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Research paper

A combined X-ray scattering and simulation study of halothane in membranes at raised pressures

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ABSTRACT

Using a combination of high pressure wide angle X-ray scattering experiments and molecular dynamics simulations, we probe the effect of the archetypal general anaesthetic halothane on the lipid hydrocarbon chain packing and ordering in model bilayers and the variation in these parameters with pressure. Incorporation of halothane into the membrane causes an expansion of the lipid hydrocarbon chain packing at all pressures. The effect of halothane incorporation on the hydrocarbon chain order parameter is significantly reduced at elevated pressure.

1. Introduction

General anaesthetics (GAs) have been used for over 170 years, and millions of surgical procedures 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] attempted to rationalise the experimental findings, and proposed what is now known as the Meyer-Overton rule. It 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.

Fifty years later, Johnson and Flagler [3,4] discovered the phenomenon of pressure reversal. They found that, by increasing ambient pressure to between 14 MPa and 21 MPa (140 and 210 bar), 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 8 MPa (80 bar) [3] to 20 MPa (200 bar) [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] performed electron spin resonance experiments on spin-labelled phosphatidylcholine and halothane (1,1,1-trifluoro-2-chloro-2-bromo-ethane) solutions. 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.

However, there is also significant evidence that a number of anaesthetic molecules act at specific protein binding sites. Several distinct protein targets have been identified and may be collectively contributing to the anaesthetic state. Among those are tandem-pore-domain potassium channels, NMDA receptors [12], voltage-gated sodium channels [13] and more importantly, anionic pentameric ligand-gated ion channels [14]. The latter class (especially the $GABA_A$ receptor) was subject to extensive studies aimed at identifying the location of the GAs binding site through targeted mutagenesis [15], mutagenesis and alkyl-labelling experiments [16], and modelling studies [17], where the binding site is thought

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to be located at the lipid-accessible side of the transmembrane domain.

Recently, Chau et al. [18,19] 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 [20], the concentration of halothane used was only twice that of clinical concentration; they were able to show that there was aggregation at 20 MPa but not at 40 MPa. This effect was not observed in simulations involving another general anaesthetic, isoflurane (1,1,1-trifluoro-2-chloro-2-(difluoromethoxy)-ethane) [21].

Importantly, none of the currently suggested molecular mechanisms for anaesthetic action adequately explains the widely observed phenomenon of pressure reversal. The conformation and activity of membrane proteins (such as the $GABA_A$ ion channel which has been widely implicated in anaesthetic action) are known to be highly sensitive to the state of the membrane in which they are embedded [22,23]. Here, we have explored the combined effect of general anaesthetic incorporation and high pressure on the lipid hydrocarbon chains of model bilayer membrane systems. Using a combined experimental - simulation approach, we have been able to gain a unique insight into the changes in packing and conformational order of the lipid molecules.

2. Materials and methods

2.1. Experimental materials

DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (>98% purity, Alabaster, AL, USA) and was used without further purification. Halothane was purchased from Sigma Aldrich (Gillingham, UK).

2.2. High-pressure X-ray scattering experiments

Dry samples of DMPC were hydrated with MilliQ water (60 wt %) and subjected to at least 10 freeze–thaw-vortex cycles. For anaesthetic containing samples, halothane was added to give a molar ratio of 128:80 lipid to halothane (representing a halothane concentration approximately 12 times greater than that of a clinical concentration [24]) and mixed thoroughly immediately prior to being loaded into sealed sample holders to minimise the effect of halothane evaporation. Samples were then mounted in a custom-built high pressure cell and subjected to a minimum of two pressure cycles (0.1–100 MPa) before each experiment to ensure sample homogeneity. Full details of the pressure cell used for X-ray studies have been described previously [25]. Briefly, the cell consists of a high tensile strength stainless steel body with 1-mm thick, 5-mm diameter sapphire windows. These windows provide good X-ray transmission (at 18 keV) while offering excellent pressure stability. Wide-angle X-ray scattering (WAXS) experiments were carried out at beamline I22 at Diamond Light Source in the range 0.1–100 MPa (1–1000 bar) at 310 K. The resulting two-dimensional WAXS patterns were radially integrated to give one-dimensional scattering intensity profiles. For each pressure measured, a water scattering pattern was recorded and used for baseline subtraction. Gaussian functions were fitted to peaks in the WAXS patterns to find the peak centres (most patterns showed only a single broad peak). The real space d-spacing (d) corresponding to the peak centres was calculated as $1/S$, where $S = \sin\theta/\lambda$ (where 2θ is the angle between the incident and scattered X-ray beams and λ is the X-ray wavelength). The maximum of the broad wide angle scattering peak indicates the average alkyl chain separation within the bilayer.

2.3. Molecular dynamics simulations

All molecular dynamics simulations were performed using NAMD version 2 [26], using the CHARMM36 potential [27] for the membrane, the TIP3P potential [28] for water, and a special potential for halothane [29], using the second set of partial charges. The initial configuration of hydrated DMPC membranes came from the web-based CHARMM-GUI membrane builder [30]. This system, under periodic boundary conditions, contains 64 DMPC molecules and is fully hydrated with 2048 water molecules. For anaesthetic containing simulations, 33 halothane molecules were added, resulting in approximately ten times clinical concentration of the drug. The halothane molecules were placed randomly in the simulation box, using the soft-core potential function implemented in NAMD.

For the pure DMPC system, molecular dynamics simulations were carried out with timesteps of 2 fs, at a temperature of 310 K and at a pressure of 0.1 MPa (1 bar). Langevin dynamics were applied; the thermostat was initially set with a time constant of 0.1 ps^{-1}, but this was reduced to 0.05 ps^{-1} over the course of equilibration (varying from 60 ns to 90 ns, depending on the pressure). The barostat was initially set with a piston decay time of 1 ps and a piston period of 2 ps, but these were increased during equilibration to a piston decay time of 500 ps and a piston decay period of 1 ns. The van der Waals cut-off was 12 Å, and Ewald summation was applied to electrostatic interactions. Data collection was carried out for 20 ns; configurations were output every 20 ps.

For the halothane-DMPC system, 33 halothane molecules were added to the membrane of 64 DMPC molecules. Post-insertion, the halothane-DMPC system was equilibrated for 200 ns. Subsequently, separate equilibration simulations (from 50 ns to 100 ns, depending on the pressure) were carried out at, respectively, 0.1 MPa (1 bar), 20 MPa (200 bar) and 40 MPa (400 bar). The piston decay time was increased to 0.5 ns and the piston decay period was increased to 1 ns. Data collection was carried out for 20 ns for both systems; configurations were output every 20 ps.

The structure factor was calculated as a function of the scattering vector **S**, from the output configurations using the Debye formula [31] implemented by the software package debyer which is freely available on the internet (https://github.com/wojdyr/debyer). A Gaussian function was fitted to the structure factor peak to find the peak centre and the real space d-spacing corresponding to the structure factor peak was calculated as 1/S (as described above for the experimental WAXS results). This d-spacing can be related to the average spacing between the alkyl chains of the phospholipids.

3. Results and discussion

3.1. Wide angle X-ray scattering experiments

WAXS probes structures on the 1–10 Å length scale and so is ideal for studying the packing of lipid hydrocarbon chains. A pure fluid membrane typically shows a broad WAXS peak corresponding to approximately 4.6 Å (S = 0.22 $Å^{-1}$).

Fig. 1 shows the effect of pressure on the WAXS scattering from DMPC. Fig. 1a shows WAXS scattering patterns from pure DMPC at different pressures. At atmospheric pressure and 25 MPa, the broad scattering pattern indicates formation of a fluid bilayer structure. At 50 MPa, there is a small sharper peak at approximately 4.2 Å (S = 0.235 $Å^{-1}$) indicating coexistence of a lamellar gel phase. The coexistence of the original fluid phase is likely to be a kinetic effect