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EC NEUROLOGY
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Mobilization of Intracellular Calcium by the General Anaesthetic Halothane in Cultured, Identified Molluscan Neurons

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Received: July 10, 2020; **Published:** July 22, 2020

Abstract

- The effects of clinical concentrations of halothane (2% and 4% halothane v/v) on the intracellular calcium concentration, $[Ca^{2+}]_i$, in four types of identified *Lymnaea* neurons in single cell culture were observed both in the presence and absence of extracellular calcium (zero Ca^{2+}/EGTA).
- Intracellular Ca^{2+} levels were measured with the cell-permeable ratiometric Ca^{2+} indicator Fura-2-acetoxymethyl ester (Fura-2 AM).
- In the presence of external calcium there was a rapid increase in $[Ca^{2+}]_i$ at both concentrations of halothane, which appeared to be both time and concentration dependent in all cell types. These effects were clearest in the neurons VV1 or 2 and RPD1.
- Similar effects were observed in zero external calcium, but the clearest concentration dependency was in RPeD1, whose $[Ca^{2+}]_i$ remained above control levels at washout, unlike those of the other neurons.
- These data indicate that halothane causes release of calcium from internal stores, even in the absence of extracellular calcium.
- We compare these findings with those from previous studies with caffeine and ryanodine, neither of which increased intracellular free calcium concentration in the absence of external calcium.

Keywords: *Intracellular Calcium; Molluscan Neurons; Lymnaea Neurons*

Introduction

Studies on *Lymnaea* using halothane and the unconventional anaesthetic menthol have demonstrated that general anaesthetics significantly reduce the duration of the calcium dependent action potential plateau and the amplitude of the after-hyperpolarization (AHP) [1,2]. These effects are fully reversible by washing the preparation in normal saline [1,2]. Furthermore, anaesthetics block chemical transmission [3,4] and modulate electrical synapses [5, 6]. These studies indicate that the actions of anaesthetics are at least partially due to inter-

ference of the neuronal ability to utilize calcium, probably by blocking Ca^{2+} channels. This view was further supported by Yar and Winlow [7], who demonstrated that high-voltage-activated calcium currents are depressed in a dose-dependent manner in cultured *Lymnaea* neurons by halothane. Similar results have also been reported in recombinant cardiac L-type channels expressed in human embryonic kidney cells (HEK293) [8], cultured neocortical astrocytes [9], cortical area 1 (CA1) neurons in hippocampal slices [10] clonal pituitary cells [11,12], isolated chromaffin cells [13-15], rat pheochromocytoma (PC12) cells [16] Xenopus oocytes [17], human neuronal cell lines [18], guinea pig myocytes [19], rabbit myocardial cells [20] and dogs [21,22]. Furthermore, both volatile and systemic anaesthetics diminish low-voltage activated Ca^{2+} currents of rat dorsal root sensory neurons [23]. In a preliminary report we also demonstrated that halothane raises intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured giant *Lymnaea* neurons [24]. In addition, we recently reported that clinical concentrations of the systemic anaesthetic, pentobarbital, also significantly increased $[Ca^{2+}]_i$ in cultured neurons of the *Lymnaea* (RPeI) right pedal I cluster [25,26], but that these effects are cell specific and some other identified neurons are unaffected.

Although volatile anaesthetics are known to act as intracellular Ca^{2+} mobilizing agents they are thought to act by different mechanisms from those activated by caffeine and ryanodine. We previously showed that low concentrations of caffeine [27] and ryanodine [28] raised $[Ca^{2+}]_i$ in four types of cultured molluscan neurons, but were dependent on the presence of extracellular calcium. Here, we examine the actions of the general anaesthetic halothane on $[Ca^{2+}]_i$ in the same cultured, identified neuron types from *Lymnaea* as in our previous studies: the giant dopamine containing neuron right pedal dorsal 1 (RPeD1), the identical cells visceral ventral 1 and 2 (VV1/2), right parietal dorsal neurons 1 and 2 (RPD1 and RPD2) whose locations and properties are reviewed elsewhere [27-29] as are their responses to applied volatile and systemic anaesthetics [25,30]. All these neurons have type 2 action potentials with a marked calcium pseudoplateau on the falling phase [31].

Materials and Methods

Specimens of *Lymnaea stagnalis* (L) were supplied by Blades Biological, Kent, kept in a controlled temperature room at 14 - 16°C in aerated tap water and fed on lettuce. In the present study, four types of identified *Lymnaea* neurons were studied and these were: Right Pedal Dorsal 1 (RPeD1); Right Parietal Dorsal 1 and 2 (RPD1 and RPD2); and Visceral Ventral 1 or 2 (VV1or2, which are physiologically indistinguishable from one another) [31]. These neurons were isolated and cultured as previously described [27,32].

Individual neurons were loaded with the cell-permeable ratiometric Ca^{2+} indicator Fura-2 AM and intracellular calcium concentration was measured as described by Ahmed., *et al* [27,28]. Control levels of $[Ca^{2+}]_i$ were recorded for individual neurons after 2 - 3 hours of incubation in Fura2/AM in standard snail saline [31]. Experiments were carried out either in standard snail saline or in calcium free saline (0 Ca^{2+}/5 mM EGTA from Sigma).

Halothane

2% and 4% solutions of halothane were prepared and delivered as previously described [7]. The neurons were observed in either standard saline or zero calcium saline until the resting $[Ca^{2+}]_i$ was achieved, about 20 - 30 min. A reading was taken at that point and the experiment started 5 min later, using 2% halothane. $[Ca^{2+}]_i$ was measured at 1, 3- and 5-min intervals in each neuron after which the preparation was washed in standard saline or zero calcium saline, as appropriate, and readings were taken at 5- and 10-min intervals before addition of 4% halothane solution. Measurements of $[Ca^{2+}]_i$ were again taken at 1, 3, and 5 min intervals in 4% halothane after which there was a further wash in standard saline with readings taken at 5, 10- and 15-min intervals before washout, again in the appropriate saline.

Data analysis

Raw $[Ca^{2+}]_i$ data were presented as mean (± S.E.M.) and arranged in a tabular form. The data were then normalised and expressed as a mean percentage (± S.E.M.) change in $[Ca^{2+}]_i$ and plotted against time in a series of graphs using "Fig. P" software (Figure P Software corporation, version 6.0a 91/11). The use of percentage change in $[Ca^{2+}]_i$ allowed us to compare resting $[Ca^{2+}]_i$ levels, despite variations in $[Ca^{2+}]_i$ within the same cell type from one animal to the next.

Results

Effects of halothane on $[Ca^{2+}]_i$

The effects of both 2% and 4% halothane on $[Ca^{2+}]_i$ occurred within about 5 - 10 minutes of application and are summarised in table 1 and figure 1.

	Time (min)	$[Ca^{2+}]_i$ in (nM) (Mean ± S.E.M.)			
		VV1/2	RPD1	RPeD1	RPD2
0%	Control	98 ± 29	136 ± 67	67 ± 21	142 ± 44
2%	1	125 ± 36	172 ± 84	84 ± 25	186 ± 57
	3	148 ± 42	187 ± 91	128 ± 41	227 ± 68
	5	174 ± 51	228 ± 110	166 ± 55	294 ± 89
1st Wash	5	143 ± 44	184 ± 89	97 ± 27	220 ± 69
	10	132 ± 41	173 ± 85	104 ± 35	203 ± 62
4%	1	158 ± 46	274 ± 127	118 ± 36	230 ± 71
	3	177 ± 48	335 ± 166	147 ± 39	301 ± 95
	5	222 ± 61	349 ± 172	174 ± 44	344 ± 106
2nd Wash	5	190 ± 56	291 ± 144	109 ± 31	265 ± 78
	10	163 ± 48	265 ± 127	90 ± 26	225 ± 67
	15	156 ± 46	242 ± 127	94 ± 29	235 ± 72
Total	n	7	6	4	6

Table 1: *Mean values of the concentration of intracellular calcium $[Ca^{2+}]_i$ in cultured, identified neurons both before (control) and after application of 2 and 4% halothane in the presence of extracellular calcium. The washes were carried out in standard snail saline. The number of experiments is given by n. There is some variability in the control level of $[Ca^{2+}]_i$ between the types of neuron as well as within the same type.*

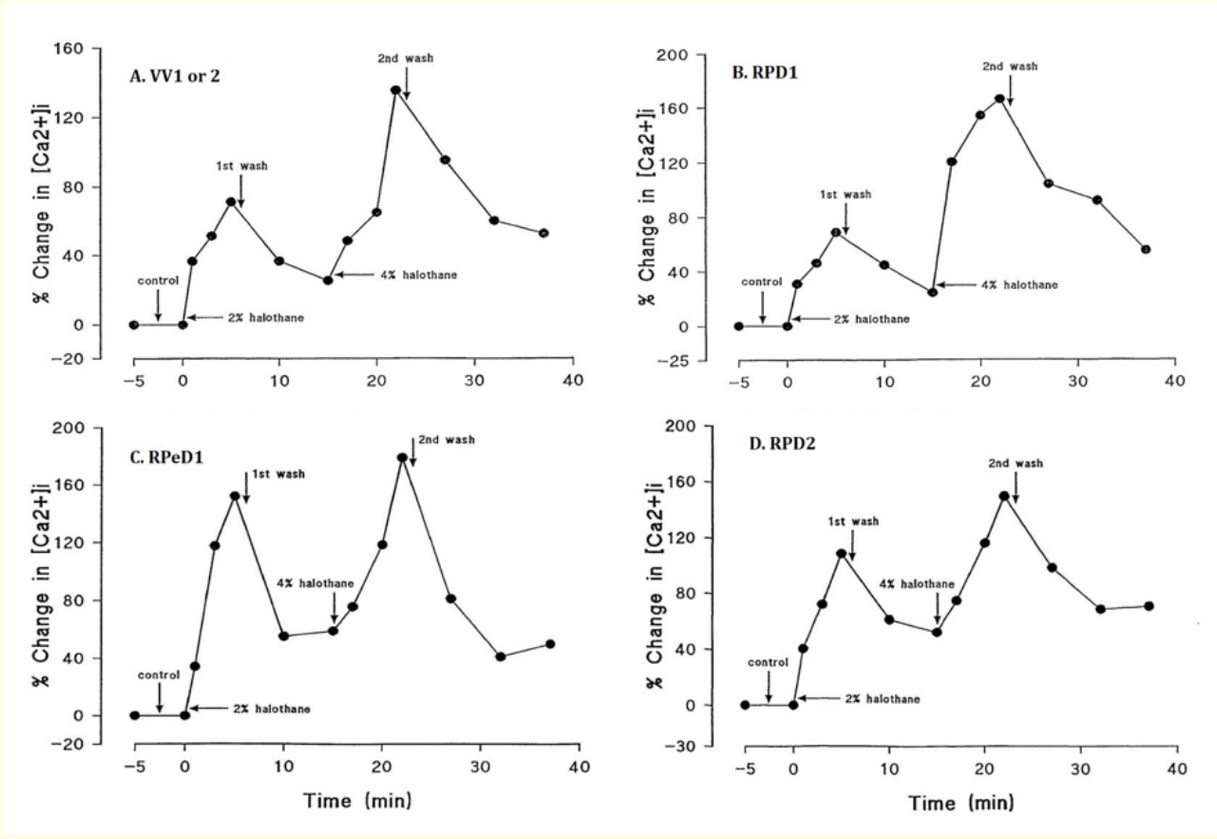

Figure 1: *Time course of action of halothane on % change from baseline control in $[Ca^{2+}]_i$ in individual neurons. Both VV1or2 (A) and RPD1 (B) exhibited concentration dependent responses to application of 2% and 4% halothane, but this effect was less clear in RPeD1 (C) and RPD2 (D). The mean percentage changes in $[Ca^{2+}]_i$ are tabulated below. The number of experiments is given by n.*

	2%	1st Wash	4%	2nd Wash
VV1/2, n = 7	73.85 ± 3.13	32.74 ± 2.10	135.30 ± 4.37	52.4 7± 2.63
RPD1, n = 6	69.96 ± 3.69	25.86 ± 2.29	157.73 ± 6.66	66.52 ± 5.06
RPeD1, n = 4	143.08 ± 14.3	52.52 ± 5.79	172.86 ± 14.77	40.25 ± 3.31
RPD2, n = 6	113.05 ± 6.10	44.30 ± 3.80	144.69 ± 4.42	67.81 ± 1.78

Table A

In both VV1/2 and RPD1 $[Ca^{2+}]_i$ appeared to increase in a time and concentration dependent manner. However, there was no clear concentration dependency in the increase in RPeD1 and RPD2. The actions of halothane on RPD2 are also illustrated in the ratioflurometric images in figure 2. These images visualise the temporal changes in $[Ca^{2+}]_i$ after application of both concentrations of halothane and show the spatial distribution of the free Ca^{2+} ions close to the cell membrane. In all four cell types there was a partial reversal of the changes in $[Ca^{2+}]_i$ following washout, but its level remained elevated above control values in all cases.

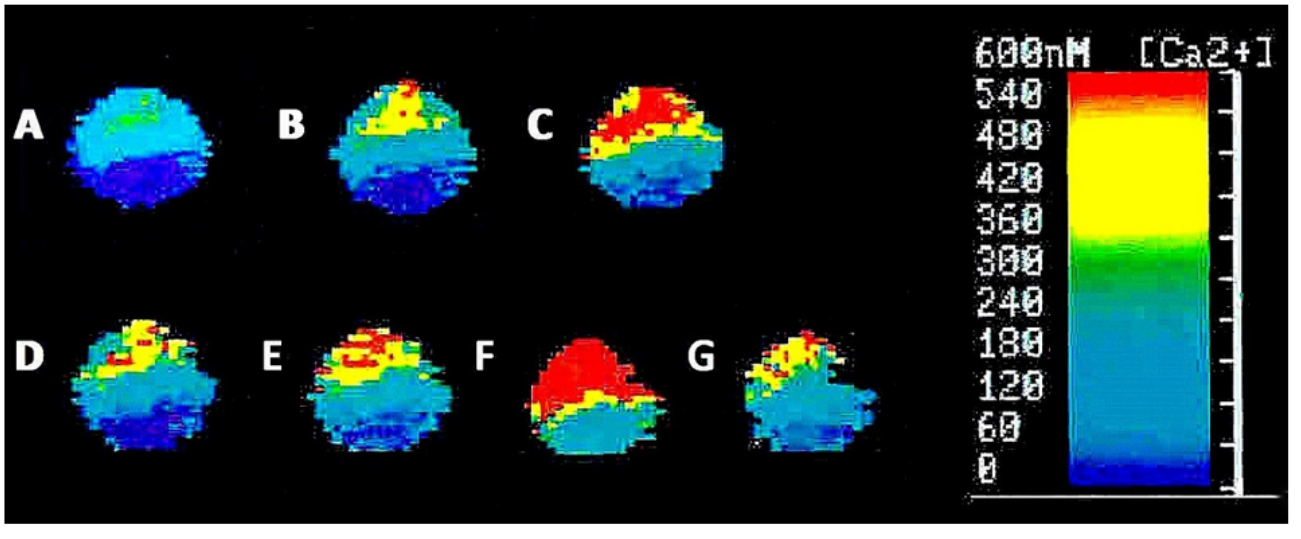

Figure 2: *Effect of halothane on the $[Ca^{2+}]_i$ in a RPD2 neuron. These ratioflurometric images illustrate the temporal effects of 2 and 4% of halothane on the free $[Ca^{2+}]_i$ in a RPD2 neuron. The calcium concentration scale bar is at right. A) Control before addition of halothane (101 nM Ca^{2+}); B) 1 minute after addition of 2% halothane (174 nM); 5 minutes after 2% halothane (287 nM); D) 10 min after 2% halothane (184 nM); E) 1 minute after 4% halothane 233 nM); F) 5 minutes after 4% halothane (414 nM); G) 15 min after washout of 4% halothane (212 nm). Notice the apparent localization of the internal Ca^{2+} release at one peripheral region in this particular cell.*

Effects of halothane on $[Ca^{2+}]_i$ in the absence of extracellular calcium (0 Ca^{2+}/EGTA)

In experiments with caffeine [27] we showed that there was no increase in $[Ca^{2+}]_i$ in the absence of extracellular calcium so we repeated this approach with 2% and 4% halothane, whose actions are summarised in table 2 and in figure 3 and 4. Unlike the situation with caffeine and ryanodine, we recorded an increase in $[Ca^{2+}]_i$ in all four cell types, but in all but RPeD1 there was no suggestion of concentration dependency. Furthermore, the time scale was similar to that in the presence of extracellular calcium and after washout all but RPeD1 showed a decline of $[Ca^{2+}]_i$ to below control values. In RPD2, the elevated $[Ca^{2+}]_i$ was markedly reduced both after the first and second

Halothane conc.	Time (min)	$[Ca^{2+}]_i$ in (nM) (Mean ± S.E.M.)			
		VV1/2	RPD1	RPeD1	RPD2
0%	Control	556 ± 53	41 - 57	68 ± 60	12 - 48
2%	1	612 ± 53	46 - 67	80 ± 4.	15 - 56
	3	657 ± 44	50 - 73	86. ± 4	17 - 63
	5	711 ± 49	54 - 80	92 ± 5	21 - 79
1st Wash	5	578 ± 47	69 - 47	67 ± 2	10 - 36
	10	494 ± 32	39- 55	64 ± 5	9 - 32
4%	1	526 ± 40	46 - 62	86 ± 5	12 - 43
	3	656 ± 50	51 - 74	114 ± 10	20 - 74
	5	751 ± 51	59 - 86	143 ± 14	25 - 95
2nd Wash	5	532 ± 40	51 - 69	80 ± 4	10 – 36
	10	497 ± 40	43 - 59	77 ± 5	8 - 30
	15	480 ± 40	39 - 54	83 ± 5	8 - 28
Total	n	3	2	3	2

Table 2: *Mean values of $[Ca^{2+}]_i$ in different neuron types both before (control) and after application of 2 and 4% halothane in the absence of extracellular calcium (0-Ca^{2+}/ EGTA). The data are given as mean (± S.E.M.) in VV1/2 and RPeD1 and range in RPD1 and RPD2. The washes were carried out using zero Ca^{2+}/EGTA saline. The number of experiments is given by n. VV1/2 shows the highest control level of $[Ca^{2+}]_i$ (556 ± 53 nM) and RPD2 is the lowest (range 12- 48 nM).*

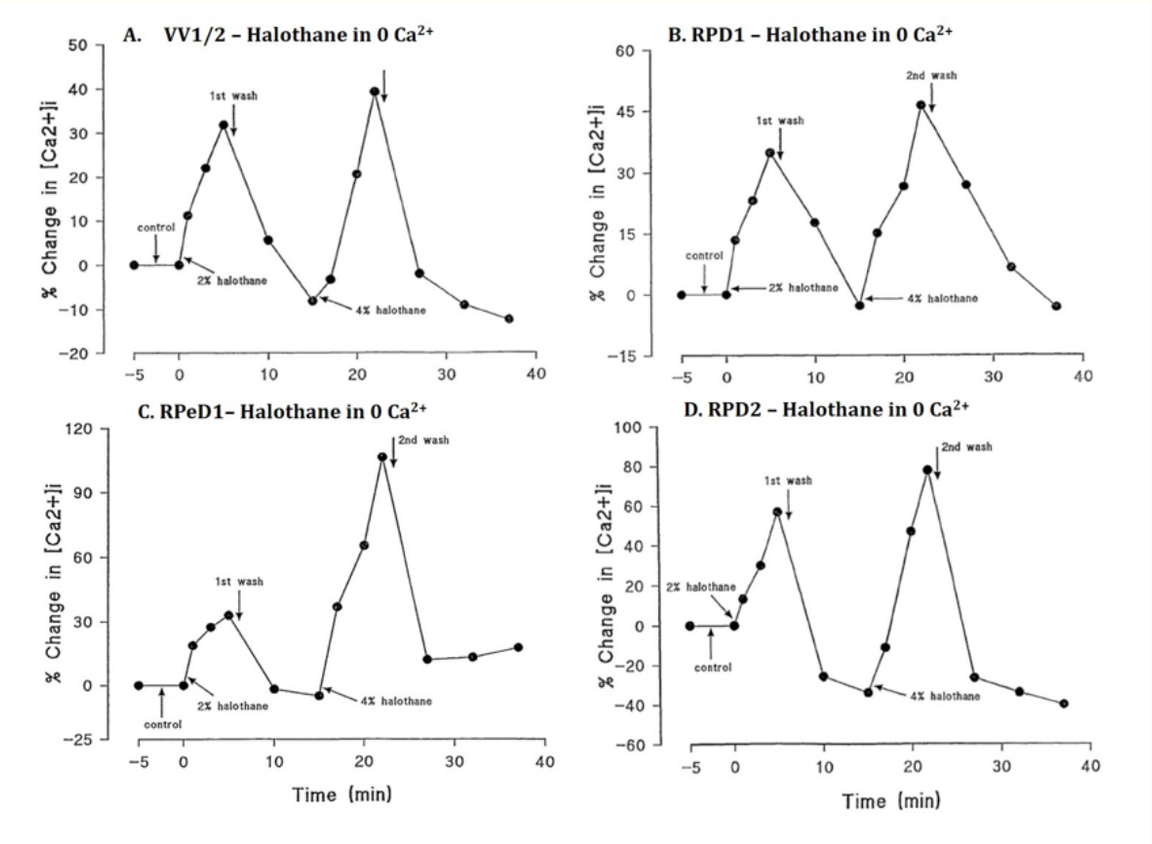

Figure 3: *Effects of halothane on % change in [Ca2+]i in four types of identified neuron in the absence of extracellular Ca^{2+} (0-Ca^{2+}/EGTA). The actions of 2% and 4% halothane on individual neurons are presented as follows: A) VV1 or 2; B) RPD1; C) RPeD1; D) RPD2. In A, B, and D there was only a small increase in $[Ca^{2+}]_i$ following the addition of 4% halothane, but in the case of RPeD1 there was apparent concentration dependency, as seen in the mean percentage changes tabulated below. The number of experiments is given by n. The data are expressed as mean ± S.E.M. in VV1/2 and RPeD1 and mean range in RPD1 and RPD2.*

	2%	1st Wash	4%	2nd Wash
VV1/2, n = 3	28.35 ± 3.44	-10.82 ± 2.61	35.61 ± 3.58	-13.50 ± 0.9
RPD1, n = 2	39.74 - 34.64	-4.63 to -2.87	50.00 - 46.09	-5.68 to -3.37
RPeD1, n = 3	36.40 ± 4.37	-5.53 ± 1.35	109.16 ± 3.99	21.62 ± 3.60
RPD2, n = 2	66.40 - 56.70	-26.83 to -34.02	86.99 - 77.32	-31.95 to -40.21

Table B

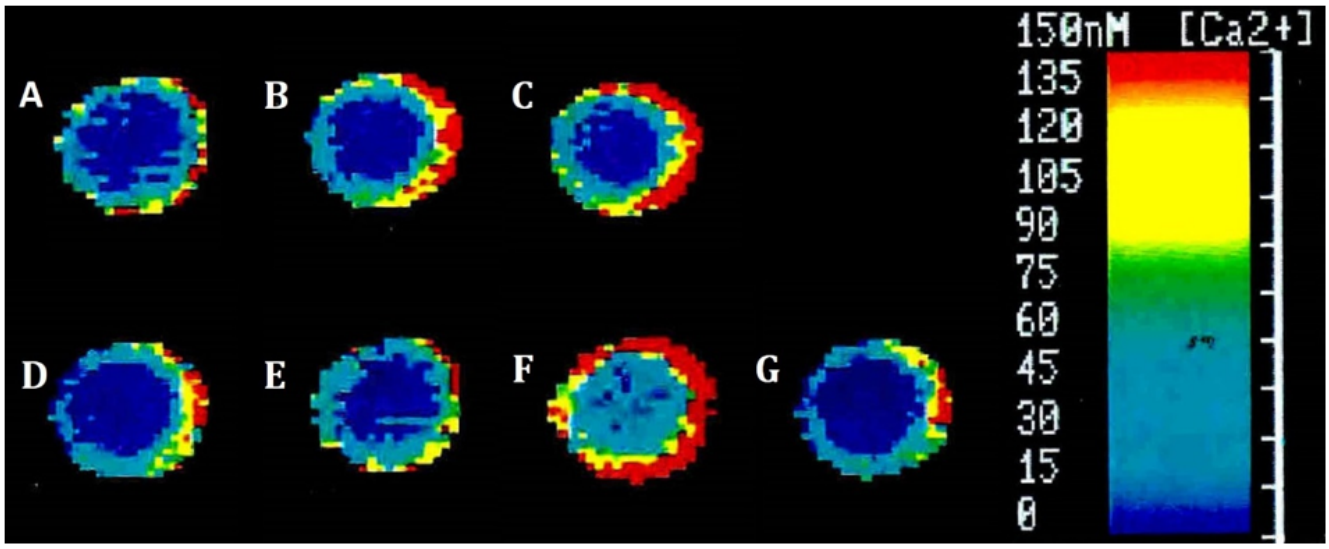

Figure 4: *Effect of halothane on the level of $[Ca^{2+}]_i$ in RPD2 in the absence of extracellular Ca^{2+}. These ratioflurometric images illustrate the time course of action of halothane on $[Ca^{2+}]_i$ in RPD2 in the absence of extracellular Ca^{2+} (0-Ca^{2+}/EGTA). Notice the progressive increase of $[Ca^{2+}]_i$ starting from the cell periphery towards the centre. The calcium concentration scale bar is at right. A) The $[Ca^{2+}]_i$ was increased from a resting level of 36 nM to B) 48 nM (+ 32.3%) 1 min after addition of 2% halothane followed by C) a further rise to 58 nM (61.3%) after 5 min perfusion with 2% halothane. D) The $[Ca^{2+}]_i$ then fell to below resting level (31 nM) after 10 min wash. E) Perfusion with 4% halothane raised the $[Ca^{2+}]_i$ by only 3.2% (38 nM) after 1 min and then F) to a peak of 127.4% (83 nM) within 5 min. G) The $[Ca^{2+}]_i$ was drastically reduced (29 nM) after 15 min wash, well below control. Note that all $[Ca^{2+}]_i$ are substantially reduced compared with figure 2.*

wash to below the resting level (Figure 3), as further demonstrated in the ratioflurometric images shown in figure 4. Again, high levels of free cytosolic calcium are shown in close proximity to the extracellular membrane.

Discussion

The present results show that halothane (2 and 4%) produced a substantial increase in $[Ca^{2+}]_i$ in all four studied neuron types (n = 23). This effect of halothane on $[Ca^{2+}]_i$ was found to be time-dependent in all neurons and apparently dose-dependent in only 2 out of 4 neuron types (VV1/2 and RPD1), and it was partially reversible after washing with saline. This may be due to the fact that, in these experi-

ments, the low dose of halothane was always added before the high dose and the wash in between did not always return the $[Ca^{2+}]_i$ level to baseline. Similar results were also obtained when halothane was tested on these neurons (in 10 out of 12 cells; 83.3%) in the absence of external Ca^{2+}, whereas, in the two remaining neurons (RPD1 and RPD2) there was no appreciable response.

The initial increase in $[Ca^{2+}]_i$ induced by halothane was very rapid (within tens of seconds) and this may be due to the lipid-soluble nature of the volatile anaesthetics in general which enables them to reach their targets very quickly both at the cell membrane and at intracellular sites. Following washout of 2% halothane, the elevated response to the higher concentration took about 10 minutes to develop and appeared to be concentration dependent in VV1 or 2 and RPD1 (Figure 4A and 4B) but less obviously so in RPeD1 and RPD2 (Figure 4C and 4D). In zero extracellular calcium there were reduced absolute responses to halothane, but all neurons responded to both doses in about the same time as in normal saline. Furthermore, there was a much more marked concentration-dependent response in RPeD1 than in any of the other neurons (Figure 3C). From previous data [28], it is clear that, RPeD1 is less responsive to ryanodine than other neurons, but responsive to halothane both in the presence and particularly in the absence of calcium in extracellular saline.

The influx of Ca^{2+} into the cytoplasm can occur through the plasma membrane as well as by release of Ca^{2+} from internal stores. The primary mechanisms that facilitate Ca^{2+} influx through the plasma membrane are the "slow" voltage-sensitive Ca^{2+} channels and the Na^+-Ca^{2+} antiporter when the physiological Na^+ gradient is reversed [33]. But in fact this reported probability of Ca^{2+} channels blockage or depression of Ca^{2+} currents by halothane [7,10,34] might well be a reflection of halothane-induced elevation in $[Ca^{2+}]_i$ which mediates calcium-dependent inactivation of Ca^{2+} channels [10,35]. These studies may also imply that Ca^{2+} channels are more sensitive to anaesthetic agents than any other membrane channels. Therefore, the degree to which Ca^{2+} channels are selectively affected by general anaesthetics obviously requires a more detailed study. However, we can reasonably assume that the reduced responses to halothane in zero Ca^{2+} and the return to baseline on washout indicate a significant component of $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+}. In external zero calcium in the presence of halothane there will be blockade of L-type calcium channels and the unavailability of external Ca^{2+} during washout to activate calcium induced calcium release from the endoplasmic reticulum [36].

Cell calcium pumps maintain a large inward calcium gradient into cells and in most cytosols the level of free calcium oscillates between 0.1 and 0.2 μM according to Carafoli [37], but occurs in millimolar concentrations in extracellular fluid of vertebrates and also in *Lymnaea stagnalis* [38], our chosen model preparation. Similar high concentrations of calcium are also found within the endoplasmic reticulum of various cell types [39] and can be made available to the cytosol by a number of calcium mobilizing agents including caffeine [27,40], ryanodine [28,41] and volatile anesthetics [25,42-45]. Thus, the low cytosolic calcium concentration may be modulated by calcium entry across the extracellular membrane or from the endoplasmic reticulum, whose size, location and effectiveness may vary from one cell type to the next.

Many vertebrate neurons and cells [46] contain more than one type of voltage-sensitive Ca^{2+} channel [47] and L-, R - and T-type channels are significantly inhibited by volatile anesthetics and some intravenous agents such as pentobarbital [47]. A similar range of channels exists in molluscan neurons [48] and in buccal neuron B5 of *Helisoma*, the low voltage-activated (LVA) and high voltage-activated (HVA) Ca^{2+} have been shown to be electrophysiologically and pharmacologically distinct [49]. Both types of current are diminished by volatile anesthetics with varying potencies [46] and it is now believed that voltage-gated calcium channels are particular targets for general anesthetics. In *Lymnaea*, halothane [7], isoflurane [30] and sodium pentobarbital have been shown to diminish L-type calcium currents in pedal I cluster neurons in a concentration dependent manner, while raising $[Ca^{2+}]_i$ [50,51] (Table 3). Since most voltage-gated calcium channels appear to be blocked by general anesthetics in a concentration dependent manner, the most likely source of intracellular free calcium is likely to be the endoplasmic reticulum given its close proximity to the cell membrane in many neurons (See figure 2 and 4).

It is interesting to note that while halothane is capable of raising $[Ca^{2+}]_i$ in the absence of extracellular calcium in the cells studied here, a parallel study [50,51] of neurons in similar locations has revealed that sodium pentobarbital cannot do so (Table 3). Thus, the calcium

	300 µM Caffeine		5 µM Ryanodine		2% Halothane		2 mM Pentobarbital	
Neuron type	**HW**	**↑ $[Ca^{2+}]_i$ in zero $[Ca^{2+}]o$**	**HW**	**↑ $[Ca^{2+}]_i$ in zero $[Ca^{2+}]o$**	**HW**	**↑ $[Ca^{2+}]_i$ in zero $[Ca^{2+}]o$**	**HW**	**↑ $[Ca^{2+}]_i$ in zero $[Ca^{2+}]o$**
VV1/2	+++	No	+++	No	-	Yes	-	No, in several different cell types
RPD1	++	No	NC	No	-	Yes	-	
RPeD1	+++	No	NC	No	-	Yes	-	
RPD2	NS	No	NC	No	-	Yes	-	

Table 3: *Comparison of the actions of intracellular calcium mobilizing agents on action potential half width (HW) and whether $[Ca^{2+}]i$ rises in zero external calcium, $[Ca^{2+}]o$. Half width is reliable measure of action potential duration half-amplitude and indicates changes in the calcium dependent pseudoplateau of type 2 action potentials. It can be used to differentiate cellular responses to applied drugs. Data on caffeine and ryanodine are from Ahmed et al [28, 52]; halothane and pentobarbital data are from Moghadam, et al [26,30]. The pentobarbital data on zero external calcium (orange column at right) are derived from several different cell types, not those shown here [26] but are included to illustrate the differential actions of inhalational and systemic anaesthetics in the absence of external calcium. +++: Maximal increase in HW, ++: Moderate increase in HW, -: Strong decrease in HW; NS: No significant change; NC: No consistent change.*

release properties of pentobarbital are more similar to those of caffeine and ryanodine in this respect [28,52], but it resembles halothane in its ability to diminish the half width of action potentials [25].

Conclusion

Halothane promotes increased intracellular calcium concentration in four types of isolated, cultured *Lymnaea* neurons, both in the presence and absence of extracellular calcium in a cell-specific manner. The most probable source of the free calcium is believed to be the endoplasmic reticulum.

Acknowledgements

IAA was partially supported on a Sudan Government Graduate studentship while at the University of Leeds, UK. Equipment was provided through an MRC grant to PMH and WW. WW was in receipt of grants from the Nuffield Foundation and Ohmeda UK, formerly at Steeton, Keighley, West Yorkshire.

Ethical Committee Approval

Approval was not required for experiments on invertebrate preparations.

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Volume 12 Issue 8 August 2020
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