were recording after superfusion with halothane. Changes in [Ca\(^{2+}\)]i in cell bodies depends on both membrane Ca\(^{2+}\) channels and intracellular Ca\(^{2+}\) stores. Transient changes of free [Ca\(^{2+}\)]i are a prerequisite for a variety of cell functions including control of membrane conductances. Thus, it is probable that voltage-dependent calcium channels in particular are directly altered by anaesthetic action, and also that effects on intracellular calcium release may then adjust the permeabilities of both these and other channels. If halothane application was repeated for longer periods of time, then with every application there was a further reduction of calcium
channel current. This could be because of cumulative or time dependent effects: for example, sustained intracellular Ca\(^{2+}\) overload can disrupt the mechanisms that regulate Ca\(^{2+}\) homeostasis [50,73,74] or activate certain biochemical processes that may cause extensive cell damage [75].

Administration of higher concentrations of halothane (4.0%) was invariably associated with disturbances of seal with glass pipette as indicated by large shifts of membrane voltage and may have had the efficacy of the buffer because of leakage. Under the microscope, some of the cells appeared relatively fragile and lost their healthy look. In most of the experiments with 4.0% halothane it was possible to get a partial recovery of Ca\(^{2+}\) channel current to around 65 - 70% of the control, but complete recovery was not obtained. It could be that higher concentrations of halothane induce a release of Ca\(^{2+}\) from intracellular stores which activates those enzymes that cause degradation of membrane proteins or enhance the Ca\(^{2+}\) channel run-down. Thus, enhanced inactivation and accelerated run-down of Ca\(^{2+}\) channel currents by halothane could be intrinsically related and could be mediated by an effect on the phosphorylation/ dephosphorylation machinery and other Ca\(^{2+}\) dependent enzymes.

**Anaesthetics may accelerate inactivation of calcium currents:** Gundersen, et al. [76] suggested that in Xenopus oocytes barbiturates exerted their action on HVA Ca\(^{2+}\) channel currents by accelerating the inactivation process. The same may be true for inhalation anaesthetics. An accelerated calcium channel inactivation by certain general anaesthetics such as barbiturates has been reported in neurones of invertebrates including Helix [77] and Aplysia [78], and in vertebrate preparations like mouse dorsal root ganglion neurones [79,80]. Furthermore, isoflurane has been shown to accelerate inactivation of L-type Ca\(^{2+}\) currents in rat cardiac myocytes [81] and propofol inhibits them in human myocytes by enhancing voltage dependent inactivation [82]. Volatile anaesthetics have also been reported to enhance the inactivation of L-type current in cardiac ventricular myocytes [24] and cardiac Purkinje cells [25]. Gross & MacDonald [83] suggested that the barbiturate-induced reduction in L-type current was because of prevention of channel activation, whereas the reduction in N-type current was thought to be as a result of enhanced inactivation. Swandulla, et al. [84] described the facilitation of inactivation of HVA current by menthol in identified Helix neurones and also [85] reported a similar acceleration of inactivation of calcium channel currents under the influence of menthol in chick and rat sensory neurones, while Krnjevic & Puil [14] reported an increased rate of decay of calcium currents along with a depression of calcium currents in CA1 neurones in hippocampal slices when exposed to halothane. Depression of HVA calcium channel currents and an accelerated inactivation of these by halothane has been demonstrated in clonal pituitary cells [1] rat sensory neurones [86] and bovine adrenal chromaffin cells [87]. Finally, Terrar & Victory [88] also observed a speeding of the decay of calcium inward current in guinea-pig ventricular myocytes under the influence of halothane and isoflurane. Most of these studies implicated an accelerated inactivation as the principal mode of action of anaesthetics on calcium channels, though other processes were also thought to have contributed substantially to the overall effect. Taken together these findings suggest that general anaesthetics decrease the amplitude of the Ca\(^{2+}\) current by reducing the opening probability of single calcium channels. The results can be interpreted such that halothane, acting on single population of HVA channels, shifts the relative distribution among inactivated, open and closed states, or favours certain modes of gating [89]. This supposition is supported by patch-clamp studies on single human neuronal L-type channels using the human neuronal cell line SH-SY5Y which revealed that halothane decreased the likelihood of channel opening and enhanced the rate of channel closure and inactivation [21].

**Are calcium channels blocked in the closed state?** As halothane depressed the peak Ca\(^{2+}\) channel current in a dose-dependent manner, it probably blocked the channels in closed state. To examine the possibility that the blocking action of halothane on calcium current might only develop after opening of the Ca\(^{2+}\) channel, the rising phase of current was examined both in the control and in the presence of halothane. When the current in the presence of halothane was scaled to match the peak amplitude of the control current (Figure 4) the Ca\(^{2+}\) currents seemed to be depressed but the rate of activation was not slowed down. Rather, in some cases there was a slight increase in the rate of activation which could be because of an early onset of inactivation. This is consistent with the view that there is no time-dependent aspect to the blocking action of halothane during the rising phase of the calcium current and the blockade by halothane is at equilibrium at the time of the first measurable current flow. On the other hand, the faster relaxation of calcium current under the influ-

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ence of halothane is consistent with drug-induced blockade of open channels but not with certain other kinetic interpretations such as drug binding to inactivated channels only. Therefore, it is suggested that halothane not only facilitates the Ca²⁺ current inactivation but also affects the channel in its closed state. It is possible that halothane acts in a way so as to shift the dynamic balance between phosphorylation and dephosphorylation so that a lesser number of channels are available to conduct Ca²⁺ currents at the beginning of a test pulse. Halothane probably acts on the enzymes involved in the process of phosphorylation and enhances the dephosphorylation either by stimulating certain enzymes directly or through the calmodulin/Ca²⁺ complex. Channels are thus allowed to remain in the open state for a relatively shorter period of time to conduct divalent ions. Further investigations of the actions of volatile anaesthetics on individual channel subunits are required to clarify this issue and to determine the precise binding sites for halothane. HVA calcium channel currents may undergo inhibition by a number of mechanisms, including direct channel blockade, receptor-coupled G-protein-induced modulation, Ca²⁺-induced inactivation, or run down [90]. From the present data, little can be inferred about the location of halothane’s binding sites. It could possibly act by modifying membrane structure or fluidity [91]. It could either bind to the receptors on the external side of the membrane channel or, since it is lipid soluble, it could reach deeper structures of the channel protein, such as the inner inactivation gate, by hydrophobic pathways as described for certain uncharged local anaesthetics [90]. In this case one would however, expect that halothane should also act from the inside of the membrane, a view supported by Fanchaouy, et al [70]. This aspect could be explored further by internally perfusing the neurones with halothane and looking at its effects on Ca²⁺ currents.

**Halothane may have multiple targets:** When halothane was used in experiments on Ca²⁺ channel currents there was a steady decrease in Ca²⁺ channel current amplitude over a period of time that was most evident when 2 & 4% halothane were used. As discussed before halothane decreased the amplitude of the current as well as enhanced inactivation. On removal of halothane, the rate of inactivation returned to control levels immediately though the amplitude of the current did not recover fully. This supports the suggestion that halothane exerts its action at more than one target site that includes a direct effect on the channel itself and another effect on the phosphorylation/dephosphorylation mechanism possibly through either a rise in [Ca²⁺]i [58,59,60,72] or a direct effect on some enzyme (such as adenylate cyclase) or a component of a second messenger system. Recent studies on the actions of isoflurane on individual channel subunits have been reviewed by Rajagopal, et al. [92], who indicate that our understanding of the molecular actions of volatile anaesthetics on VGCCs will not be complete until their actions on the configuration of the protein folding of the subunits within the channels is determined.

**How does temperature affect clinically useful anaesthetics concentration range?** Volatile anaesthetics are quite toxic with a low therapeutic index, and it is essential to do experimental work in the clinically useful range. A number of earlier investigations carried out to study the mechanism of action of anaesthetics used unrealistic doses of anaesthetics and those results are unlikely to be applicable to clinical situations [93]. One criticism of experiments with volatile anaesthetics on isolated tissues carried out at room temperature is that the anaesthetic concentration is much higher than that at body temperature since halothane is more soluble at lower temperatures. However, when recording from hippocampal slices, Hagan., et al. [94] showed that there was no significant difference in synaptic depression when recording at either 22°C or 35°C in the presence of 1 MAC (minimum alveolar concentration) halothane (equivalent to 0.33 mM in their experiments). Similar concentrations of 0.39 mM [95] and 0.32 mM [96] have previously been reported. The MAC of an anaesthetic is defined as the concentration at one atmosphere that produces immobility in 50 % of patients or animals exposed to a noxious stimulus [97]. For humans, the MAC for halothane is 0.75% v/v and for *Lymnaea* it is 0.83% v/v [30]. Anaesthesia is usually achieved in the vast majority of patients (or animals) at alveolar concentrations equivalent to 1.3 MAC or above [98]. In experiments on dogs Gil-Rodrigues et al. [99] demonstrated a clear linear relationship between the inspired halothane concentration in the carrier gas (v/v) and arterial blood concentration. This was supported in experiments on L-type calcium currents in human atrial cardiomyocytes, where Hunek., *et al.* [100] clearly demonstrated a linear relationship between the volume% of anaesthetic gas applied to solution and its concentration (mM) for both halothane and sevoflurane. Hong., *et al.* [101] indicated that that 0.5 - 2.0% v/v corresponds to 0.6 - 2.2 mM halothane in plasma at 37°C and we [36] measured stable bath concentrations of 0.43mM for 1% halothane and 0.77 mM for 2% halothane, which are close to the values of halothane in human arterial blood of 0.275 to 0.69 mM for six of the seven patients studied by Davies., *et al.* [102] – the

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seventh patient became hypotensive during operation and the data was then excluded. In vitro experiments on rat hippocampal slices at 35°C used halothane concentrations of 0.4 to 0.9 mM (0.5-1.5% v/v) [96], very similar to those used in our experiments at 21°C.

Conclusions

This study was confined to investigation of the slowly inactivating high-threshold calcium current that was elicited by depolarizing voltage steps from -50 mV. The marked reduction of the voltage-dependent calcium current by halothane could be expected to have multiple direct and indirect effects on neuronal function. The selective reduction of calcium currents by halothane, applied within the accepted clinical range, may underlie its ability to inhibit neurotransmission. The concentration dependence of the inactivation of the HVA Ca²⁺ channel current in Lymnaea neurones is consistent with the hypothesis that anaesthetic action of halothane is partly mediated by a reduction in calcium currents.

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