Differential effects of halothane and sevoflurane on hypoxia-induced intracellular calcium transients of neonatal rat carotid body type I cells†

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Background. The purpose of this study was to investigate the effects of halothane and sevoflurane on the magnitude of the increase in intracellular calcium with hypoxia in carotid body type I (glomerus) cells. We wished to ascertain if the effects of these agents in single cells paralleled their known effects on the human hypoxic ventilatory response, where halothane depresses this response more than does sevoflurane.

Methods. We studied single glomus cells from neonatal rat carotid bodies. Halothane and sevoflurane were administered in concentrations ranging from 0.18 to 1.45 and 0.1 to 2 minimum alveolar concentration (MAC), respectively (rat values). The intracellular calcium response to a ~90 s period of hypoxia was measured using indo-1 dye. We also assessed if these agents influenced the calcium response to exposure to potassium 100 mM.

Results. Halothane depressed the increase in intracellular calcium with hypoxia more than did sevoflurane (P=0.036). Although halothane was depressive at all concentrations tested, sevoflurane depressed the calcium response only at 2 MAC. Both agents also depressed the calcium response to elevated extracellular potassium—halothane more so than sevoflurane (P=0.004).

Conclusions. The actions of the agents in single cells reflect their known influence on human hypoxic ventilatory response, consistent with the notion that the cellular process underlies the whole-body effect. The responses to elevated extracellular potassium, which depolarizes the cell membrane, indicate that (in addition to molecular mechanisms previously proposed), voltage-activated calcium channels may also be involved in the anaesthetic effect.

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It is well established that even at low concentrations of ~0.1 minimum alveolar concentration (MAC), volatile anaesthetics depress the human ventilatory response to hypoxia by 50–70%. At >1 MAC, the hypoxic response is virtually abolished. However, different agents appear to depress the hypoxic ventilatory response to different degrees. Halothane is the most depressive, diminishing the hypoxic response by ~50% at 0.1 MAC. Sevoflurane is one of the least depressive, obtunding the response by just ~20% at the same concentration.

Volatile agents have also been found to depress chemoreflex responses in intact animals, but an important limitation of much whole animal work is that integrity of all elements of the respiratory control system makes it difficult to establish whether the primary effect of anaesthetic occurs at the peripheral chemoreceptors or at some central point in the chemoreflex loop. Also, possible differential effects of the agents on ventilatory control have not been fully explored in animals.

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A recent review of the literature on animal studies whose methodology had functionally isolated the carotid body concluded that volatile anaesthetics do indeed depress carotid body response to hypoxia. This review was, however, unable to confirm the notion—raised by the human work referred to above—that the carotid body was differently sensitive to various agents.

Developments in our understanding of mechanisms of oxygen sensing in the carotid body, and the potential for anaesthetics to interact with these mechanisms, offer an opportunity to re-examine this question from a fresh perspective. For example, Buckler and colleagues described a background TASK-like K⁺ channel in the carotid body glomus (type I) cell that is sensitive to hypoxia, acid, and halothane. Background K⁺ channels are important in determining resting membrane potential, and the reduction of their open probability by hypoxia (and acid or CO₂) results in depolarization of the cell and voltage-gated Ca²⁺ influx. If this channel responds similarly with other volatile anaesthetics, it would make a compelling case that it underlies the mechanisms involved in the effect of all volatile anaesthetics on the human chemoreflex response.

Regardless of the specific molecular mechanisms involved in oxygen- or anaesthetic-sensing at the carotid body, it is important first to establish if an anaesthetic depresses glomus cell hypoxic response and further, if the variation in anaesthetic effect known in humans is reflected in a similar variation in effect at the glomus cell.

The aim of this study, therefore, was to investigate the effect of the agents halothane and sevoflurane on the response of the carotid body glomus cell to hypoxia (measured by the magnitude increase in intracellular calcium concentration, [Ca²⁺]i, with hypoxia—the ‘Ca²⁺ transient’). We chose halothane and sevoflurane to approximate to the known ‘extremes’ of response. The former is arguably the most depressive in humans and (notwithstanding one report that desflurane depresses the acute hypoxic response in humans very little) the latter is consistently shown by a number of reports as being one of the least depressive. We wished to establish ‘dose–response’ relationships for their effects. If the glomus cells were sensitive to volatile anaesthetics, we would expect both halothane and sevoflurane to depress the hypoxia-induced Ca²⁺ transient more than would sevoflurane.

Methods

Cell isolation

Experiments were performed on glomus cells enzymatically isolated from neonatal Sprague–Dawley rat pups (10–13 days old), as described elsewhere. Briefly, rat pups were anaesthetized using halothane 4% in O₂ and the carotid bodies excised and placed in ice-cold saline (the rats were then killed by decapitation, in accord with United Kingdom Home Office animal licence procedures and supervised by the Committee on Animal Care and Ethical Review, University of Oxford). The carotid bodies were incubated in a phosphate-buffered saline containing collagenase (0.5 mg ml⁻¹, Type I, Worthington, Freehold, NJ, USA) and trypsin (0.2 mg ml⁻¹, Sigma-Aldrich, St Louis, MO, USA) at 37°C and then mechanically dispersed by the use of forceps followed by trituration through fire-polished glass pipettes. The cell suspension was then centrifuged, resuspended in culture medium (Ham’s F-12 supplemented with 10% v/v heat-inactivated fetal calf serum, 100 IU ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 84 unit litre⁻¹ insulin), and plated out onto glass coverslips coated with poly-n-lysine (Sigma-Aldrich). Cells were maintained in culture medium for 2–4 h at 35°C in CO₂ 5% in air until use. The time difference between anaesthesia and experimentation was at least 4 h, more than sufficient for any halothane acting on the cells at the time of animal preparation to have completely dissipated.

Microspectrofluorimetry

A microspectrofluorimeter was constructed from an inverted microscope (Nikon Diaphot, Nikon, Tokyo, Japan). The fluorescence-excitation light source was provided by a 100 W xenon lamp. Fluorescent emissions were detected by a pair of tri-alkali photomultiplier tubes (PMT; EMI 9658R) cooled to between approximately −17 and −20°C. The output from each PMT was fed through a current-to-voltage converter and then digitized (250 Hz; CED 1401) and stored on the hard disk of a computer. The collected data from each PMT were integrated over 500 ms intervals, ratioed, and calibrated as described below.

Measurement of intracellular calcium

Cells were loaded with indo-1 by incubation with 2.5 μM indo 1-AM (the acetoxyethyl ester form) in culture medium at room temperature (20–24°C) for 1 h (indo 1-AM was added from a 1 mM stock solution in dimethyl sulphoxide). Indo-1 fluorescence was excited at 340 (sd 5) nm and measured at 405 (16) and 495 (10) nm (using PMTs). [Ca²⁺] was calculated with the following equation:

\[ [Ca^{2+}]_i = K_d \times \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \]

where \( R \) is the ratio fluorescence at 405 nm/fluorescence at 495 nm, \( R_{\text{min}} \) the fluorescence ratio of the free form of indo-1, \( R_{\text{max}} \) the fluorescence ratio of the Ca²⁺-bound form.
of indo-1, $F_{495,\text{free/bound}}$ the ratio fluorescence of free form at 495 nm/bound form at 495 nm, and $K_d$ the dissociation constant for indo-1. The calibration constants, $R_{\text{min}}$, $R_{\text{max}}$, and $F_{495,\text{free/bound}}$ for indo-1, were obtained from an in situ calibration protocol using ionomycin in a separate group of cells; $K_d$ was assumed to be 250 nM.23 24

Solutions

The standard control Tyrode solution contained (mM): 117 NaCl, 4.5 KCl, 23 NaHCO3, 1 MgCl2, 2.5 CaCl2, and 11 d-glucose. High K+ (100 mM) Tyrode solutions were made by adding 95.5 mmol of KCl per litre (over and above the usual 4.5 mmol) and reducing NaCl correspondingly by 95.5 mmol. Calcium-free Tyrode was made by omitting CaCl2 from the solution and adding ethylene glycol tetra-acetic acid 50 μM rigorously to chelate any Ca$^{2+}$ in impurities.

Gas control

Euvoxic solutions were made by equilibrating the above Tyrode with CO2 5% in air, and hypoxic solutions by equilibrating Tyrode with CO2 5% in N2 95%. After gas equilibration, pH was 7.4–7.45 at 35°C and in others to reduce [Ca$^{2+}$]$\text{in}$.

Protocols

The prepared coverslips containing the cells were transferred to a perfusion chamber of ~100 μl volume, mounted on the stage of the inverted Nikon Diaphot microscope (described above). Solutions were delivered from gas-impermeable glass bottles at 35–37°C under gravity via medical grade stainless steel tubing (also gas impermeable) to a low-volume tap and from there via a very short length (5 cm) of low gas permeability narrow bore (1/32 in.), thick walled (3/32 in.) Pharmach tubing (Saint-Gobain Glass, London, UK) to the experimental chamber. The volume of the experimental chamber was <100 μl, solution flow rate was ~4 ml min$^{-1}$ resulting in a solution exchange half-time of <2 s.

Solutions were either Tyrode equilibrated with CO2 5% in air (‘euvoxia’) or Tyrode equilibrated with hypoxic gas (‘hypoxia’). Both euoxic and hypoxic Tyrode were equilibrated with anaesthetic for the protocols described below. We have previously confirmed that, when using Tyrode bubbled with nitrogen, there is little leak of oxygen into the perfusate along the length of this apparatus (i.e. the $P_O_2$ achieved in the cell perfusion chamber was found to be <0.26 kPa).33 Consequently, there is unlikely to be any significant leak of anaesthetic out of solution along the length of our apparatus, consistent with previous reports of the impermeability of the tubing we used.34

Halothane was administered in concentrations of 0.2% (corresponding to 0.18 MAC in rats), 0.4% (0.36 MAC), 1% (0.91 MAC), and 1.6% (1.45 MAC). Sevoflurane was administered in concentrations of 0.25% (0.10 MAC), 0.5% (0.20 MAC), 2.5% (1 MAC), and 5% (2 MAC). These values were determined by the accuracy of the vaporizer dial settings on the two vaporizers. A minimum of six exposures were conducted at each concentration in different cells on different days.

Each experimental period consisted of the cells being exposed to the following steps: first at least 90 s exposure to euoxia; then ~90 s exposure to hypoxia (a shorter exposure was used if the cells demonstrated a decline with time in the magnitude of the Ca$^{2+}$ transient with sustained hypoxia); then at least 120 s period of equilibration with euoxia plus anaesthetic; then a ~90 s exposure to hypoxia plus anaesthetic; then at least 90 s washout with euoxia; and finally a ~90 s exposure to hypoxia.

We also conducted experiments to assess anaesthetic influence on the increase in [Ca$^{2+}$]$\text{i}$ in response to 100 mM K+$. All experiments were conducted with background euoxic gas, with or without anaesthetic. Each experimental period consisted of the following steps: first at least 90 s exposure to standard Tyrode; then a ~30 s exposure to high-K+ Tyrode; then at least 120 s exposure to standard Tyrode equilibrated with anaesthetic; then a ~30 s exposure to high-K+ Tyrode equilibrated with anaesthetic; then at least 90 s washout with standard Tyrode; and finally a ~30 s exposure to high-K+ Tyrode. The anaesthetics used for this part of the experiment were either halothane 2% (1.81 MAC) or sevoflurane 5% (2 MAC).

Volatile anaesthetics have been shown in some tissues to increase [Ca$^{2+}$]$\text{i}$ and in others to reduce [Ca$^{2+}$]$\text{i}$.36 Although it has been demonstrated that glomus cells do not produce a Ca$^{2+}$ transient in Ca$^{2+}$-free solution,37 38 we...
wished to exclude the possibility that a direct anaesthetic influence on \([\text{Ca}^{2+}]_{i}\) might confound the results. Therefore, we conducted a series of control experiments. First, we located cells that responded robustly to hypoxia (~90 s) in standard (i.e. with Ca\(^{2+}\)) Tyrode. Then, we exposed these cells to at least 120 s Ca\(^{2+}\)-free Tyrode in euoxia, followed by ~90 s of hypoxia (in Ca\(^{2+}\)-free solution). Finally, we exposed the cells to at least 120 s of Ca\(^{2+}\)-free Tyrode in euoxia equilibrated with anaesthetic and then perfused them for ~90 s with Ca\(^{2+}\)-free Tyrode in hypoxia equilibrated with anaesthetic. The anaesthetic concentrations used were either halothane 2% (1.81 MAC) or sevoflurane 5% (2 MAC); levels that would elicit a clear result, if the phenomenon of anaesthetic-induced Ca\(^{2+}\) changes existed.

**Data analysis**

Raw fluorimetric data were collected continuously on the Spike 2 (version 4.24) program (Cambridge Electronic Design, Cambridge, UK) with a 2 Hz sampling frequency. These raw data were converted to \([\text{Ca}^{2+}]_{i}\) values using the calibration method described above.

All experimental periods demonstrated recovery of the original response to hypoxia and K\(^{+}\) after anaesthetic exposure. For each hypoxic exposure, we calculated using the Spike 2 program the magnitude of the increase in \([\text{Ca}^{2+}]_{i}\) (the Ca\(^{2+}\) transient) with hypoxia (i.e. the difference between the mean[Ca\(^{2+}\)] in the 90 s before hypoxic exposure and the mean \([\text{Ca}^{2+}]_{i}\) during 90 s hypoxia). The ratio of these magnitudes with and without anaesthetic (i.e. Ca\(^{2+}\) transient with anaesthetic/Ca\(^{2+}\) transient without anaesthetic) represented the anaesthetic effect. Thus, the ratio would be 1.0 if anaesthetic had no effect; if anaesthetic abolished the response to 100 mM K\(^{+}\) completely, the ratio would be zero. We assessed the statistical significance of differences between the means of the two agents, and between the means of each agent vs control using Student’s \(t\)-tests.

**Results**

Figure 1 shows the results of a typical experimental period using halothane. \([\text{Ca}^{2+}]_{i}\) increased rapidly on induction of hypoxia, this response being attenuated in a dose-related manner by halothane (Fig. 1). The magnitude of the Ca\(^{2+}\) response to hypoxia after anaesthetic washout was similar to that before any anaesthetic administration.

Figure 2 shows the patterns of responses were similar in the protocols using sevoflurane, but this agent seemed to
attenuate the hypoxic response somewhat less than did halothane at an equivalent concentration.

Figure 3A shows the ranges of absolute $[\text{Ca}^{2+}]_i$ values for the six test conditions. The baseline euoxic and peak values in hypoxia are consistent with measurements of previous reports in these cell preparations. In the absence of background anaesthetic, there was an approximately nine-fold range (1.2–11.2) in the increase in $[\text{Ca}^{2+}]_i$ from baseline (mean 3.2-fold, SD 2.2, inter-quartile range 1.8–3.5-fold) as is typical for this cell preparation and paralleling approximately eight-fold ranges (mean 3–4-fold) reported in the human hypoxic ventilatory response.

Figure 4 shows the effect of the two anaesthetics on this response to hypoxia, plotted as a ‘dose–response’ relationship normalized to the mean control value. The relationship for halothane appeared generally steeper than that for sevoflurane. ANOVA confirmed significant effects of both ‘agent’ $P<0.001$ ($n=76$, d.f. 1) and ‘concentration’ $P<0.001$ ($n=76$, d.f. 3), indicating that both agents depressed the hypoxic response and that this effect was dependent on their concentration. In the ANOVA, the interaction of ‘agent’ and ‘concentration’ was also significant ($P=0.036; n=76$, d.f. 3), indicating that the effect of concentration varied with the agent used, that is, that halothane caused more profound depression of $[\text{Ca}^{2+}]_i$ response with concentration than did sevoflurane (Fig. 1). Post hoc $t$-tests with the Bonferroni correction confirmed that the effect of halothane was depressive at all concentrations (e.g. $P=0.001$ at 0.2%; $n=8$), but the depressive

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**Fig 2** Representative trace of $[\text{Ca}^{2+}]_i$, (nM) during hypoxic protocols for sevoflurane. The panels are rat values of (a) 0.2, (b) 1, and (c) 2 MAC. Within each panel, the traces are sequentially: control, with background anaesthetic, after washout of anaesthetic. The periods of hypoxia and of anaesthetic exposure are shown by the horizontal bars.

**Fig 3** (a) Boxplots showing distribution of absolute $[\text{Ca}^{2+}]_i$, (nM) for each of the six test conditions. Hypoxia generally produces a large increase in $[\text{Ca}^{2+}]_i$, the calcium transient, which is greatly attenuated by halothane (all concentrations shown) but less so by sevoflurane (all concentrations shown). (b) Boxplots of the $[\text{Ca}^{2+}]_i$ transients for control condition and each of four concentrations of halothane and sevoflurane, indicating the dramatic effect of halothane vs more modest effects of sevoflurane. The middle line of each box represents the median value, the edges of the box the inter-quartile range, the error bars the 10th and 90th deciles, and outliers are shown by single points.
The effect of sevoflurane was only significant at the highest concentration ($P=0.001$ at 5%; $n=7$).

Figure 5 shows results from the control Ca$^{2+}$-free experiment. Cells that demonstrated a large response to hypoxia in standard Tyrode showed no convincing response to hypoxia in Ca$^{2+}$-free solution. Figure 6 shows the range of [Ca$^{2+}$], values obtained for the eight test conditions in this part of the experiment. The presence of either halothane or sevoflurane did not appear to change this (lack of) response to hypoxia in Ca$^{2+}$-free solution. ANOVA confirmed this impression: neither the effect of Ca$^{2+}$-free solution ($P=0.912$) nor anaesthetic ($P=0.880$) was significant, thus excluding the possibility of direct anaesthetic-induced changes in [Ca$^{2+}$].

Figure 7 shows the results of anaesthetic effect on the response to high external [K$^+$]. The results were broadly similar to those with the hypoxic responses. Both halothane (1.81 MAC) and sevoflurane (2 MAC)—that is, concentrations sufficient to obtund the Ca$^{2+}$ transient to hypoxia in both agents—significantly depressed the [Ca$^{2+}$]i response to 100 mM K$^+$ ($P<0.001; n=16$). The magnitude of [Ca$^{2+}$]i response also differed between the two agents ($P=0.004; n=16$).

**Discussion**
Our main result that halothane and sevoflurane both significantly depress the type I cell Ca$^{2+}$ response to hypoxia is probably the first demonstration that volatile anaesthetics can attenuate glomus cell activity. These effects on the hypoxic response vary with concentration for both agents and these effects on hypoxic response and high K$^+$ response differ for the two agents, with halothane attenuating the responses more than sevoflurane. Our observations in single cells are consistent with previous reports on the
similar to the reduction in $[\text{Ca}^{2+}]$ of the $[\text{Ca}^{2+}]$ response. Again this seems consistent with the persistence of such a mechanism in the single cell response (Fig. 4). Sjögren and colleagues reported that in contrast to halothane, high-concentration (0.8 MAC in background poikilocapnia) of sevoflurane (0.2 MAC in background poikilocapnia) reduces human hypoxic ventilatory response. At a concentration of 0.1–0.2 MAC, halothane reduces human hypoxic ventilatory response by $0.2 \text{ MAC}$ (Fig. 4). At a concentration of 0.1–0.2 MAC, halothane reduces human hypoxic ventilatory response by 0.1–0.2 MAC (Fig. 4). Higher concentrations ($>1 \text{ MAC}$) of halothane virtually abolish the human hypoxic ventilatory response, and this is also consistent with the single cell response (Fig. 4). Sjögren and colleagues reported that in contrast to halothane, high-dose sevoflurane (0.8 MAC in background poikilocapnia) does not alter the human acute hypoxic ventilatory response. Again this seems consistent with the persistence of such a mechanism in the single cell response (Fig. 4). All this makes it very tempting to conclude that the anesthetic effects on type I cell activity that we report underlie their effects on whole-body hypoxic ventilatory response (i.e. the primary site of action of anesthetic when depressing the hypoxic response in humans is at the carotid body). Although this conclusion does not preclude an additional anesthetic effect on the chemoreflex arc more centrally in the brain, it does make such an action somewhat redundant. If anesthetics inhibit the response to hypoxia at carotid body level, then little or no information can travel along the afferent pathway to the brain.

**Molecular basis of anesthetic action**

It is pertinent to make some comment on possible molecular mechanisms of the effects we describe. Halothane, as previously reported, may exert its effect via the hypoxia–acid–anesthetic-sensitive TASK-like $K^+$ channel, and this may be the case also for sevoflurane. If so, then one implication of our result is that the affinity, the efficacy, or both of these two agents differ for this channel. Our results justify the need to assess this possibility more directly using voltage-clamp techniques. Indeed, an investigation of any such differential effects of anesthetics may also be helpful in determining which two-pore $K^+$ channel subtypes constitute the TASK-like channels in glomus cells. For example, it is well known that TASK-1 and TASK channels can be differentiated on the basis of their sensitivity to halothane (which activates TASK-1, TASK-3, and TREK-1) and chloroform and diethyl ether (which only activate TREK-1). A recent report indicated that halothane, sevoflurane, and isoflurane all open human TASK-1 channels expressed in oocytes (and that this response differs from the i.v. agents propofol and etomidate), but dose-related effects between the volatile agents were not examined. In a preliminary report, Cotten and Miller observed that 1 MAC of agent potentiated the TASK-3 mediated current in Fisher rat thyroid epithelial monolayer preparations in the order halothane $>$ enflurane $>$ desflurane $>$ nitrous oxide. They correctly noted that this was also the order of potency of these agents in depressing the human hypoxic ventilatory response. Our results help bridge the gap between these anesthetic effects on channels from non-respiratory tissue, and show similar differential effects on the glomus cell response, indicating that the channel effects could plausibly explain whole-body ventilatory effects.

We discovered that both halothane and sevoflurane also reduce the $[\text{Ca}^{2+}]_i$ response to high $K^+$-induced membrane depolarization. With 100 mM $[K^+]_o$, the Nernst equation predicts the membrane to depolarize to approximately greater than $-9 \text{ mV}$ (i.e. the calculated equilibrium potential for $K^+$, assuming that $[K^+]_o$ is $-140 \text{ mM}$). This protocol mimics a voltage clamp. Under these depolarized conditions, the principal determinants of the $[\text{Ca}^{2+}]_i$ response are theoretically: (i) the activity of the...
In other words, if a certain novel drug influences a neur-
onal K+ channel in a manner similar to that of known
anaesthetics, it does not necessarily mean that this novel
drug will have anaesthetic properties. The carotid body is
different: for all agents that reduce glomus cell [Ca2+]i,
response to hypoxia, we can be absolutely certain that (toxi-
city apart) they will depress the organism’s ventilatory
response to hypoxia. This very direct link between mol-
ecular/cellular physiology and phenotype can be exploited
to gain important insights into both anaesthetic action and
channel function.

Conclusions
In summary, we describe the direct, depressive influences
of volatile anaesthetic agents on glomus cell responses to
hypoxia. We further report that agents can differ in their
effects on this response. The results we obtain are remark-
ably similar to those in humans and so suggest that these
cellular responses underlie the whole-body ventilatory
response.

It would be desirable for future studies to extend our
observations to acid or carbon dioxide stimuli and to
investigate the effect of other anaesthetics (e.g. isoflurane)
and also i.v. agents (e.g. propofol) on glomus cell
responses. Jonsson and colleagues58 found that propofol
antagonized both hypoxia- and nicotine-induced increases
in afferent carotid sinus nerve activity in an isolated
carotid body preparation, suggesting that this drug acts at
the synapse to prevent acetylcholine released from carotid
body cells binding with nicotinic receptors of the afferent
glossopharyngeal nerve terminal. Our cell preparation
could be used to test the alternative hypothesis that propo-
fol exerts an inhibitory action directly on the glomus cell,
in a manner similar to volatile anaesthetics that we
describe here. It would further be important to establish if
the effects we describe for volatile anaesthetics are
mediated by differential sensitivity of the hypoxia- and
anaesthetic sensitive TASK-like K+ channel to various
agents, or if other mechanisms (such as voltage-gated
Ca2+-channels) are more important.

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