Competitive interactions between halothane and isoflurane at the carotid body and TASK channels

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Abstract

Background: The degree to which different volatile anesthetics depress carotid body hypoxic response relates to their ability to activate TASK potassium channels. Most commonly, volatile anesthetic pairs act additively at their molecular targets. We examined whether this applied to carotid body TASK channels.

Methods: We studied halothane and isoflurane effects on hypoxia-evoked rise in intracellular calcium ($Ca^{2+}i$, using the indicator Indo-1) in isolated neonatal rat glomus cells, and TASK single-channel activity (patch clamping) in native glomus cells and HEK293 cells transiently expressing TASK1.

Results: Halothane (5%) depressed glomus cell $Ca^{2+}i$ hypoxic response (mean ± SD 94 ± 4% depression; $p < 0.001$ vs control). Isoflurane (5%) had a less pronounced effect (53 ± 10% depression; $p < 0.001$ vs halothane). A mix of 3% isoflurane/1.5% halothane depressed cell $Ca^{2+}i$ response (51 ± 17% depression) to a lesser degree than 1.5% halothane alone (79 ± 15%; $p = 0.001$), but similar to 3% isoflurane alone (44 ± 22%; $p = 0.224$), indicating sub-additivity. Halothane and isoflurane increased glomus cell TASK1/TASK3 activity, but mixes had a lesser effect than that seen with halothane alone: 4% halothane/4% isoflurane yielded channel open probabilities (nPopen) 127 ± 55% above control, vs 226 ± 12% for 4% halothane alone ($p = 0.009$). Finally, in HEK293 cells, progressively adding isoflurane (1.5%-5%) to halothane (2.5%) reduced TASK-1 channel activity from 120 ± 38% above control, to 88 ± 48% ($p = 0.034$).

Conclusions: In all three experimental models, the effects of isoflurane and halothane combinations were quantitatively consistent with the modelling of weak and strong agonists competing at a common receptor on the TASK channel.

Introduction

Volatile anesthetics are widely used during surgery to maintain unconsciousness but amongst undesirable side effects is the depression of breathing, especially postoperatively, contributing to increased morbidity and mortality, particularly in patients with pre-existing cardiorespiratory disease.[1] This is exacerbated by simultaneous use of opioids and residual neuromuscular blockade.[2] In the UK alone, there are ~3 million general anesthetic procedures annually.[3] With an incidence of postoperative hypoxemia as high as 30%,[6] even small risk reductions could yield large benefits.

Under normal conditions hypoxemia evokes hyperventilation. This protective hypoxic ventilatory response is suppressed by volatile anesthetics even at the very low levels that can persist long after surgery.[4] Understanding this mechanism could facilitate the development of agents without respiratory side effects and/or other drugs that can selectively antagonise these depressive effects.[5]

Hypoxic ventilatory response is mediated by the carotid bodies. Under normoxic conditions the resting membrane potential of their type I (glomus) cells is maintained negative by constitutively active potassium channels (TWIK-related acid-sensitive potassium, TASK, channels; where TWIK is 'tandem pore domains in a weak inwardly-rectifying potassium' channel). Hypoxia decreases TASK channel activity causing membrane depolarisation, activating voltage gated calcium (Ca^{2+}) entry and neurosecretion. These neurotransmitters excite afferent (glossopharyngeal) nerve terminals, which project to the ventilatory control centres in the brainstem and induce hyperventilation.[6-10]

In rat and mouse glomus cells the predominant channel present is a heterodimer of TASK1 and TASK3 (termed TASK1/3) together with some homodimeric TASK1 and TASK3, all of which are *inhibited* by hypoxia.[8-10] In contrast TASK channels are typically *activated* by

volatile general anesthetics.[10-12] These opposing effects on TASK channels could explain the depressive effect of anesthetics on hypoxic response. Furthermore, the observation that TASK channels appear differently sensitive to volatile agents (i.e., activated more strongly by some agents than others)[11-13] could underlie established differences between volatile anesthetics on hypoxic response depression at organismal level.[4, 14-17] Our initial aim in this study was to test the simple hypothesis that anesthetic agents (we selected halothane and isoflurane) differ in their efficacy of supressing hypoxic response of the cell and TASK channel activity.

A second aim was to test the hypothesis that the less efficacious of the two agents might antagonise the more efficacious in a manner predicted by classical drug-receptor theory. This would be consistent with the current paradigm that anesthetics act on discrete target receptors.[18] However for pairs of volatile inhaled anesthetics, only additive effects have hitherto been observed using immobility as the outcome,[19-21] and molecular target studies (albeit not investigating TASK channels) produce similar findings.[22,23] We therefore employed mixtures of these agents in different concentrations to test this second hypothesis at three hierarchies of biological organisation: (a) Ca^{2+} responses to hypoxia, (b) activation of endogenous TASK1/3 channels and (c) activation of cloned TASK-1 channels.

Materials and methods

The methods have been described in detail elsewhere[9,10,24-26] and we provide abbreviated details.

Single glomus cell isolation

Carotid bifurcations were surgically removed from isoflurane anesthetized rat pups day 10-15. Animals were then killed by decapitation or exsanguination whilst still deeply anesthetised, and ethical and regulatory approvals were obtained in accordance with project and personal licences (UK Animals (Scientific Procedures) Act, 1986). The bifurcations were placed in ice-cold phosphate-buffered saline, carotid bodies sub-dissected and placed in 37ºC F12-medium containing trypsin and collagenase. Enzymatic digestion was followed by trituration, leading to a single cell suspension in fetal bovine serum and insulin supplemented F12 Ham's or F12 Ham's/Dulbecco's Modified Eagle Medium which was plated out on poly-L-lysine coated glass coverslips in a culture dish. After adding further culture media (2 hrs), cells were stained with rhodamine-peanut-agglutinin (10 µg/ml, Vector Labs, UK; incubated for at least another hour); a bright orange fluorescence when excited at 540 nm aids cell identification. Cells were used from 2 to 8 hrs after plating.

Transient expression of TASK-1 in HEK293

Cells from the human embryonic kidney cell line (HEK293) were grown in Dulbecco's Modified Eagle Medium high glucose plus sodium pyruvate (110mg/l) supplemented with 10% fetal calf serum, 100 units/ml penicillin/streptomycin and 6mM L-glutamine (all Fisher Scientific/Gibco, Loughborough, UK). Rat TASK-1 inserted in the internal ribosome entry site- enhanced green fluorescent protein plasmid (pIRES-EGFP, Clontech, Takara Bio, Saint-Germain-en-Laye, France). This was transiently transfected using lipofectamine and PLUS™ reagent (Life Technologies, Paisley, UK): following the manufacturers recommendations, 24-

48 hrs following transfection cells were re-plated onto poly L-lysine-coated glass coverslips and used for recordings. Transfected cells expressing TASK-1 were identified by their green fluorescence.

Measurements of $[Ca^{2+}]_i$ by spectrofluorimetry

Rat glomus cells were loaded with Indo-1-AM in culture media at room temperature and incubated for 1 hr (2.5 μM added from a dimethyl sulfoxide (DMSO) stock: Invitrogen by Fisher Scientific/Gibco, Loughborough, UK). Indo-1 was excited at 340 nm light; $[Ca^{2+}]$i was calculated from the ratio of Indo-1 fluorescence intensities at 405 and 495 nm, corrected for minimum (zero Ca^{2+}) and maximum (2.5 mM Ca^{2+}) fluorescence ratios measured in each cell.

Anesthetics were introduced into the gas mixtures used to equilibrate Tyrode solutions using calibrated vaporizers with a range of up to 5%. Tyrode solutions contained, in mM: 117 sodium chloride, 4.5 potassium chloride, 1 magnesium chloride, 23 sodium bicarbonate, 11 glucose, 2.5 calcium chloride and were equilibrated with 95% air/5% CO_2 (control) or 1% O_2 / 5% CO_2 / 94% N_2 (hypoxia). At the start of each recording peanut agglutinin labelled glomus cells (see above) were identified and exposed to a brief (e.g. 30 sec) hypoxic stimulus to confirm a robust $[Ca^{2+}]_i$ response. Next the cell was exposed to halothane and/or isoflurane for ~1 min in normoxia, followed by a second exposure to hypoxia (30 sec) in the presence of halothane and/or isoflurane. This was followed by a recovery period in normoxia without anesthetic of ~1 min. At the very end of a recording, a control ~30 sec exposure to hypoxia was performed without anesthetic to ensure appropriate recovery in magnitude of the original hypoxic response. Only cells that demonstrated this recovery were included in our analysis.

For each hypoxic exposure, the magnitude of the Ca^{2+} -transient was calculated as: the absolute difference between the mean $[Ca^{2+}]_i$ in the period before hypoxic exposure and the

mean $[Ca^{2+}]_i$ during hypoxia. The hypoxic response in the presence of anesthetic was then expressed as a % of the response to hypoxia in the absence of anesthetic.

Electrophysiology

Cell-attached patch clamp recordings on native glomus cells and HEK293 cells were performed using an Axopatch 200B (Molecular Devices LLC, Sunnyvale, US). Single-channel recordings were filtered at 2–5 kHz and current was digitized and recorded at 20 kHz. Voltage clamp control, data acquisition and analysis were performed using Spike2 (Cambridge Electronic Design, Cambridge, UK).

Recordings were made with borosilicate pipettes (Harvard Apparatus Ltd, Kent, UK) which were sylgard-coated and fire-polished before use. The pipette filling solution contained, in mM, 140 potassium chloride, 1 magnesium chloride, 1 EGTA, 10 HEPES, 10 tetra-ethyl-ammonium and 5 4-aminopyridine (included to inhibit other, non-TASK potassium channels), pH was adjusted to 7.4 at 37˚C. Cells were placed in a recording chamber and initially superfused with a standard Tyrode solution equilibrated with 5% CO_2/95% air in which seal formation was attempted. Upon obtaining a gigohm seal the superfusate was changed to a Tyrode containing 100 mM potassium chloride (with an equimolar reduction in sodium chloride and no calcium chloride) but otherwise identical to the standard Tyrode. Pipette potential was then set to +80mV. Under the recording conditions used in this study, the glomus cell background potassium channel conducts inward currents exhibiting short, flickering openings interspersed with clear sections of baseline The protocol was (all in normoxic solutions and in high potassium Tyrode), (1) no anesthetic (2) anesthetic for (~30-90 sec) (3) no anesthetic.

Analysis of channel activity was performed on 20 sec sections of recording obtained at least 10 sec after any solution exchange, to allow full exchange of solutions within the

recording chamber. For each condition in each cell/patch we constructed an all points histogram to determine the modal single channel current. This modal current was then used to analyse channel nPopen (the product of the number of channels in the patch and the probability of a channel being open opening) using a 75% threshold crossing method (with 175% representing double openings). For each condition/anesthetic, measurements of nPopen were also expressed as % increase relative to nPopen during the control period. So that if a stimulus resulted in unchanged channel activity, the increase in channel activity compared with control would be 0%.

Monitoring of anesthetic delivery

For all experiments the gas phase above the bubbled solutions was constantly monitored by an infrared analyzer (Capnomac Ultima, Datex, Sevenoaks, UK). This, in turn, was calibrated against a quadropole mass spectrometer (Airspec MGA 3000, Fareham, UK) using calibration gases. Moreover the relationship between vapor levels/setting on the vaporizer and anesthetic levels in solution was linear.[27] Mixtures of agents were delivered by arranging the vaporizers in series (with the isoflurane vaporizer sited first in the pathway of gas flow). We verified using infra-red analysis that, with one vaporizer empty, the output of the other was not influenced by turning the dial of the empty vaporizer (i.e., to exclude any potential back-pressure effect). We also confirmed, using mass spectrometry, that when using two vaporizers in series (one with halothane the other with isoflurane) the anesthetic concentrations in the headspace gas were as dialed on the vaporizer (i.e., there was no significant loss of agent from the gas phase due to arranging the vaporizers in series). Finally, we confirmed using gas chromatography-mass spectrometry (GC-MS) that the concentrations of each anesthetic in solution was identical when using a mixture, to the concentration when each agent was bubbled separately (i.e., there was no selective loss of one agent over another within the Tyrode solution). The concentrations of anesthetic in solution sampled in both the bottles containing

the Tyrode and in the perfusion chamber were not significantly different (i.e. there was no measurable anesthetic loss from the perfusion system, for either agent). Separately we have confirmed that our gas delivery system and perfusion system is mostly gas-tight: nitrogen equilibrated solutions have a P_{O_2} in the perfusion chamber of just ~2-3 mmHg (i.e., there is minimal ingress of oxygen). A detailed analysis of the performance of this system, especially the gas chromatography-mass spectrometry data in relation to mixtures of agents, has been published elsewhere.[27]

Statistical analysis

Sample size: We were guided by the approach of Johnstone et al.[28] who modelled methods for ion channel studies based on a minimum of three separate concentrations (plus the zero point) with a minimum of three repeats (in independent cells from different animals) at each concentration, to construct a suitable dose-response curve. The exact concentration we used at any time was guided by the results of the effects of a previous concentration (where we had no prior knowledge of likely drug effect). For studies of mixtures of agents, we examined at least three concentrations, with studies conducted in at least five cells (each from different animals) at each concentration level, in paired studies (i.e, each agent plus mix in the same cell preparation).[29] This exceeded the minimum sample size per data point indicated by the 'resource equation'.[30] Based on the *a priori* estimate of the degrees of freedom for the error term in an analysis of variance, and the number of groups under study (three in our study; one for each agent, plus the mix), the formula for minimum sample size is (10/3) + 1. On average, 1-2 data points are achievable in a single day's experimentation, for either Ca^{2+} imaging or single channel recording, reflecting the workload involved.

Where concentration-response relationships were plotted, lines of best fit were constructed using non-linear least squares regression, with models fit empirically to a simple ligand binding equation:

$$Y = \frac{[Bmax].[X]}{[Kd]+[X]} \qquad \text{Equation 1}$$

Where Bmax is the estimated maximum effect of drug, [X] is drug concentration (%), Kd is the dissociation constant (%). For drug mixtures, especially where one drug appeared to behave as a partial agonist, we predicted the result of drug admixtures according to the method proposed by Howard and Webster:[29]

$$Y = \frac{\left(Bmax_A \frac{[A]}{K_A}\right) + \left(Bmax_B \frac{[B]}{K_B}\right)}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B}} \qquad \text{Equation 2}$$

Where A and B are the respective drugs in their concentrations with their respective maximum effect alone (Bmax), and K_A and K_B are their respective dissociation constants.

Data are presented as mean ± SD. Anesthetic concentrations are expressed as % delivered; these can be converted to mM in aqueous solution using the data of Franks and Lieb,[18] where 1% halothane equates to 0.24mM and 1% isoflurane to 0.28 mM (see online supplement for presentation of results in terms of mM). We conducted statistical analysis using factorial analysis of variance (SPSS for Windows version 25, SPSS Inc, Chicago, IL, USA), where the 'response' was, respectively for each of the experiments described: (a) the [Ca2+]i transient, (b) the normalised nPopen for native TASK channels in glomus cells, (c) normalised nPopen of TASK-1 channels in HEK293 cells. The comparisons of interest were first, between

halothane and isoflurane (to assess if these agents differed in effect) and second, between halothane and the mix of agents (to assess if the mix produced an infra-additive response). This was therefore a more stringent approach than comparing the results of mix with the results of a predicted additive effect between the two agents. There were two factors: ‘agent’, a nominal factor (one level for each anesthetic/mix), and ‘dose’ (a scalar factor, one level of each concentration). The locations of any significant effects were explored using post hoc Bonferroni test, and $p<0.05$ (two tailed) considered significant.

Results

Effect of single anesthetic agents on hypoxia-induced $[Ca^{2+}]_i$ transients in glomus cells

Baseline $[Ca^{2+}]_i$ was unaffected by either anesthetic at any concentration used in this study ($[Ca^{2+}]_i$ was 70 ± 15 nM control, 55 ± 18 nM with halothane (1-5%), 56 ± 13 nM with isoflurane (0.5-5%); ANOVA for 'agent', 'dose' and interactive term being p = 0.517, 0.796, 0.595 respectively). In contrast, the $[Ca^{2+}]_i$ response to hypoxia (the 'Ca^{2+} transient') was depressed by both agents; halothane virtually abolishing it at concentrations of ~3% and higher (Figure 1A and B). Isoflurane also depressed glomus cell hypoxic response in a concentration-dependent manner, but this was more modest such that ~50% of the response remained, even at supra-clinical isoflurane concentrations of 5% (Figure 1A and B). Quantitatively, halothane 5% depressed glomus cell Ca^{2+}i hypoxic response by 94 ± 4% ($p < 0.001$ vs control). Isoflurane 5% had a less pronounced effect: 53 ± 10% depression ($p < 0.001$ vs halothane).

Statistically, the effect of 'dose' was significant, confirming that drug dose had a depressive effect ($p<0.001$, $F = 9.77$, $df = 7$). The difference between the agents was also significant ($p < 0.001$, $F = 114.2$, $df = 1$ for factor 'drug' in the ANOVA model). The interactive term 'dose*agent* was not significant ($p = 0.200$, $F = 1.50$, $df = 5$), indicating that the effect of dose did not differ across the agents. Post hoc testing for comparison of specific data points was not necessary for further confirmation of the conclusion that both agents were depressive, with halothane more so than isoflurane.

When this data was plotted as a function of 'anesthetic effect' from 0-100% (Figure 1B; with 100% being complete abolition of a response) the effects of both anesthetics appeared to show saturability but with differing maximal efficacies. Thus isoflurane appeared to have a lower intrinsic activity than halothane. We applied Equation 1 which assumes a ligand binding

to a single receptor, to estimate a Kd for halothane of 0.65 ± 0.07% and Bmax 109% ± 7%; and for isoflurane a Kd of 0.80 ± 0.18% and Bmax of 60 ± 6%.

Effect of anesthetic combinations on hypoxia-induced $[Ca^{2+}]_i$ transients in glomus cells

We selected a fixed background level of halothane of 1.5% so we could detect additive, or infra-additive effects of adding increasing concentrations of isoflurane (1%, 1.5% and 3%). Figure 2 shows that the degree of inhibition of the hypoxic $[Ca^{2+}]_i$ response caused by 1.5% halothane was progressively diminished – not enhanced - with the addition of increasing amounts of isoflurane to the mix. Thus a mix of 3% isoflurane/1.5% halothane depressed cell Ca^{2+}i response (51 ± 17% depression) to a lesser degree than 1.5% halothane alone (79 ± 15%; p = 0.001), but similar to 3% isoflurane alone (44 ± 22%; p = 0.224), suggesting antagonism by isoflurane towards halothane.

Statistically, comparison between the halothane results and mix results was significant (p = 0.001, F = 14.49, df = 1, for 'agent', ANOVA); as was comparison for effect of progressive concentration of isoflurane (p = 0.002, F = 6.09, df = 3 for 'dose', ANOVA). Post hoc testing for comparison of specific data points was not necessary for further confirmation of the conclusion that isoflurane was infra-additive to halothane in the mix.

We next used the individual estimates for Kd and Bmax (see above) and applied Equation 2, which assumes competition of a partial with a full agonist at a single receptor. Figure 2 (green line) shows the results of this modelling which predicts that, over the range of anaesthetic concentrations tested, adding isoflurane will progressively antagonise the effects of halothane. This model prediction (Equation 2) is in reasonable agreement with the data - albeit to a slightly greater degree than the model (see Discussion).

Electrophysiology: anesthetic effect on native TASK channel activity

We next investigated the effects of halothane and isoflurane on the native TASK channels in glomus cells, which mediate the cellular effects of both hypoxia and volatile anesthetics on [Ca2+]i. Figure 3A shows cell attached patch recordings of TASK-channel activity in rat glomus cells, which typically display short flickery openings, increased in frequency by both halothane and isoflurane. Across the concentration ranges examined, halothane and isoflurane again differed in their efficacy with halothane being the more efficacious (Figure 3B). The response to halothane did not show saturation, whereas for isoflurane it did. Statistically (ANOVA), there was a significant effect of 'dose' ($p<0.001$, $F = 0.452$, $df = 7$; indicating for both agents there was a dose-related effect), and 'agent' ($p<0.001$, $F = 16.43$, $df = 1$; reflecting the difference between agents), but not of the interactive term 'dose*agent' ($p = 0.331$, $F = 1.16$, $df = 4$; indicating that dose did not differentially influence the agent effect). Post hoc testing for comparison of specific data points was not necessary for further confirmation of the conclusions that the agents activated the native channel, and that they did so to different degrees.

Modelling to Equation 1 thus resulted in a very high estimated Bmax for halothane of 830 ± 356% and a Kd of 10.8 ± 6.3%. For isoflurane the respective values were 156 ± 33% and 1.5 ± 1.2% respectively for Bmax and Kd.

Electrophysiology: effect of anesthetic combinations on native TASK channel activity

We next examined the effects of combining isoflurane and halothane on TASK channels in glomus cells, applied in equal amounts (1.5, 2.5, 4% of each). Again there was a concentration dependent increase in channel activity with the progressive total concentration in mixes of these anesthetic agents, but the response to 'halothane plus isoflurane' produced a

far lesser increase in nPopen across the dose range, than that to 'halothane alone' (Figure 3B green symbols).

For the mix condition, post hoc testing revealed (a) that the effects of 'isoflurane alone' and of 'isoflurane plus halothane' were significantly less than the effects of 'halothane alone' ($p<0.001$, respectively, with Bonferroni correction for multiple testing), and (b) that the effects of 'isoflurane plus halothane' and of 'isoflurane alone' were similar ($p = 0.602$). In quantitative terms, 4% halothane/4% isoflurane yielded channel open probabilities (nPopen) 127 ± 55% above control, vs 226 ± 12% for 4% halothane alone ($p = 0.009$). Moreover, we employed Equation 2 using the estimated values of Kd and Bmax for the individual agents (see above). The resulting line for predicted effect, assuming competition by a partial agonist at a single receptor, lay very close to the actual data points for the mix (Figure 3, green line).

We analysed the all points histograms for the channel activities displayed in Figure 3A. The predominant form of TASK channel present in the glomus cell is a heterodimer of TASK1 and TASK3 but there is also evidence of smaller numbers/sub-populations of TASK1 and TASK3 homodimeric channels.[8,9] To complicate single channel analysis, channel density is high in these cells so most patches contain multiple channels and these channels often display more than one conductance state. Consequently, all-points histograms derived from these recordings often show a single broad peak at ~2.6 pA under the conditions used in this study (see Figure 4A) reflecting the predominant activity of the heterodimer. There was an increase in channel openings by isoflurane and halothane and of the mix at all conductance levels. In one recording (Figure 4B) however, we also captured another type of channel which was probably a homodimeric TASK-1 channel judging by its peak channel current of ~1.2 pA.[8,9] This channel seemed to be little affected by isoflurane alone but was activated by halothane. Moreover, this activation by halothane was antagonised by isoflurane suggesting competition

between different volatile anesthetic agents. We therefore investigated the effects of halothane and isoflurane on homodimeric TASK-1 channels expressed in HEK293 cells.

Electrophysiology: effect of single anesthetic agents on TASK-1 channels expressed in HEK293 cells

We next investigated the effects of isoflurane and halothane on homodimeric TASK-1 channels heterologously expressed in HEK293 cells. These channels exhibited similar short openings with an average single channel current of 1.2 pA. Halothane clearly increased TASK-1 channel activity, isoflurane less so (Figure 5A). This was confirmed in quantitative analysis over a wide range of concentrations, with the response to halothane saturating at a maximum increase in channel activity (nPopen) of 122 ± 38% (Figure 5B). Isoflurane increased channel activity much less, saturating at a maximum increase of just 51 ± 27% (Figure 5B). Statistically, the effect of 'dose' was significant ($p < 0.001$, $F = 5.22$, $df = 6$, ANOVA), indicating dose-related effects. The comparison between agents was also significant ($p < 0.001$, $F = 27.8$, $df = 1$, ANOVA), indicating agents differed. The interactive term 'dose*agent' was not significant ($p = 0.425$, $F = 0.94$, $df = 3$), indicating dose effects did not differ across agents. Post hoc testing for comparison of specific data points was not necessary for further confirmation of the conclusion that halothane and isoflurane both activated the channel yet differed in their efficacies.

The shape of these dose-response curves in transiently expressed single channels was similar to those obtained in whole cell Ca^{2+} transients (compare with Figure 5 with Figure 1). It was notable that the increase in channel activities of expressed TASK-1 (c.50% to c.120% for isoflurane and halothane respectively) were less than observed in native TASK1/TASK3 channels (c.100% to c.220% for the same concentration range; see Figure 3). Modelling to

Equation 1 yielded estimates of, for halothane Kd 1.15 ± 0.45% and Bmax 160 ± 36%; and for isoflurane Kd 1.2 ± 0.54% and Bmax 66 ± 20%.

Electrophysiology: effect of anesthetic combinations on TASK-1 channels expressed in HEK293 cells

We next investigated the effects of combining isoflurane with halothane in expressed TASK-1 channels. We applied a slightly different approach to that used in the previous experiment on native TASK channels, in that we selected a fixed concentration of halothane (2.5%) that caused significant but not maximal TASK1 activation (see Figure 5B). Against this background halothane concentration, we tested the effects of adding isoflurane in varying concentrations (1.5%, 2.5%, 4% and 5%; Figure 6). The effect of isoflurane under these conditions is not as striking as for the glomus cell or native channel (compare with Figure 3B), but the addition of isoflurane with halothane clearly reduced, rather than increased, the level of channel activity compared to that seen with halothane alone. The overall reduction of activity from nPopen 122 ± 38% (with 2.5% halothane only) to 88 ± 48% (with 2.5% halothane plus 5% isoflurane) was statistically significant ($p = 0.034$, $F = 4.91$, $df = 1$, ANOVA by 'agent').

Moreover, we employed Equation 2 using the estimated values of Bmax and Kd for the individual agents at the expressed channel (see above). The resulting line for predicted effect lay very close to the actual data points for the mix (Figure 6, green line), consistent with the model assumption of competition by a partial agonist at this single receptor,

Discussion

Competitive behaviour between anesthetics

The major finding of this study is that two volatile anesthetic agents – both agonists - exhibit competition by the weaker antagonising the effects of the stronger at both whole cell (Ca^{2+} signalling) and molecular level (TASK channels). This conclusion was strengthened by a quantitative approach in fitting data to classic binding isotherms to derive apparent Bmax and Kd values, then using these to calculate predicted effects of weak and strong agonist combinations in a competitive receptor-mediated effect model. These measured pharmacological effects potentially link to the important clinical effect of volatile anesthetics suppressing hypoxic response. However, the clinical relevance of these results will need whole-animal studies.

This result of infra-additivity is notable when viewed in context of the extensive review of anesthetic interactions by Hendrickx et al.[21] They reported that for pairs of volatile inhaled agents only additive effects are found, using immobility as the outcome.[19,20] They also found experimentally, that many candidate molecular targets produced similar additive findings (but TASK channels were not examined).[21-23] Additive or synergistic anesthetic interactions, rather than sub-additive, are also observed when intravenous agents are included in other animal/molecular studies.[31-33] As these authors discussed, the ability of studies to distinguish whether two drugs compete for occupancy of a common receptor sites[34] is dependent on the occupancy of those receptors at the endpoint studied. If the typical behavioral endpoints used to characterize general anesthetics are associated with low site occupancy, then sub-additivity may not be detectable.

A considerable body of work indicates anesthetics bind to specific sites on a range of channels, which implies that competition between agents is to be expected (although this has not been directly reported for isoflurane/halothane combinations). Andres-Enguix et al.

reported that in truncated TASK-1 channels a single point mutation (lysine 159 to arginine) caused reduction of anesthetic sensitivity to both agents, indicating a common binding site.[13] Luethy et al. found that mutations close to a proposed binding site rendered TASK3 resistant to activation by both trichloroethanol and isoflurane.[35] There is also evidence for site-specific anesthetic pharmacology in $GABA_A$ receptors, restricted to intravenous agents, supported amongst other observations by the finding of a novel drug (naphthalene–etomidate) with little or no efficacy with regards to $GABA_A$ activation, but which antagonizes the actions of etomidate, pentobarbital and propofol.[32] So on the one hand, our result of sub-additivity is consistent with receptor theory; on the other hand it differs from the general findings of additivity reported in many other channels for anesthetic combinations.

The generally additive interactions between anesthetics has historically been interpreted, together with the correlation of anesthetic potency with lipid solubility (the Overton-Meyer rule), as implying a non-receptor, physico-chemical mechanism of action (e.g., the lipid solubility theory).[36] Shafer's group modelled additivity and synergy of agent interactions within a framework of anesthetic binding to single (or multiple) receptors, but they did not address the possibility of an anesthetic behaving as a partial agonist at a receptor, in the manner we now describe for isoflurane at the TASK channel.[23]

Possible molecular mechanisms for competitive antagonism between anesthetic agents.

We are unaware of any existing theory, other than classic ligand binding models to receptors, that would be consistent with our data. A non-receptor theory akin to lipid solubility theory might be developed post hoc, given our new data, but we cannot imagine what that might be. The attempts to reconcile the classical lipid hypothesis with receptor theory should be noted. Several proposed binding sites are hydrophobic (or accessible to drugs only via prior dissolution in lipid).[37] This could lead to sufficient disruption of cell membrane 'rafts' by

anesthetic as to influence (TREK potassium) channel function.[38] Pavel et al. have recently reported that modulation of raft-related phospholipase D2 can confer anesthetic sensitivity on a previously insensitive potassium channel (TRAAK): a putative receptor could therefore lie within the associated membrane raft site, and not necessarily on the protein moiety of the channel, at least for some channels.[39]

The most parsimonious interpretation is therefore that our results strongly support general anesthetics acting through specific target sites to exert their effects. Our quantitative modelling is not absolute proof that interactions must reflect competition at a single channel; but rather that our observations are consistent with this proposition.

Brief discussion is needed as to what is meant by a 'receptor' in this context. Although a channel might be conceived as a whole (including its related lipid elements[39]) as a single *receptor*, it may consist therein of more than one *binding site*. Particularly so where the functional channel is made of multiple subunits (two in the case of TASK). The simplest explanation of our data is to assume only one *type* of binding site which may or may not be present in multiple identical *copies* per channel. At these putative sites either halothane or isoflurane can bind but have differing efficacies with respect to increasing channel open probability. Thus, when present together, these agents compete to bind to this single type of site and the lower efficacy agent acts as a competitive inhibitor to the higher efficacy agent. A single site model is probably best suited to studies conducted in expressed TASK1 channels (Figure 6). In native heterodimeric TASK1/3 channels (Figure 3B) there could be two different sites, given two different subunits per channel. With regards to Ca^{2+}-signalling at cell level (Figure 2), anesthetics may have additional effects on voltage-gated Ca^{2+}-channels and other channels, or on other mechanisms putatively involved in oxygen sensing.[40,41] From such considerations we would therefore predict that the best fit of a single-site model (Equation 2)

would be seen for data obtained with pure TASK-1, and this is what we observed (compare the proximity of mix data results to the predicted line for Figure 6 vs Figures 3B or 2).

Potential limitations of the study

Our results on the effects of single agents on TASK channels agree with Patel et al.[42] and Putzke et al.[12] and Sirois et al.[43] who collectively reported that both halothane and isoflurane activate TASK-1 (in various cell preparations). However, Berg et al. found almost no effect of isoflurane on rat TASK-1 channels in HEK293 cells (although this would also predict possible antagonism between isoflurane and any other stronger activator of this channel) .[11]

A limited the number of data point intervals at delivered concentrations <1% limited the precision of our Kd estimates. Conversely, we could not deliver concentrations >5% which limited the precision of Bmax estimates; the constraint being the upper and lower limits and calibration intervals on the vaporizers. Our experimental set-up is designed primarily to study the effects of hypoxia on isolated chemoreceptor cells in vitro, requiring tight control of oxygen, and continuous bubbling of gas mixtures and sustained perfusion of cells achieves the gas equilibria in an 'open' system.[27] A 'closed' delivery system using directly dissolved agents might have permitted a wider concentration range to be assessed, but not allowed rapid switches into and out of hypoxia. That said, our Kd and Bmax estimates for individual agents in Equation 2, resulted in predictions that agreed reasonably well with the data across all experiments.

Whilst we have observed competitive interactions between halothane and isoflurane in both the carotid body TASK1/3 channel and the TASK1 channel we do not yet know what role the TASK3 subunit plays. It is entirely feasible that this may have a slightly different anesthetic binding site to that of TASK1.

We do not have a complete molecular explanation for the interactions we describe, and detailed molecular modelling as used by other groups will be required.[13,32-34,35] A hierarchy of organisation also absent from this work is the study of intact animal hypoxic response. Although the literature on animal hypoxic responses and anesthesia has been reviewed elsewhere,[44] the effect of halothane/isoflurane combinations in both wide type and TASK knockout animals would be important. However, our observations detecting sub-additive effects of drug combinations are mostly at high (supra-clinical) anesthetic concentrations. The inferred competitive interactions may be harder to detect under clinical or subanesthetic conditions due to presumed lower receptor occupancy.[21,23]

Implications of our results

Our discovery was prompted by reports of apparent selectivity between volatile anesthetics in both their depression of hypoxic response[4,14-17] and activation of TASK.[11-13,18] It will be interesting to see if other agents behave like isoflurane, as partial agonists, at TASK channels and so in turn are able to compete with other more efficacious anesthetics.

Exploiting competitive behaviour at TASK channels could be important in managing postoperative hypoxia.[1,2] Some TASK channel inhibitors offer promising results in cell/channel preparations[45,46] as new 'respiratory stimulants'. The potential of an antagonist to anesthetic binding could offer an alternative approach: anesthetic-induced depression of hypoxic ventilatory response could be reversed in a targeted manner, with fewer side effects (e.g., as compared with more-centrally acting respiratory stimulants). A similar approach to the reversal of general anaesthesia has been previously suggested for $GABA_A$ receptor antagonists; the notion of reversing general anesthesia 'on demand'.[31-33] It is proposed by others that two-pore K+ channels are involved in mediating the hypnotic/narcotic effects of anesthesia[47,48]; it

would be further interesting if a putative antagonists to anesthetic at the TASK channel also restored consciousness.

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References

1. Dunham CM, Hileman BM, Hutchinson AE, Chance EA, Huang GS: Perioperative hypoxemia is common with horizontal positioning during general anesthesia and is associated with major adverse outcomes: a retrospective study of consecutive patients. *BMC Anesthesiol* 2014; 14:43

2. Pandit JJ, Eriksson LI: Reversing neuromuscular blockade: not just the diaphragm, but carotid body function too. *Anesthesiology* 2019; 131:453-55

3. Sury MR, Palmer JH, Cook TM, Pandit JJ: The state of UK anaesthesia: a survey of National Health Service activity in 2013. *Br J Anaesth* 2014; 113:575-84

4. Sarton E, Dahan A, Teppema L, van den Elsen M, Olofsen E, Berkenbosch A, van Kleef J: Acute pain and central nervous system arousal do not restore impaired hypoxic ventilatory response during sevoflurane sedation. *Anesthesiology* 1996; 85: 295-303

5. Bayliss DA, Barrett PQ: Emerging roles for two-pore-domain potassium channels and their potential therapeutic impact. *Trends Pharmacol Sci* 2008; 29:566-75

6. Nurse CA: Synaptic and paracrine mechanisms at carotid body arterial chemoreceptors. *J Physiol* 2014; 592:3419-26

7. Weir EK, Lopez-Barneo J, Buckler KJ, Archer SL: Acute oxygen-sensing mechanisms. *N Engl J Med* 2005; 353:2042-55

8. Kim D, Cavanaugh EJ, Kim I, Carroll JL: Heteromeric TASK-1/TASK-3 is the major oxygen-sensitive background K+ channel in rat carotid body glomus cells. *J Physiol* 2009; 587:2963-75

9. Turner PJ, Buckler KJ: Oxygen and mitochondrial inhibitors modulate both monomeric and heteromeric TASK-1 and TASK-3 channels in mouse carotid body type-1 cells. *J Physiol* 2013; 591:5977-98

10. Buckler KJ, Williams BA, Honore E: An oxygen-, acid- and anaesthetic-sensitive TASK-like background potassium channel in rat arterial chemoreceptor cells. *J Physiol* 2000; 525:135-42

11. Berg AP, Talley EM, Manger JP, Bayliss DA: Motoneurons express heteromeric TWIK-related acid-sensitive K+ (TASK) channels containing TASK-1 (KCNK3) and TASK-3 (KCNK9) subunits. *J Neurosci* 2004; 24:6693-702

12. Putzke C, Hanley PJ, Schlichthörl G, Preisig-Müller R, Rinné S, Anetseder M, Eckenhoff R, Berkowitz C, Vassiliou T, Wulf H, Eberhart L: Differential effects of volatile and intravenous anesthetics on the activity of human TASK-1. *Am J Physiol Cell Physiol* 2007; 293:C1319-26

13. Andres-Enguix I, Caley A, Yustos R, Schumacher MA, Spanu PD, Dickinson R, Maze M, Franks NP: Determinants of the anesthetic sensitivity of two-pore domain acid-sensitive potassium channels: molecular cloning of an anesthetic-activated potassium channel from Lymnaea stagnalis. *J Biol Chem* 2007; 282:20977-90

14. Pandit JJ: The variable effect of low-dose volatile anaesthetics on the acute ventilatory response to hypoxia in humans: a quantitative review. *Anaesthesia* 2002; 57:632-43

15. Karanovic N, Pecotic R, Valic M, Jeroncic A, Carev M, Karanovic S, Ujevic A, Dogas Z: The acute hypoxic ventilatory response under halothane, isoflurane, and sevoflurane anaesthesia in rats. *Anaesthesia* 2010; 65:227-34

16. Pandit JJ, Manning-Fox J, Dorrington KL, Robbins PA: Effects of subanaesthetic sevoflurane on ventilation. 2: Response to acute and sustained hypoxia in humans. *Br J Anaesth* 1999; 83:210-6

17. Pandit JJ, Moreau B, Donoghue S, Robbins PA: Effect of pain and audiovisual stimulation on the depression of acute hypoxic ventilatory response by low-dose halothane in humans. *Anesthesiology* 2004; 101:1409-16

18. Franks NP, Lieb WR. Selective actions of volatile general anaesthetics at molecular and cellular levels. *Br J Anaesth* 1993; 71: 65-76

19. Eger EI 2nd, Tang M, Liao M, Laster MJ, Solt K, Flood P, Jenkins A, Raines D, Hendrickx JF, Shafer SL, Yasumasa T, Sonner JM: Inhaled anesthetics do not combine to produce synergistic effects regarding minimum alveolar anesthetic concentration in rats. *Anesth Analg* 2008; 107:479-85

20. Eger EI 2nd, Xing Y, Laster M, Sonner J, Antognini JF, Carstens E: Halothane and isoflurane have additive minimum alveolar concentration (MAC) effects in rats. *Anesth Analg* 2003; 96:1350-3

21. Hendrickx JF, Eger EI, Sonner JM, Shafer SL: Is synergy the rule? A review of anesthetic interactions producing hypnosis and immobility. *Anesth Analg* 2008; 107:494-506

22. Jenkins A, Lobo IA, Gong D, Trudell JR, Solt K, Harris RA, Eger EI 2nd: General anesthetics have additive actions on three ligand gated ion channels. *Anesth Analg* 2008; 107:486-93

23. Shafer SL, Hendrickx JF, Flood P, Sonner J, Eger EI 2nd. Additivity versus synergy: a theoretical analysis of implications for anesthetic mechanisms. *Anesth Analg* 2008; 107: 507-24

24. Buckler KJ: A novel oxygen-sensitive potassium current in rat carotid body type I cells. *J Physiol* 1997; 498:649-62

25. Pandit JJ, Buckler KJ: Differential effects of halothane and sevoflurane on hypoxia-induced intracellular calcium transients of neonatal rat carotid body type I cells. *Br J Anaesth* 2009; 103:701-10

26. O'Donohoe PB, Turner PJ, Huskens N, Buckler KJ, Pandit JJ: Influence of propofol on isolated neonatal rat carotid body glomus cell response to hypoxia and hypercapnia. *Respir Physiol Neurobiol* 2019; 260:17-27

27. Huskens N, O'Donohoe P, Wickens JR, McCullagh JS, Buckler KJ, Pandit JJ: A method for continuous and stable perfusion of tissue and single cell preparations with accurate concentrations of volatile anaesthetics. *J Neurosci Methods* 2016; 258:87-93

28. Johnstone RH, Bardenet R, Gavaghan DJ, Mirams GR: Hierarchical Bayesian inference for ion channel screening dose-response data. *Wellcome Open Res* 2016; 1: 6

29. Howard GJ, Webster TF: Generalized concentration addition: a method for examining mixtures containing partial agonists. *J Theor Biol* 2009; 259: 469-77

30. Charan J, Kantharia ND: How to calculate sample size in animal studies? *J Pharmacol Pharmacother* 2013; 4: 303 -8

31. Forman SA, Miller KW: Mapping general anesthetic sites in heteromeric γ-aminobutyric acid type A receptors reveals a potential for targeting receptor subtypes. *Anesth Analg* 2016; 123: 1263-1273

32. Ma C, Pejo E, McGrath M, Jayakar SS, Zhou X, Miller KW, Cohen JB, Raines DE: Competitive antagonism of anesthetic action at the γ-aminobutyric acid type A receptor by a novel etomidate analog with low intrinsic efficacy. *Anesthesiology* 2017; 127: 824-837

33. Kent DE, Savechenkov PY, Bruzik KS, Miller KW: Binding site location on GABAA receptors determines whether mixtures of intravenous general anaesthetics interact synergistically or additively in vivo. *Br J Pharmacol* 2019; 176: 4760-4772

34. Franks NP, Lieb WR: Do general anaesthetics act by competitive binding to specific receptors? *Nature* 1984; 310:599-601

35. Luethy A, Boghosian JD, Srikantha R, Cotten JF: Halogenated ether, alcohol, and alkane anesthetics activate TASK-3 tandem pore potassium channels likely through a common mechanism. *Mol Pharmacol* 2017; 91: 620-629

36. Pandit JJ: When anaesthetics collide: antagonism of one agent by another? *Anaesthesia* 2017; 72:555-60

37. Vemparala S, Saiz L, Eckenhoff RG, Klein ML: Partitioning of anesthetics into a lipid bilayer and their interaction with membrane-bound peptide bundles. *Biophys J* 2006; 91: 2815-25

38. Petersen EN, Pavel MA, Wang H, Hansen SB: Disruption of palmitate-mediated localization; a shared pathway of force and anesthetic activation of TREK-1 channels. *Biochim Biophys Acta Biomembr* 2020; 1862: 183091

39. Pavel MA, Petersen EN, Wang H, Lerner RA, Hansen SB: Studies on the mechanism of general anesthesia. *Proc Natl Acad Sci USA* 2020; 117:13757-13766.

40. Brunelle JK, Bell EL, Quesada NM, Vercauteren K, Tiranti V, Zeviani M, Scarpulla RC, Chandel NS: Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. *Cell Metab* 2005; 1:409-14

41. Waypa GB, Guzy R, Mungai PT, Mack MM, Marks JD, Roe MW, Schumacker PT: Increases in mitochondrial reactive oxygen species trigger hypoxia-induced calcium responses in pulmonary artery smooth muscle cells. *Circ Res* 2006; 99:970-978.

42. Patel AJ, Honoré E, Lesage F, Fink M, Romey G, Lazdunski M: Inhalational anesthetics activate two-pore-domain background K+ channels. *Nat Neurosci* 1999; 2:422-6

43. Sirois JE, Lei Q, Talley EM, Lynch C 3rd, Bayliss DA: The TASK-1 two-pore domain K+ channel is a molecular substrate for neuronal effects of inhalation anesthetics. *J Neurosci* 2000; 20: 6347-54

44. Pandit JJ: Volatile anaesthetic depression of the carotid body chemoreflex-mediated ventilatory response to hypoxia: directions for future research. *Scientifica* 2014; 2014: 394270

45. Cotten JF: TASK-1 (KCNK3) and TASK-3 (KCNK9) tandem pore potassium channel antagonists stimulate breathing in isoflurane-anesthetized rats. *Anesth Analg* 2013; 116:810-6

46. O'Donohoe PB, Huskens N, Turner PJ, Pandit JJ, Buckler KJ: A1899, PK-THPP, ML365, and Doxapram inhibit endogenous TASK channels and excite calcium signalling in carotid body type-1 cells. *Physiol Reports* 2018; 6: e13876

47. Franks NP, Lieb WR: Background K+ channels: an important target for volatile anesthetics? *Nat Neurosci* 1999; 2:395-6

48. Steinberg EA, Wafford KA, Brickley SG, Franks NP, Wisden W: The role of K_2p channels in anaesthesia and sleep. *Pflugers Arch* 2015; 467: 907-16

FIGURE LEGENDS

Figure 1. A: Representative traces of depression of Ca^{2+} transients in response to hypoxic exposures against different background halothane (top trace) and isoflurane (bottom trace) concentrations. The top panel is from one cell and the bottom from another, different cell. After each anesthetic exposure, response returned to control levels after washout (results not shown).

B: Quantitative effect of halothane and isoflurane across a range of concentrations tested on depressing the glomus cell $[Ca^{2+}]i$ response to hypoxia. Each point is mean ± SD, with a total of 44 recordings for halothane and 52 for isoflurane (different cells for each agent; a maximum of four concentrations in any one cell for any single agent). This is plotted as a conventional 'dose-response' relationship, where 'response' is the agent's ability to diminish the $[Ca^{2+}]i$ response to hypoxia (100% being complete abolition of the response). This plot makes clearer how isoflurane appears to have a lower intrinsic activity for this effect. The lines represent the application of Equation 1 to the data. See Supplementary Figure S1 for the data plotted against aqueous concentrations.

Figure 2. The effect anesthetic combinations on depression of the hypoxic $[Ca^{2+}]_i$ transient. The depression of the hypoxic $[Ca^{2+}]_i$ transient by halothane 1.5% (see Figure 1B) is shown by the single red symbol. At this constant background level of 1.5% halothane, the effect of progressively adding isofurane to the mix (1%, 1.5% and 3%) in separate experiments is shown by the green symbols. For clarity, also shown (purple) is the best fit line using Equation 1 transposed from Fig. 1B for the effects of isoflurane alone to illustrate the fact that as more isoflurane is added to the mix, the effect tends towards that for isoflurane alone. Also shown (solid green line) is the estimated plot from Equation 2, assuming the agents compete at a single receptor, using individual agent Kd and Bmax values from the data in Fig1B. Each symbol is

mean ± SD with n = 25 total for the mix. There were two conditions per cell (halothane and mix). See Supplementary Figure S2 for the data plotted against aqueous concentrations.

Figure 3. A; Representative traces of single channel activities in rat glomus cells. Note the large increase in overall activity with halothane and a more modest increase from control with isoflurane alone. Also shown (lowest trace) is the effect of a mix of agents (2.5% of each agent is shown) from which it appears that the activities are perhaps a little more than isoflurane alone but clearly less than halothane alone. In each recording washout restored the activities back to the level of control.

B: Quantitative analysis of nPopen activities for halothane (red) and isoflurane (purple) and mix of equal concentrations of agent administered (1.5% + 1.5%; 2.5% + 2.5% and 4% + 4%; green symbols: see Supplementary Figure S3 for total aqueous concentrations). Values are mean ± SD (unidirectional bars for clarity), n = 176 patches in separate cells performed. The x-axis is plotted to indicate the concentration of total agent. The red and purple lines (for halothane and isoflurane respectively) are the result of applying Equation 1 to the data, from which individual agent Kd and Bmax were estimated. The green line is the result of applying Equation 2 using these Kd and Bmax values.

Figure 4. All points histograms from cell attached patch recordings shown in Fig. 3. The x-axis represents the current amplitude (pA) in 50 fA bins and the y-axis is the number of recorded data points in each bin (from 0-1800). **A**: A typical/characteristic recording as was observed in most patches. The control (pink) data shows a single peak at ~2.6pA, representing the predominant TASK-1/3 heterodimer channel found in rat glomus type-1 cells. Isoflurane

2.5% (purple) modestly increased the activity of this channel, whereas halothane 2.5% (red) markedly increased the activity. A mixture of 2.5% halothane and 2.5% isoflurane (green) resulted in reduced (not increased) channel activity compared with 2.5% halothane alone. **B:** A more unusual where the control (pink) histogram shows two peaks, one probably representing a TASK-1 homodimeric channel (at ~1.2 pA) and the other a heterodimeric TASK1/3 channel (at ~2.5 pA). Isoflurane 2.5% (purple) increased the activity of the heterodimer but had minimal effect on the TASK-1 channel. However, halothane 2.5% (red) markedly increased the activity of both channels. The concentration mix (green) of agents, 2.5% halothane and 2.5% isoflurane, resulted in reduced (not increased) channel activity compared with that of 2.5% halothane alone for both TASK-1 and TASK-1/3 channels.

Figure 5. A: Representative traces of single TASK-1 channels expressed in HEK293 cells. Top to bottom: control, halothane 2.5%, isoflurane 2.5% and a mixture of 2.5% halothane and 2.5% isoflurane. In each recording washout restored the activities back to the level of control.

B: Results of quantitative analysis of TASK-1 channel activation expressed in HEK293 cells (nPopen values, mean ± SD; n = 81 separate recordings in separate cells). Halothane (red) is a stronger activator than isoflurane (purple) with both agents reaching apparent saturation, and isoflurane (as in Figure 1) appearing to exhibit partial agonist behaviour. The red (halothane) and purple (isoflurane) lines are the result of applying Equation 1 to the data, and estimating individual Kd and Bmax values. See Supplementary Figure S4 for the data plotted against aqueous concentrations.

Figure 6. The effect of anesthetic combinations on TASK1 open probabilities expressed in HEK293 cells. Compare with Figure 2. Halothane 2.5% (single red symbol) activates TASK1 channels to just under 120% (see also Figure 5B). At this constant background level of 2.5% halothane (red symbol), progressively increasing concentrations of isofurane were added to the mix in concentrations of 1.5%, 2.5%, 4% and 5% in separate experiments (green symbols). Also for clarity is shown (purple) is the plotted line from Equation 1 for ligand binding copied from Fig. 5B for isoflurane, to show that as isoflurane is added to the mix, the result tends towards that for isoflurane alone, and not away from it. The green line is the line plotted from Equation 2, using the estimated values of Bmax and Kd for halothane and isoflurane. Each symbol is mean ± SD, n = 30 paired recordings in separate cells for the mix line. See Supplementary Figure S5 for the data plotted against aqueous concentrations.