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Research Article

Comparative Effects of Halothane and Isoflurane on K^+ and Ca^{2+} Currents in Isolated, Cultured Neurons of *Lymnaea stagnalis*

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Abstract

Halothane and isoflurane are halogenated volatile anaesthetics. Here we report on their actions on the gross potassium current and on the high-voltage activated L type-like calcium current of isolated, cultured right pedal I cluster neurons of the pond snail *Lymnaea stagnalis*. The effects of both anaesthetics are concentration-dependent. At higher clinical concentrations (2% v/v) both anaesthetics diminish these currents, but, unlike halothane, a low concentration of isoflurane (0.5% v/v) enhances the L-type calcium current.

Keywords: *Potassium Currents; Calcium Currents; Molluscan Neurons; Whole Cell Patch Clamp; Halothane; Isoflurane; Lymnaea stagnalis*

Introduction

Halothane (fluothane) was first introduced in 1956 [1] but has fallen out of favour in the USA due to its hepatotoxic actions, although these may also occur with other volatile agents [2,3]. However, Halothane continues to be widely used in developing countries as it is effective inexpensive and fast acting [4]. Isoflurane remains a commonly used inhalational anesthetic and has an excellent safety record, since its introduction in the USA in 1979 [5]. Both induction and recovery from isoflurane are rapid [6]. In our experiments on identified neurons *Lymnaea stagnalis*, which provides us with an excellent model system for studying the effects of anaesthetic agents, we have worked well within the clinical range for each of these compounds as discussed elsewhere [7,8].

We recently compared the actions of general and systemic anaesthetics on the activity patterns of identified motor neurons and interneurons in of *Lymnaea stagnalis* both *in situ* and in isolated cell culture [9] and demonstrated significant differences in responses between cell types. Here we compare the actions of the volatile anaesthetics halothane and isoflurane on isolated, cultured neurons from the asymmetrical right pedal I (RPeI) cluster neurons of *Lymnaea*. These cells are yellow, easily identifiable, have a diameter of 30-40 μM [10] and often project via the penis nerve of the right cerebral ganglion [11]. In the intact brain, they have an irregular firing pattern, type 2 action potentials with a pseudoplateau, are weakly electrically coupled with their ipsilateral neighbours and have common excitatory inputs with the pedal A cluster neurons [11,12]. In addition, analysis of earlier work indicates that they are capable of generating paroxysmal depolarising shifts (PDS) [11], implying that they may be motor neurons [13].

In previous studies we demonstrated the presence of up to four potassium currents [13] and a single high-voltage activated L-type calcium current [14] (similar to Ca^{2+} currents recorded in *Lymnaea* caudodorsal cells by Brussard., *et al.* [15]) in isolated, cultured RPeI cluster neurons. We have also described the dose-dependent reduction of the L-type Ca^{2+} current by the volatile, general anaesthetic halothane in the clinical concentration range [8]. Here we compare the actions of halothane and isoflurane on potassium currents and the calcium current of isolated, cultured RPeI cluster neurons. We also demonstrate for the first time a significant difference between the effects of low concentrations of halothane and isoflurane on the calcium current in these neurons.

Materials and Methods

Animals, cell culture techniques and methods for recording whole cell potassium currents were as described by Moghadam and Winlow [13] as were the techniques for data analysis. The recording protocol for Ca^{2+} current measurements was similar to that for K^+ currents, except that the command depolarization step time was reduced to 180 ms [8,14], rather than the 500 msec used for K^+ currents (see figure 2B).

Bath and Pipette solutions for whole cell patch clamp

a) **K^+ currents:** Bath solution contained NMDG 51.6, $CaCl_2$ 4, $MgCl_2$ 1.5, HEPES 10 and Glucose 10 mM. Pipettes had a resistance of 2 - 5 MΩ and contained KCl 50, NaCl 1.6, $MgCl_2$ 1.5; HEPES 10, Mannitol 10, EGTA 5, $CaCl_2$ 4.7, ATP 3 mM.

b) **Ca^{2+} currents:** The bath solution for Ca^{2+} current recording contained: TEACl, 40; 4AP, 10; $MgCl_2$, 1.5; Glucose, 10; HEPES, 10; $CaCl_2$, 4. The pipette solution contained (mM): CsCl, 40; EGTA, 5; $MgCl_2$, 5; HEPES, 10; ATP, 3. The osmolarity of both solutions was adjusted to 150 mosm by adding sucrose and pH was adjusted to 7.9 by CsOH. The pipette had a similar resistance to those used to study gross K^+ currents [5]. Since Ca^{2+} inactivation is dependent on Ca^{2+} entry rather than voltage [16] we chose Ca^{2+} as a charge carrier, for comparison with the actions of barium as previously used [8, 14].

Halothane and isoflurane were prepared using appropriate vaporizers and the gas mixture was dissolved in the bathing solutions, which were kept in air tight glass flasks until immediately before the experiment, and then continuously superfused over the cultured cells. Their concentrations in the preparation dish were in the clinical concentration range (for discussion see [8]).

Using the CED Patch and Voltage Clamp Software (V clamp, version 5.0) potassium currents were measured at the sustained current on each trace but calcium currents were measured at the peak current of each trace. The recorded data (n = 6 in each case) were averaged and were then leakage subtracted. Experimental values presented here were analysed by two way analysis of variance and a P value of $\leq$ 0.005 was considered to be a significant alteration of normal currents by the applied anaesthetic.

Results

K+ currents

2% halothane reduced gross K^+ currents by 50% immediately (Figure 1), but no significant change was recorded after continuous application of halothane solution for 5 minutes. After continuously washing the cell for 10 minutes with bath solution the gross K^+ current increased significantly but not to the pre-control level. To ensure that K^+ currents had recovered, 1 mM $CdCl_2$ was then applied and significantly reduced the K^+ current (P value $\leq$ 0.005), by blocking the Ca^{2+} dependent K^+ current (P value $\leq$ 0.005). The proportion of gross K^+ current blocked by halothane was similar to that blocked by $CdCl_2$. Thus, halothane depresses Ca^{2+} dependent K^+ currents as well as other K^+ channels. Application of 1 mM $CdCl_2$ decreased gross K^+ current by 18% with respect to the post-control wash, which was significant, and suggested that $CdCl_2$ had blocked calcium dependent potassium currents in this particular cluster neuron. A previous report [17] has shown that Ca^{2+} activated K^+ currents, which are present in snail neurons, are superimposed on the delayed outward current. Half of this calcium dependent K^+ current was blocked by halothane, but half of the Ca^{2+} dependent K^+ current was unaffected by halothane. The effects of halothane on K^+ currents were not reversible after 10 minutes continuous wash out, requiring additional washing for complete reversal.

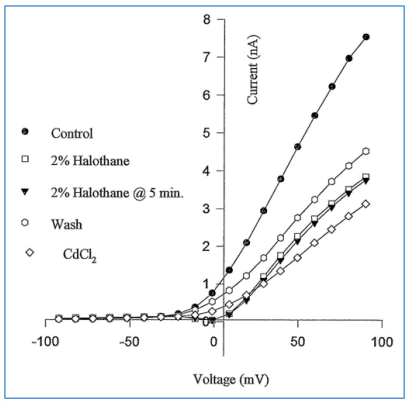

Figure 1: *Effects of 2% halothane on K^+ currents of pedal I cluster neurons of Lymnaea (n = 6). Control data were obtained in standard bath solution. 2% halothane data were obtained within 30s of adding halothane saline. The 5 minute data were recorded 5 min later during continuous 2% halothane perfusion. The preparation was then washed in standard bath solution (wash) and finally 1 mM CdCl2 was applied in bath solution to block calcium dependent K^+ currents. Sequence of application of solutions: 1) Pre-control in normal bath solution; 2) 2% halothane; 3) 2% halothane after 5 min; 4) Wash in normal bath solution; 5) 1 mM CdCl2. The amplitude and duration of the command pulses are as in figure 2.*

Similar effects were obtained with 2% isoflurane (Figure 2 and see [18]). Long term (5 min) application of halothane had little further effect (P value ≤ 0.005). After 5-10 min wash out the K^+ currents partially recovered, which is statistically significant if compared with the effect of halothane, after 5 minutes. Similar results were obtained by Tas., *et al.* [19] on Ca^{2+} dependent K^+ channels of rat glioma C6 cells when they were exposed to halothane. Since there are at least three different Ca^{2+} dependent K^+ currents [20], further work will be required to identify which of them are sensitive to halothane.

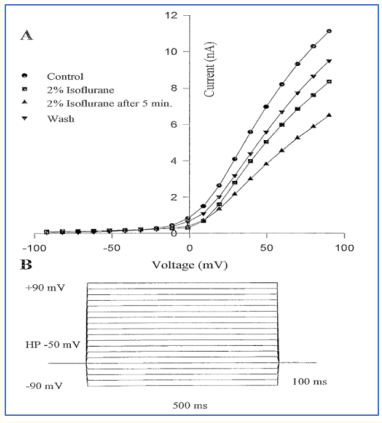

Figure 2: *Effects of 2% isoflurane on the K+ currents of pedal I cluster neurones of Lymnaea stagnalis (n = 6). A) Sequence of application of solutions: 1) Pre-control in normal bath solution; 2) 2% Isoflurane; 3) 2% Isoflurane after 5 min; 4) Post-control. Each of these currents is significantly different from the others. Recovery is quite good after continuous wash out for 10 min. This figure was reproduced from Winlow., et al. [18] under Creative Commons Attribution License (CC BY). B) The stimulus protocol showing the amplitude and duration of the command pulse.*

Isoflurane decreased K^+ currents significantly (P value ≤ 0.005) at +90 mV depolarization by 24% immediately after application, and this current decreased by a further 17% after 5 minutes of continuous application of 2% isoflurane, a total reduction of gross current by 41%. Comparison of the effects of halothane and isoflurane shows that halothane at 2% concentration is more effective than isoflurane at the same concentration on gross K^+ currents, reducing the gross current by 50%.

Calcium currents

Low concentrations of isoflurane (0.5%) significantly increase (P value ≤ 0.005) Ca^{2+} currents (Figure 3A), by 40% at +10 mV peak current, immediately after application. No further alteration in current was recorded after continuous application of 0.5% of isoflurane for 5 minutes. With continuous wash out of isoflurane not only did the Ca^{2+} currents not return to the normal level but were significantly enhanced after wash II.

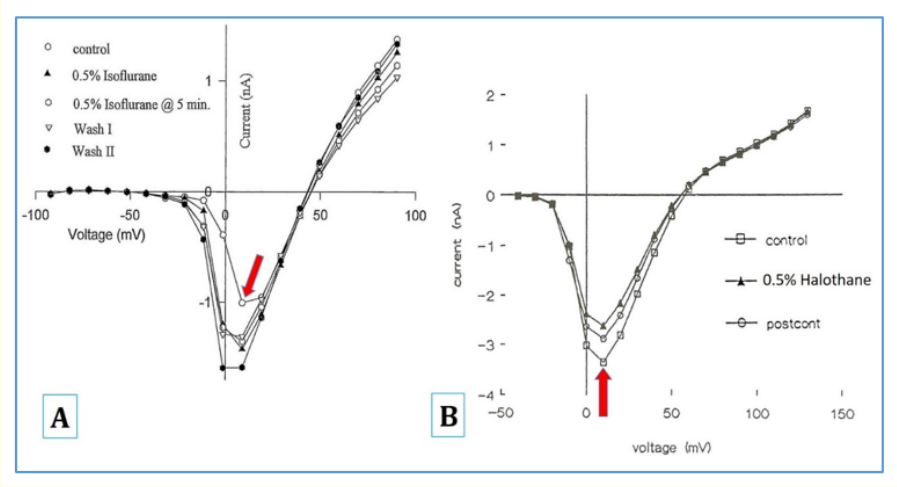

Figure 3: *Effects of 0.5% isoflurane (n=6) (A) and 0.5% halothane (n=6) (B) on peak Ca^{2+} currents of isolated pedal I cluster neurons. A) Isoflurane significantly increases calcium currents at -10 to +20 mV. Sequence of application of solutions: 1) Pre-control in normal bath solution; 2) 0.5% Isoflurane; 3) 0.5% Isoflurane after 5 min; 4) post control I (wash I); 5) post control II (wash II). At low concentrations of isoflurane and even after washout, Ca^{2+} currents are activated as compared to control (red arrow) which is opposite to the effects of 0.5% halothane (see B) or 2% isoflurane (see Figure 5 for comparison). B) For comparison this data was reproduced from Yar and Winlow [8] under Creative Commons Attribution License (CC BY). In this case the charge carrier was barium and the neuron was voltage-clamped for 180 msec to voltages between -30 to +30 mV from a holding potential of -50 mV and there was a clear decrease from the control current (red arrow) in the presence of halothane. It should be noted that peak sustained current is known to increase ca. 3-fold in the presence of barium as the charge carrier [8,21], which explains the difference in the amplitude of the currents recorded here.*

Application of high concentrations (2%) of isoflurane decreases Ca^{2+} currents (P value ≤ 0.005) (Figure 4 and 5), as has previously been demonstrated by Yar and Winlow [8]. Calcium currents decreased immediately by 30% at the peak current and decreased by 41% after 5 minutes of continuous application of isoflurane. With continuous wash out of isoflurane significant differences were found between the isoflurane effects and wash out, but significant differences found between control and wash-out data. These data suggest that the effects of isoflurane are partially reversible, but full recovery does not take place in the short term.

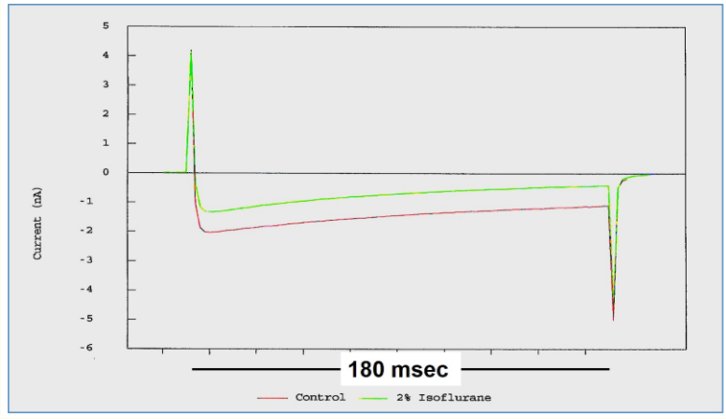

Figure 4: *Data record - Actions of 2% isoflurane on the Ca^{2+} current in a single isolated neuron. Membrane potential was clamped at -50 mV and a test potential of +60 mV was applied. Membrane potential reached to +10 mV. The duration of the command pulse was 180 msec. Calcium currents were recorded in normal bath solution (lower trace; red line) and in the presence of 2% isoflurane (upper trace). Thus, the calcium current was reduced by 35% at +10 mV membrane potential.*

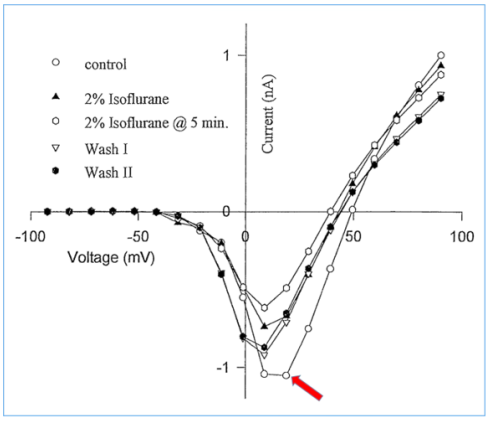

Figure 5: *2% Isoflurane decreases Ca^{2+} current in cultured pedal I neurons (n = 6). A control Ca^{2+} current (red arrow) was elicited from a holding potential of -50 mV by 180 ms depolarization at 10 mV steps to the indicated test potentials (mV) with sample rate of 0.5 KHz delivered every 1 second. Ca2+ currents were recorded in the presence of extracellular 2% isoflurane both immediately and after 5 minutes of anaesthetic application. The preparation was than washed for 5 - 10 minutes with 20 - 30 ml normal bath solution.*

Yar and Winlow, [8,22] have already shown halothane generates a dose-dependent decrease of calcium current with increasing concentrations (0.5 to 4.0%). However, this is not the case with isoflurane, because 0.5% isoflurane was found to enhance the calcium current (Figure 3A) and is compared with the effects of 0.5% halothane from Yar and Winlow [8], where the charge carrier was barium, which results in a larger currents [14,21]. However, increasing concentrations of isoflurane above 0.5% produced a dose dependent decrease in calcium current as shown for 2% isoflurane in figures 4 and 5.

Discussion

It is may be significant that we have demonstrated that 2% halothane is more effective at blocking gross potassium and calcium currents than is 2% isoflurane, since this has also been shown to be the case in behavioural experiments on the *Lymnaea* whole animal withdrawal reflex [7]. 2% isoflurane is also less effective at blocking the peak L-type calcium current in these neurons than is 2% halothane. Here we found that 2% isoflurane significantly depressed peak inward calcium currents (P value ≤ 0.005) initially by 29% and then 43% after 5 minutes (n = 6) (see Figure 5). In comparison our previous data using 2% halothane [8], showed that the peak calcium current was reduced by 52% (n = 6).

Krnjevic and Puil [23] originally suggested that voltage sensitive Ca^{2+} channels were a possible site of action of anaesthetics and we have recently demonstrated that halothane decreases L-type Ca^{2+} currents in the RPeI cluster neurons of *Lymnaea* [8] in a dose dependent manner using barium as the charge carrier. From the results presented here, it is clear that the actions of isoflurane are also dose dependent, but at low concentrations (0.5%) it increases calcium currents unlike halothane (Figure 3). At higher concentrations isoflurane acts in a similar way to halothane on HVA calcium currents. This finding apparently contradicts our previous assertion that the actions isoflurane were not concentration dependent [9], but these data were obtained from recording activity patterns of neurons in in the intact brain where many synaptic influences are present, rather than from the isolated cells where such influences as absent. Interestingly, subhypnotic, low doses of isoflurane may be associated with stimulation of the central nervous system [24] perhaps by potentiating the action of the postsynaptic receptors as has been demonstrated for peptides where halothane enhanced the actions of met-enkephalin on isolated cultured pedal A cluster neurons from *Lymnaea* [25-27].

In 1937 Guedel [28] first described the excitation phase of anesthesia, now described as Stage 2 anesthesia [29], in which unconscious, uncoordinated movements often occur. These movements are masked in current clinical practice by more rapid induction of anesthesia, the use of neuromuscular blocking agents (NMBAs) and other anaesthetic adjuvants. This phase of uncoordinated behaviour is observed in *Lymnaea* during induction of anesthesia with volatile anesthetics [7]. Furthermore, concentration dependent effects of halothane (0.25 - 4.0%) have been demonstrated in the isolated brain of *Lymnaea* [9]. In interneurons and giant motor neurons the common responses to low doses of halothane (0.5 - 1.0%) can be generalized as an initial increase in patterned discharge, which we have previously suggested to be the cellular equivalent of Guedel's early excitatory phase of anaesthesia followed by a decline in activity [9].

Previous experiments on the whole animal withdrawal response [7] indicated that low doses of halothane generated several uncoordinated behavioural responses which included mouthing and biting movements. Detailed studies on the feeding system of *Lymnaea* [29] had already demonstrated that these movements were accompanied by increased spiking and patterned activity in the buccal motor neurons and the cerebral giant cells (CGCs), which are interneurons. This activity declined over time and many of the cells exhibited paroxysmal depolarizing shifts (PDS) *in situ*. More recently, we demonstrated that application of increasing concentrations of halothane (1 - 4%) first enhances depolarizing responses to applied serotonin in isolated, cultured neurons from the *Lymnaea* feeding system [30]. Following this effect there is dose-dependent decline of these responses. In neurons exhibiting hyperpolarizing responses to 5HT, both the amplitude and duration of these actions were first decreased at low anaesthetic concentrations, but then increased in a dose dependent manner [30]. Previous studies on the effects of halothane on the feeding system of *Lymnaea* [30], demonstrated that its application initially caused an increase in biting and mouthing movements accompanied by increased spiking and patterned activity which declined over time in the buccal motor neurons and in the cerebral giant cells (CGCs), which are interneurons. Many of the cells exhibited paroxysmal depolarizing shifts *in situ* and also do so in isolated cell culture [31].

Interestingly, a key finding of Girdlestone., *et al.* [32] was that the calcium dependent components of the CGC action potentials were more sensitive to halothane than its other ionic components. Our findings here have demonstrated that 0.5% isoflurane increases the L-type calcium current while 0.5% halothane decreases it. Furthermore, PDS has not previously been observed in *Lymnaea* neurones in the presence of isoflurane [9], but halothane can trigger it in zero calcium saline [33] even though rising levels of intracellular calcium

may be sufficient to trigger it [18,34,35]. PDS is clearly a complex phenomenon and more detailed studies on its generation are required with respect to different volatile anesthetic agents.

Conclusions

The effects of clinical concentrations of the volatile anesthetics halothane and isoflurane on isolated cultured RPeI neurons are as follows:

- Low concentrations (0.5%) of isoflurane enhance the HVA Ca^{2+} current, unlike 0.5% halothane which depresses it.
- Both 2% halothane and 2% isoflurane decreased the HVA Ca^{2+} significantly.
- 2% (v/v) halothane is more effective than 2% (v/v) isoflurane in depressing the gross K^+ and the gross Ca^{2+} currents of pedal I cluster neurons.
- Halothane partially depresses Ca^{2+} dependent K^+ currents.

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