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Isoflurane does not aggregate inside POPC bilayers at high pressure: Implications for pressure reversal of general anaesthesia

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ABSTRACT

We placed isoflurane, a general anaesthetic, inside palmitoyloleoylphosphatidylcholine (POPC) bilayers at clinical concentration, and performed molecular dynamics simulations at atmospheric and raised pressures, using two different thermodynamic ensembles. We also performed a simulation of this system with isoflurane at ten times the clinical concentration. We found that isoflurane did not aggregate inside POPC membranes at 20 MPa, nor at 40 MPa. The implications of these findings for pressure reversal is discussed, in light of the high-pressure neurological syndrome.

1. Introduction

General anaesthetics (GAs) have been used for over 170 years, and literally millions of surgical operations have been performed using these drugs, but their site and mechanism of action are still not entirely clear. In the beginning of the 20th century, Meyer [1] and Overton [2] proposed what is now known as the Meyer–Overton rule, which stated that the logarithm of the efficacy of an anaesthetic was proportional to the logarithm of its lipophilicity. Since this rule applied to a large variety of general anaesthetics, it suggested that a unified mechanism of action might exist.

Half a century later, Johnson and Flagler [3,4] discovered the phenomenon of pressure reversal. They found that, by increasing ambient pressure to between 2000 psi and 3000 psi (1.4×10^7 Pa and 2.1×10^7 Pa), general anaesthesia by ethanol could be reversed in tadpoles. Paton and his co-workers [5,6], and Halsey and Wardley-Smith [7] extended the work by using several different anaesthetics and different animals, and the reversal phenomena were observed in all the general anaesthetics they used. The pressure where pressure reversal was observed depended on the species and the drug administered, and varied from 80 atm (8×10^6 Pa) [3] to 200 atm (2×10^7 Pa) [6]. This pressure reversal effect has since been observed in many species and using different kinds of general anaesthetics [8,9].

Subsequently, Trudell et al. [10,11] measured the electron spin resonance spectra of spin-labelled phosphatidylcholine and halothane solutions. They attached a nitroxide group to the terminal methyl of the lipid tails, and measured the angular deviation of the nitroxide $2p\pi$ orbital axis from the axis of magnetic symmetry. They defined a bond order parameter S'_n based on the angular deviation, and found that applying halothane decreased S'_n but increasing pressure would increase S'_n. These results implied that the phospholipid cell membrane was involved in general anaesthetic action. Later, Cantor [12] suggested that drugs could induce pressure change in the lipid membrane; these changes would shift the conformational equilibrium of membrane proteins and thus cause anaesthesia [13]. Scientists have certainly observed the effect of membrane on ion channel gating [14], but there still is a debate about the relative contributions of the membrane and the protein to general anaesthetic effects [15].

Griepernau and Böckmann [16] investigated the links between protein conformations and pressure changes by molecular dynamics (MD) simulations. They used 1-alkanols as anaesthetics and hydrated dimyristoylphosphatidylcholine (DMPC) bilayer as the model cell membrane. By comparing the lateral pressure profiles of systems at 10^5 Pa and 10^8 Pa, they found no significant changes in the protein conformational equilibrium unless the bent helix model was assumed.

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Unfortunately, at 10^8 Pa, pressure reversal is reversed; that is, the general anaesthetics would be able to exert their anaesthetic effects if the animal were to survive pressures of that magnitude. Johnson and Flagler [3,4] observed that non-anaesthetised tadpoles swimming in a tank would increase their activity if exposed to a pressure of between 1500 psi and 2500 psi (10^7 Pa to 1.7×10^7 Pa), but at about 4500 psi (3.1×10^7 Pa), their normal swimming movements were replaced by uncoordinated twitches and spasms. For non-anaesthetised newts, the increased activity was observed at 1000 psi (6.9×10^6 Pa). In the presence of general anaesthetics, tadpoles became active at between 2000 psi and 3000 psi (1.4×10^7 Pa and 2.1×10^7 Pa), but at about 3.1×10^7 Pa, they would exhibit similar uncoordinated muscle movements. However, this kind of spastic paralysis was always preceded by a brief restoration of swimming activity. When these tadpoles were returned to atmospheric pressure, most of them died within two days of the experiment, so the spastic paralysis at 4500 psi (3.1×10^7 Pa) could be a prelude to death. When this experiment was repeated on newts, the same behaviour was observed over a similar pressure range, but very few of the animals died.

In the work of Lever et al. [5], in the absence of anaesthetics, more and more of the newts became paralysed as the pressure increased, reaching 90% at 200 atm (2×10^7 Pa). In the presence of anaesthetics, more than 90% of the newts were paralysed at 3.4×10^6 Pa, but their activity increased as the pressure was raised. A maximum of 90% of the newts exhibited activity at a pressure of about 1.3×10^7 Pa. There was then a drop in the activity level, but even at about 2.7×10^7 Pa, the activity level was over 50%. This contrasts with the non-anaesthetised cases, where there was no activity beyond 2×10^7 Pa. These authors did not report the post-experiment survival of these animals. The paralysis at around 2.7×10^7 Pa remains a mystery, but no experimental work at that pressure has been attempted again.

Recently, Chau et al. [17,18] performed molecular dynamics simulations of a membrane patch with a concentration of halothane six times that of clinical concentration, and showed that the drugs aggregated inside the membrane at raised pressures. In a subsequent simulation system [19], the concentration of halothane used was only twice that of clinical concentration; they were able to show that there was aggregation at 2×10^7 Pa but not at 4×10^7 Pa. These results suggested that the aggregation of halothane in the membrane at high pressure could be the mechanism for pressure reversal and for the reversal of pressure reversal described previously [3,5]. To generalise this conclusion, we carry out simulations on another general anaesthetic, isoflurane (1,1,1-trifluoro-2-chloro-2-(difluoromethoxy)-ethane), to determine if it behaves similarly to halothane in membranes at high pressures.

2. Methods

2.1. Molecular dynamics simulations

All molecular dynamics simulations were performed using NAMD version 2 [20], using the CHARMM36 potential [21] for the membrane, the TIP3P potential [22] for water, and a special potential for isoflurane [23]. The initial configuration of hydrated POPC membrane came from the CHARMM membrane builder freely available on the internet. This system, under periodic boundary conditions, contains 512 POPC molecules and is fully hydrated with 16691 water molecules, 44 potassium ions and 44 chloride ions, giving a KCl solution of 0.14 M. It is thereafter called the 'pure POPC' system. In a different system, nineteen isoflurane molecules were added to the membrane system, resulting in clinical concentration of the drug (see appendix for details of how this was determined). The isoflurane molecules were placed randomly in the simulation box, one by one, using an internal function of NAMD. The simulation box was equilibrated to give an isotropic pressure profile; this system is thereafter referred to as the 'POPC–isoflurane' system. See Figure 1 for a diagram showing isoflurane and the POPC–isoflurane system.

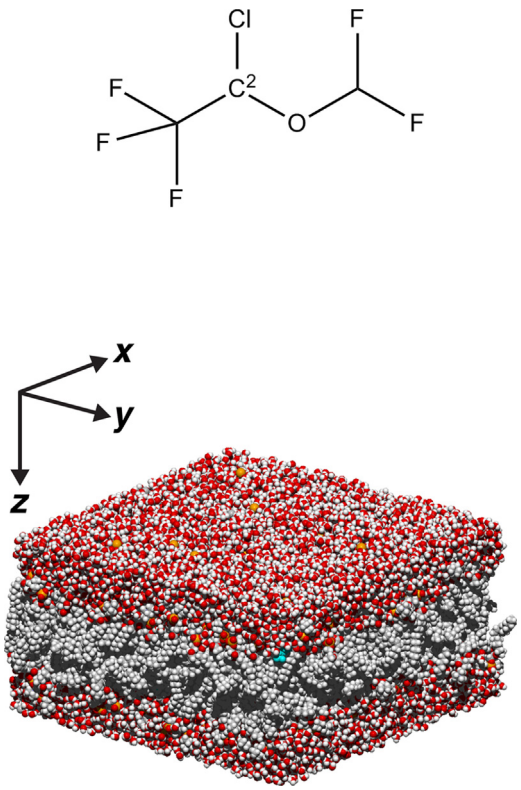

Figure 1. Diagram showing a chemical diagram of isoflurane, with the C2 atom labelled (top panel), and the simulated system with 512 POPC molecules (bottom panel). In the bottom panel, the atoms are coloured according to this scheme: grey = carbon, white = hydrogen, blue = nitrogen, red = oxygen and orange = phosphorus; all atoms of the isoflurane molecules are shown in cyan. In the centre of the simulation box lie POPC molecules, with water molecules on either side. The directions of the three axes are shown on the left; the *xy*-plane, where $z = 0$, is between the two halves of the membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Molecular dynamics simulations were carried out with timesteps of 2 fs, at a temperature of 310 K and at pressures of, respectively, 10^5 Pa (1 atm), 2×10^7 Pa (200 atm) and 4×10^7 Pa (400 atm). Langevin dynamics was applied; the thermostat was set with a time constant of 5 ps^{-1}, and the barostat set with a piston decay time of 100 fs and a piston period of 200 fs. The van der Waals cut-off was 13 Å, and Ewald summation was applied to electrostatics interactions. These NPT simulations were equilibrated for 60 ns and data collection carried out for 100 ns. This set of simulations will be called the NPT simulations.

In order to eliminate the effect of the thermostat and barostat on aggregation, we calculated the average box size from the NPT simulations, and used them to carry out simulations in the NVE ensemble at pressures of, respectively, 10^5 Pa (1 atm), 2×10^7 Pa (200 atm) and 4×10^7 Pa (400 atm), for 100 ns of simulated time. This set of simulations will be called the NVE simulations.

Lastly, to investigate the effect of concentration on aggregation, we also prepared a system consisting of 64 POPC molecules (fully hydrated with 1856 water molecules) with 24 isoflurane molecules embedded. This represents a system with ten times the clinical concentration of isoflurane. The system was equilibrated for 90 ns, and a data-production run of 50 ns was carried out. Langevin dynamics was applied; the thermostat was set with a time constant of

Table 1
This table shows the thermodynamic properties of each system.

System	E	P	T
512-POPC systems			
Pure POPC	$-3.68 \times 10^5 \pm 1250$	5.56 ± 121	310 ± 0.80
NPT 10^5 Pa	$-3.72 \times 10^5 \pm 1550$	-0.260 ± 193	310 ± 0.85
NPT 2×10^7 Pa	$-3.72 \times 10^5 \pm 1550$	202 ± 132	310 ± 0.85
NPT 4×10^7 Pa	$-3.75 \times 10^5 \pm 1550$	405 ± 133	311 ± 0.86
NVE 10^5 Pa	$-3.68 \times 10^5 \pm 88$	64.3 ± 125	310 ± 0.66
NVE 2×10^7 Pa	$-3.70 \times 10^5 \pm 88$	282 ± 125	310 ± 0.66
NVE 4×10^7 Pa	$-3.73 \times 10^5 \pm 71$	487 ± 126	310 ± 0.65
64-POPC system			
NPT 10^5 Pa	$-3.30 \times 10^4 \pm 523$	4.84 ± 378	310 ± 2.34
NPT 2×10^7 Pa	$-3.33 \times 10^4 \pm 523$	207 ± 381	310 ± 2.41
NPT 4×10^7 Pa	$-3.52 \times 10^4 \pm 594$	398 ± 383	310 ± 2.54

E = total energy (kJ/mol), P = pressure (10^5 Pa), T = temperature (K).

0.01 ps^{-1}, and the barostat set with a piston decay time of 250 ps and a piston period of 500 ps. The van der Waals cut-off was 13 Å, and Ewald summation was applied to electrostatics interactions. This set of simulations will be called the high-concentration simulations.

2.2. Deuterium order parameter

Order parameters give us the information about the structure of the phospholipids of membranes. In this work, we use the deuterium order parameter S_{CD}, which is defined in the following manner: the S_{CD} of a given carbon atom of the lipid tail can be calculated as:

$$S_{CD} = -\frac{\langle 3\cos^2\theta - 1\rangle}{2} \quad (1)$$

where θ is the angle between the C—H bond and the membrane normal, and the term in angled brackets denote ensemble averaging over all the membrane molecules, over the C atoms located at the same position in the two tails, and over both C—H bonds belonging to the same C atom. The deuterium order parameter measures roughly the same quantity as the order parameter used by Trudell et al. [10].

3. Results

3.1. Energy, temperature and pressure

Table 1 shows the thermodynamic properties of the systems. The energy and temperature are stable. The fluctuation of pressure is large due to the small size of the simulation box, which is typical for simulations of this scale.

In our simulations, the area per POPC molecule is 64.6 $Å^2 \pm 0.1 Å^2$ at 10^5 Pa and 63.8 $Å^2 \pm 0.1$ $Å^2$ at 4×10^7 Pa. Previous experiments showed that, in the liquid phase (L_α phase), the area per POPC molecule was 63 $Å^2$ at 297 K [24], 68.3 Å at 303 K [25], 66 $Å^2$ at 310 K [26], and 62 $Å^2$ at 323 K [27]. Our system is thus in the biologically relevant liquid phase at all pressures. These results are also consistent with experimental findings that, at a pressure of 4×10^7 Pa, the gel/liquid phase transition temperature of POPC is about 278 K [28], well below our simulation temperature of 310 K.

We also show the distribution of the isoflurane molecules in the z-direction in Figure 2. The isoflurane molecules are preferentially located near the headgroup, with a small number of them located in the lipid matrix. These results are consistent with those from previous work [19].

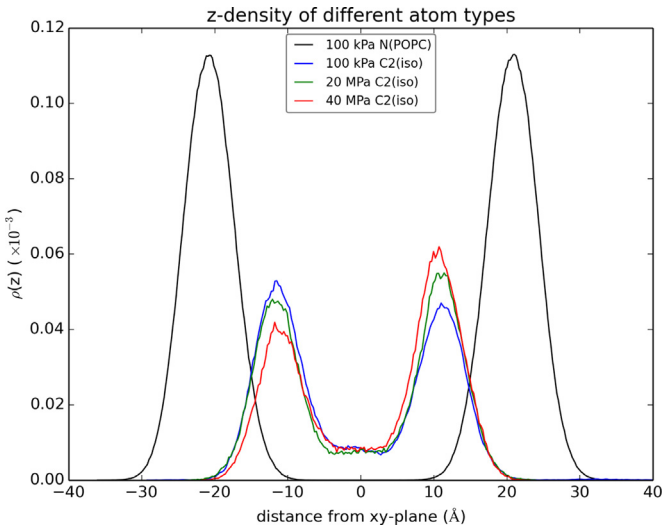

Figure 2. Diagram showing the atomic density distribution along the z-axis, $\rho(z)$, of the choline nitrogen of the POPC molecules, and that of the carbon atom of halothane. The distribution of the choline nitrogen is not affected by the change in pressure, so we show only one set of data. The distribution of halothane carbon atoms are shown for the three different pressures, colour-coded as in the legend. The values for nitrogen have been divided by 512 so that the four lines can be viewed on the same graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Radial distribution function g(r)

The radial distribution function between the C2 atom of isoflurane molecules, $g(r)$, are shown in Figures 3, 4 and 5, respectively, for the NPT simulation, the NVE simulation and the high-concentration simulation. The average and standard deviation were calculated. We take the C2 atom because it is the atom nearest the centre of mass of isoflurane.

For the NPT simulation, the first maxima of the $g(r)$ at the three pressures are indistinguishable from each other, though there are some differences in the second maximum. The first maxima all reach a value of about 4.0 when $r = 6$ Å. The first minimum is at $r = 8.5$ Å with a $g(r)$ of 1.5, and is almost identical for all three pressures. The second maximum is centred around $r = 10.5$Å; $g(r)$ is about 3 when the pressure is 2×10^7 Pa, but drops to about 1.7 when the pressure is 4×10^7 Pa.

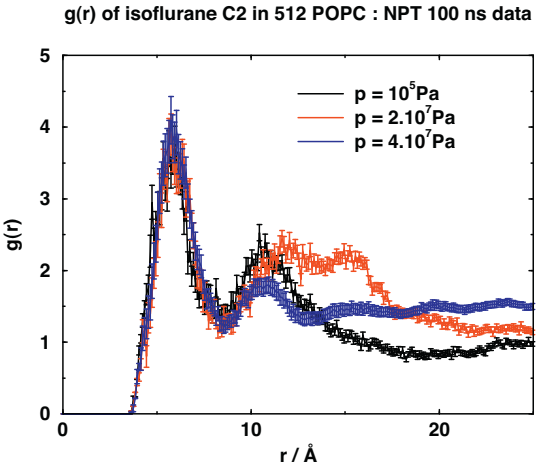

Figure 3. Diagram showing the radial distribution function of the C2 atom of isoflurane in the molecular dynamics simulation runs performed with an NPT ensemble. The concentration of isoflurane used is that of clinical concentration.

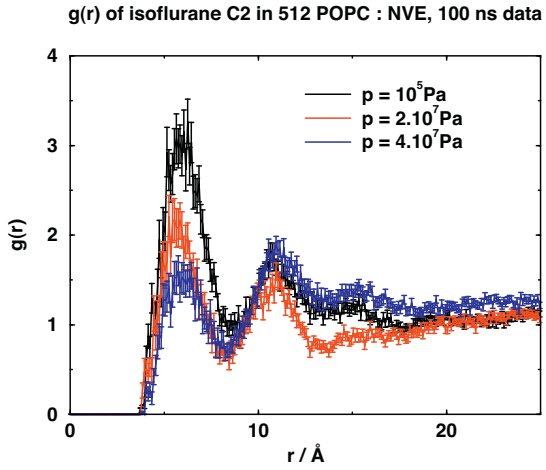

Figure 4. Diagram showing the radial distribution function of the C2 atom of isoflurane in the molecular dynamics simulation runs performed with an NVE ensemble. The concentration of isoflurane used is that of clinical concentration.

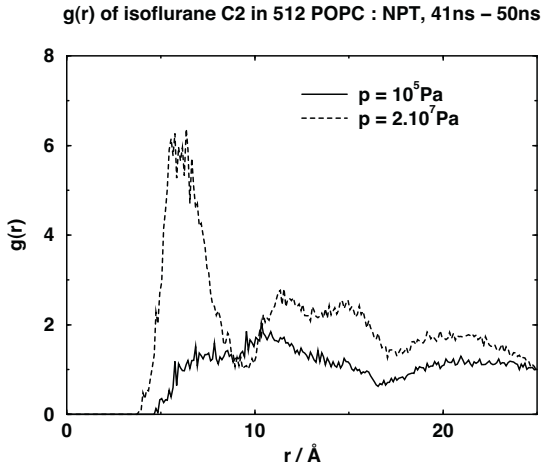

Figure 6. Diagram showing the radial distribution function of the C2 atom of isoflurane in the molecular dynamics simulation runs performed with an NPT ensemble, between the 41st nanosecond to the 50th nanosecond, at two different pressures. The concentration of isoflurane used is that of clinical concentration.

For the NVE simulation, at 10^5 Pa, the first maximum is about 3, and it decreases to 2.0 at 2×10^7 Pa, and is about 1.5 at 4×10^7 Pa. The first minimum is at $r = 8.2$ Å with a $g(r)$ of <1, and is almost identical for all three pressures. The second maximum is also at $r = 10.5$ Å, but its magnitude at different pressures is almost identical. There is then a second minimum at $r = 13$ Å; it is more obvious at 2×10^7 Pa than at other pressures.

For the high-concentration simulation, the $g(r)$ at all three pressures are very close to each other. There is a first maximum of about 2.5 at $r = 6$ Å, then a first minimum of about 1.0 at $r = 8.5$ Å, a second maximum of about 1.5 at about $r = 10.7$ Å and a second minimum of 1.1 at $r = 13$ Å.

We can thus conclude that no aggregation of isoflurane molecules is observed at 2×10^7 Pa. Using the NPT ensemble, the $g(r)$ graphs at all three pressures are quite similar; using the NVE ensemble, there is a monotonic decrease in the first maximum of the $g(r)$ as the pressure increases. In both simulations, there are large fluctuations in the $g(r)$ graphs (data not shown).

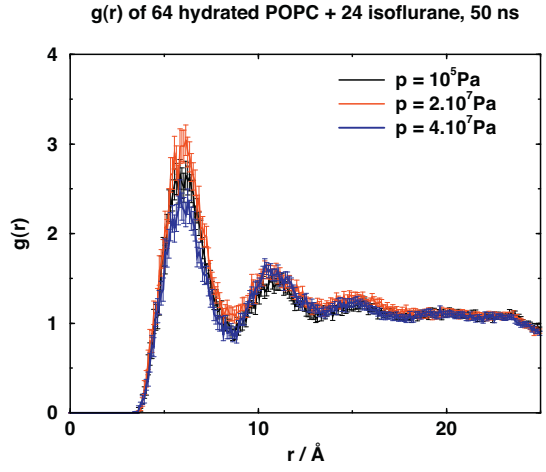

Figure 5. Diagram showing the radial distribution function of the C2 atom of isoflurane in the molecular dynamics simulation runs performed with an NPT ensemble. The concentration of isoflurane used is ten times that of clinical concentration.

3.3. Number of unclustered molecules

Since we performed some of the simulations at clinical concentration of isoflurane, we could proceed to quantify the number of molecules in the membrane under different conditions.

We calculated the number of unclustered molecules at extremes of aggregation. Figure 6 shows the $g(r)$ of the isoflurane C2 atom at 10^5 Pa and at 2×10^7 Pa, between the 41st nanosecond and the 50th nanosecond. It can be seen that there is a big difference between the first maxima at two pressures. We decided that the first solvation shell has a radius of 8 Å. Using this value, we evaluated the number of unclustered molecules in the two system. At 10^5 Pa, this is 18.6, and at 2×10^7 Pa, it is 17.0 (out of a total of 19 isoflurane molecules).

The same argument goes for the case of halothane. Although we only performed simulations at twice the clinical concentration of the drug [19], the difference in $g(r)$ when halothane aggregated and when it did not was similar to the difference observed for isoflurane.

Thus, even if we were to observe the degree of aggregation as dramatic as that shown in Figure 6, this would constitute only a reduction of the concentration of isoflurane by less than 10%. In pharmacological terms, this is negligible; the effect of drugs is not significantly attenuated with a 10% reduction in concentration. This was observed in the case of halothane, but not in isoflurane. It can thus be safely concluded that the aggregation of general anaesthetics inside the membrane is not a mechanism for pressure reversal.

3.4. Deuterium order parameter

Figure 7 shows a chemical diagram of POPC, with the location of each corresponding bond number. Panel (b) shows that the bond-order parameter at all three pressures is very similar, and that the presence of isoflurane does not greatly change the bond order parameter.

4. Discussion

Although general anaesthetics have been in use for over 160 years, their exact site and mechanism of action are still unclear. Johnson and Flagler [3,4] showed that increased pressure reversed anaesthesia, and this work has been repeated by many researchers ever since [5–7].

Significantly, Halsey and Wardley-Smith [7] showed that pressure reverses the effect not only of general anaesthetics, but also

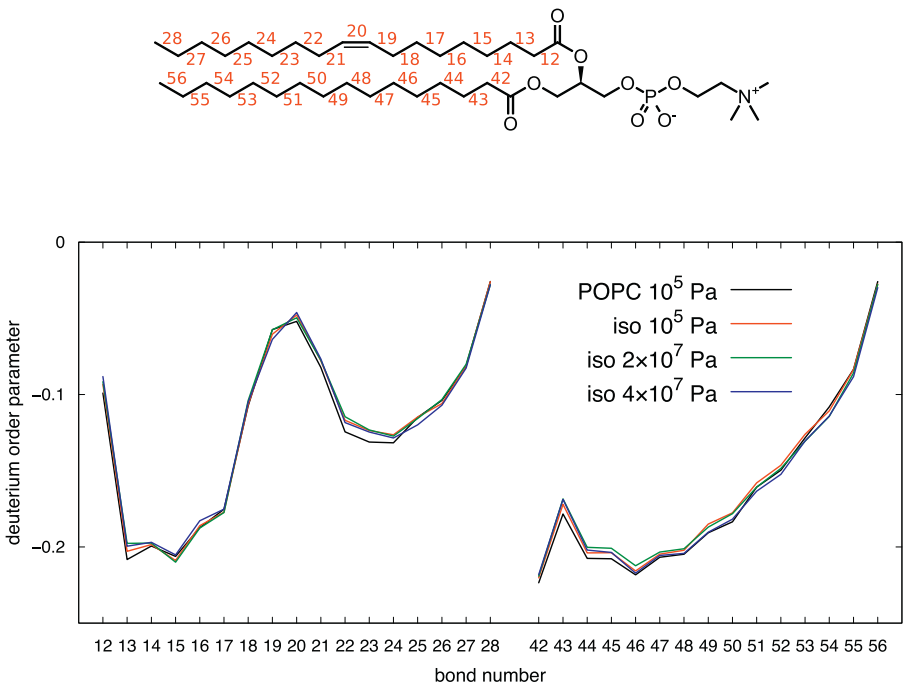

Figure 7. The top panel shows the bond numbering scheme used in our calculations. The lower panel shows the bond order parameter of the system consisting of hydrated POPC only (POPC 10^5 Pa), and of POPC with isoflurane at three different pressures (iso 10^5 Pa, iso 2×10^7 Pa and iso 4×10^7 Pa).

of other drugs such as diazepam, chlorpromazine and lignocaine, into their respective sites of action. Since the sites of action of chlorpromazine and lignocaine are known to be different from the putative site of action of general anaesthetics, it appears that pressure reversal is a pharmacokinetic effect, rather than a pharmacodynamic effect. Pharmacokinetics describes the processes whereby a drug is absorbed, distributed and transported to its site of action, so pharmacokinetic effects are not specific to any particular binding site. Pharmacodynamic effects are site-specific.

This possibility was supported by the finding that general anaesthetics were found to be enantiospecific, but this enantiospecificity cannot be accounted for by their effects on the cell membrane [29–32]. The membrane is thus unlikely to be the site of action of general anaesthetics. The putative target protein of at least the halogenated alkane general anaesthetics was postulated to be the γ-aminobutyric acid type A receptor ($GABA_A$ receptor); it has been shown that modifying the $GABA_A$ receptor would modify the response to general anaesthetics in whole animals [33,34]. Mutations of this receptor and subsequent patch-clamping of the protein also supported this hypothesis [35–37]. For a more detailed discussion of the membrane hypothesis vs the protein hypothesis, please see Chau [15].

Jenkins et al. [38] noted that different general anaesthetics were of different sizes; isoflurane is bigger than halothane, which in turn is bigger than chloroform. They also noted that mutations of the α-subunit transmembrane domain 2 amino acid Ser 270 could abolish $GABA_A$-receptor modulation by general anaesthetics. They mutated Ser 270 into amino acids of different sizes, and were able to estimate the volume of a proposed anaesthetic binding site to be 250 Å^3–370 Å^3. Their results also suggest that the sites of action for isoflurane, halothane and chloroform at least has a significant degree of overlap; it is also probable that these drugs share a common binding site. This putative binding site is only large enough to accommodate one isoflurane molecule.

In previous work, we proposed that pressure reversal occurred when halothane aggregated, so there were fewer monomeric halothane to bind to this putative binding site [17,19]. In this work, we performed simulations of nineteen isoflurane molecules inside the POPC membrane at pressures of 10^5 Pa, 2×10^7 Pa and 4×10^7 Pa. The concentration of isoflurane used was that of clinical concentration or ten times that of clinical concentration. At clinical concentration, we also tried two different thermodynamic ensembles. We did not observe any isoflurane aggregation inside the POPC membrane in the pressure ranges tested.

It is thus possible that pressure-induced aggregation is only observed with the halothane-DMPC or halothane-POPC combination. In these cases, where aggregation was observed, the halothane $g(r)$ graphs [19] showed the same trend as the isoflurane $g(r)$ graph shown in Figure 6, so a 10% reduction in free halothane would be expected. In the case of isoflurane, we quantified the reduction in unaggregated isoflurane in the membrane under transient conditions where aggregation was observed. In both cases, a 10% reduction in free general anaesthetics is not enough to cause any significant reduction in drug effect. What other mechanism could account for pressure reversal then?

High pressure causes hyperexcitability of the central nervous system (see Halsey [39], Jain [40] for reviews on the effect of high pressure on the central nervous system). The symptoms include headache, vertigo, nausea, and euphoria; the patient exhibits clinical signs such as tremor, opsoclonus, myoclonus, dysmetria, hyperreflexia and, in experimental animals, convulsions. There are also cognitive deficits and memory impairment. Experiments on the baboon [41] show that a competitive NMDA receptor antagonist can reduce body tremor and brain electroencephalogram changes, showing this receptor is involved in the high-pressure neurological syndrome (HPNS). Experiments also demonstrate the involvement of the neurotransmitter GABA in the high-pressure neurological syndrome in the baboon [42]. Drugs such as flurazepam or diaminobutyric acid, which enhanced the activation of the $GABA_A$ receptor, substantially raised the threshold for both tremor and convulsions [43]. It is now thought that the change of balance between the noradrenaline, dopamine, NMDA and GABA systems causes the HPNS disturbances [44,45].

Thus the key to pressure reversal may lie in the $GABA_A$ receptor. This receptor is most probably the target of a large number of general anaesthetics [15], and it is also pivotal to some of the effects of the high-pressure neurological syndrome. At raised pressures, the body experiences changes related to the high-pressure neurological syndrome. General anaesthetics have been shown to potentiate the effect of GABA on its $GABA_A$ receptor [46]; they might well act like flurazepam or diaminobutyric acid to increase the activity of the $GABA_A$ receptor, and thus reduce the effects of the high-pressure neurological syndrome.

If this hypothesis is true, then pressure reversal is a manifestation of the high-pressure neurological syndrome, masked by anaesthesia. We would then expect general anaesthetics not to abolish pressure effects completely, as some of the high-pressure neurological syndrome effects are mediated by other neurotransmitter systems [44,47], and thus would be insensitive to general anaesthetics. We would also expect mutations of the $GABA_A$ receptor which potentiate the effect of GABA to render the subject less susceptible to pressure reversal, as it is protected against the high-pressure neurological syndrome.

Acknowledgements

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Appendix A.

In the appendix, we determine the number of isoflurane molecules to be put into each membrane.

The oil:gas partition coefficient for isoflurane has been reported to be 90.8 [48], or between 78 and 140 depending on the temperature [49]. We take the oil:gas partition coefficient to be 100 for isoflurane. This means 1 l of lipids would contain 100 l of isoflurane gas if exposed to 100% isoflurane at a pressure of 10^5 Pa. However, the minimum alveolar concentration (MAC) of isoflurane for anaesthesia is 1.2% [50]. By Henry's Law, 1 l of lipids contain 1.2 l of isoflurane. At body temperature, 1 mol of isoflurane occupies about 24 l of space. Thus the concentration of isoflurane in lipids is 1.2 l/24 l = 0.05 mol.

We assume that the density of POPC is not far from that of water, then given its relative molecular mass of 760, 1 l of POPC contains 1000/760 mol of POPC, or about 1.32 mol of POPC. Thus, at MAC, [isoflurane]/[POPC] = 0.05/1.32 = 0.038. This means for each POPC molecule, there should be 0.038 isoflurane molecule. For 512 POPC, at clinical concentration, there should be 19 isoflurane molecules. At 10× the clinical concentration, for 64 POPC, there should be 24 isoflurane molecules.

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