Biochimica et Biophysica Acta, 736 (1983) 1–10
Elsevier

BBA 71893

A ^{1}H-NMR INVESTIGATION OF THE EFFECTS OF ETHANOL AND GENERAL ANAESTHETICS ON ION CHANNELS AND MEMBRANE FUSION USING UNILAMELLAR PHOSPHOLIPID MEMBRANES

G.R.A. HUNT and I.C. JONES

Department of Science, The Polytechnic of Wales, Pontypridd CF37 1DL, Mid Glamorgan (U.K.)

(Received May 3rd, 1983)

Key words: Lanthanide ion; Anesthetic-membrane interaction; Membrane permeability; Ion channel; Phase transition; Membrane fusion; ^{1}H-NMR; (Phospholipid membrane)

Using ^{1}H-NMR of small unilamellar vesicles in the presence of the lanthanide probe ion Pr^{3+}, the effects of ethanol, diethyl ether and chloroform on various mechanisms of channel-mediated transport were studied. The mechanisms include channel formation by the polypeptide Alamethicin 30 and vesicular lysis at the gel to liquid-crystal phase transition of the lipid. Channel stabilisation and membrane fusion induced by sub-critical micelle concentrations of Triton X-100 were also investigated. The observation that ethanol and diethyl ether increase membrane permeability and fusion while chloroform inhibits them suggests a common locus of action on the properties and structure of channel-associated water. This conclusion is discussed in terms of current theories of general anaesthesia.

Introduction

The effects of ethanol and other general anaesthetics on the central nervous system are not understood at the molecular level [1,2]. However, their physiological action seems likely to be centred on the processes involved in synaptic transmission [2,3] which include the opening of calcium channels and fusion of synaptic vesicles with the plasma membrane. Our understanding of these processes of transmembrane transport and membrane fusion has benefitted from the simplification of the systems involved by using model lipid membranes in a very large number of studies. However, investigations into the effects of ethanol or general anaesthetics on transmembrane channels using lipid bilayer membranes are surprisingly limited [4–6] and their effects on membrane fusion do not seem to have been significantly studied at all in model systems.

We have therefore investigated the action of ethanol, diethyl ether and chloroform on a variety of mechanisms of membrane permeability using unilamellar phospholipid vesicles and on their fusion stimulated by Triton X-100. The choice of these three compounds seemed suitable for an initial study since they are all small uncharged molecules and have a wide range of partition coefficient from aqueous to membrane phase [7,8].

Our previous work with small phosphatidylcholine vesicles has shown that ^{1}H-NMR in conjunction with paramagnetic probe ions such as the lanthanide ion Pr^{3+}, can be used to study a number of different mechanisms of membrane permeability. These include (a) channel formation at or near the bilayer gel to liquid-crystal phase transition temperature (T_c) which causes vesicular lysis [9,10]; (b) carrier-mediated transport by ionophores such as A23187 [11–13]; and (c) pore-mediated transport by ionophores such as the polypeptide Alamethicin 30 [13]. We here report the application of these methods to investigate the

effects of ethanol, diethyl ether and chloroform on channel formation in phosphatidylcholine vesicular membranes. Experiments using Triton X-100 have been included in order to extend the experimental conditions to study the effect of the three compounds on Triton-stabilised channels at T_c [10,14] and on vesicular fusion [10,15]. The investigation of such fusion processes by NMR has now been well established and we have made use of the line-broadening methods developed by Prestegard and co-workers [16,17].

Until recently the trend has been to explain general anaesthesia in terms of hydrophobic effects such as bilayer fluidity or expansion [18]. These lipid-based theories have now been effectively criticised [2,19] and a number of studies reported in which hydrophilic effects such as hydrogen bonding are implicated [20]. Our results support these indications of anaesthetic action at aqueous interfaces and in particular suggest that the effects of ethanol and the general anaesthetics studied on the structure and properties of the water in the channels could account for their stimulation or inhibition of the observed channel-mediated transport of Pr^{3+}. Our observations are also in keeping with recent studies which indicate the probable importance of water in both the ionic permeability of the lipid bilayer at the phase transition [21–23] and in the structure of ion channels formed by Alamethicin [24].

Materials and Methods

Chemicals

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Lipid Products, South Nutfield, Redhill, and Triton X-100 (Scintillation grade) was purchased from BDH, and an average molecular weight of 624 (9.5 oxyethylene units) was used to calculate molarities. A23187 was a gift from the Lilly Research Centre, Windlesham, Surrey. Alamethicin 30 was obtained from PHLS Centre for Applied Microbiology and Research Porton Down, Salisbury, and praseodymium chloride ($PrCl_3 \cdot 6H_2O$) from Lancaster Synthesis. Deuterium oxide (^2H_2O - 99.8% Gold Label) was purchased from Aldrich, Gillingham, Dorset. The AnalaR chloroform used was purified by passing over alumina to remove ethanol and water, distilled and restabilised by addition of 1% AnalaR methanol. Absolute ethanol (spectrotropic grade) and AnalaR diethyl ether (sodium dried to remove water) were obtained from BDH Chemicals, Poole, Dorset.

Experimental procedures

The vesicular membranes were prepared by sonication of lipid in ^2H_2O as described previously [9] to give a DPPC concentration of 10 mg/ml. The ^{1}H-NMR spectra of the vesicular solutions were obtained at 60°C, or 50°C in the case of the experiments using the ionophores, i.e. above the phase transition temperature of the DPPC vesicles. A JEOL FX60Q FT NMR spectrometer operating at 60 MHz and fitted with a calibrated temperature control was used to record the spectra. The 10-mm NMR tubes contained 1 ml of vesicle solution confined by a vortex plug and capped. Typically, 15 pulse sequences were used (π-τ-$\pi/2$) with a pulse interval, τ of about 3.0 s to minimise ^{2}HHO, ethanol and ether peaks. The desired concentration of Pr^{3+} in the extravesicular solution was obtained by adding a known volume of a stock solution of $PrCl_3$ in ^2H_2O to the vesicle solutions in the NMR tube. In experiments on phase transitions and lysis of the vesicular membranes using ethanol, ether and chloroform, appropriate volumes of these were pipetted into the NMR tubes containing the vesicles and Pr^{3+}, and incubated for 1 h before performing the experiment.

The ^{1}H-NMR spectrum of DPPC vesicles at 60°C includes high resolution signals from the lipid acyl chains (H) and choline headgroups. On adjusting the extravesicular concentration to 5 mM Pr^{3+}, the spectrum shown in Fig. 1 is obtained. Separate signals are now seen originating from the extravesicular headgroups 'O' and intravesicular headgroups 'I'. The resulting separation is mainly due to the downfield shift of signal 'O', this being concentration dependent [25]. Such shifts are now well documented [26] and are caused by pseudo-contact, dipolar interaction of Pr^{3+} in rapid exchange between ^2H_2O and the phosphate sites on the extravesicular headgroups. The ratio of the areas $O:I$ is approx. 1.7 ± 0.05 obtained by integrating the two peaks and indicates that vesicles of average diameter of 28 nm have been formed [27].

Observation of the phase transition temperature T_c, of the vesicular membranes and the effects on it of ethanol, diethyl ether and chloroform was made by monitoring the temperature dependence of the line widths in the ^{1}H-NMR spectrum as described previously [9] except that here the linewidths at half-height ($\nu_{1/2}$) of the acyl chain signal (H) was measured from temperatures above the phase transition down to the lowest temperature before the linewidth was too great to be separated from the base-line noise.

Using the changes in the spectrum shown in Fig. 1 which occur when the vesicles interact with membrane active substances we can distinguish between the following types of event: (I) Transmembrane transport of diffusion of the probe ion Pr^{3+} into all vesicles simultaneously. (II) Lysis of the membranes in which 'holes' or channels are formed in some of the vesicles so that equilibration of the 5 mM Pr^{3+} takes place across the bilayer but without the loss of vesicular integrity. (III) Fusion of the vesicles. These events have been distinguished in our previous studies [9–13] and are illustrated in the results below.

Lysed vesicles were obtained by cycling the vesicular solution from 60°C to below T_c and back to 60°C three times over 60 min in the NMR tube, spectra being taken before and after each series of cycles. Triton X-100 was introduced (to a final concentration of 0.1 mM) by pipetting a stock solution of Triton X-100 in ^2H_2O into the vesicular solution which was already equilibrated with Pr^{3+} and anaesthetic for 1 h. This was further equilibrated at 60°C for 30 min before a single cycle through T_c over a 20-min period.

The ionophores were introduced into dry NMR tubes by pipetting a known volume of a chloroform stock solution. The solvent was carefully removed under a stream of nitrogen followed by evacuation. One ml of vesicular solution was then added and incubated at 50°C for 1 h. In some experiments Alamethicin 30 was introduced into the vesicular solution by adding a known volume of ^2H_2O stock solution of the polypeptide (1 mg/ml) to 1 ml of the vesicle solution in the NMR tube, followed by incubation at 50°C for 60 min. Transport was initiated by the addition of the stock Pr^{3+} solution to give an extravesicular concentration of 5 mM and followed at 50°C by observation of the NMR spectrum at suitable time intervals. In the case of samples containing ethanol or anaesthetic these were added to the vesicular solution and co-equilibrated with the ionophore for 1 h at 50°C before the addition of Pr^{3+}.

Experimental Results

1. The effects of ethanol, diethyl ether and chloroform on the ^{1}H-NMR spectrum and phase transition of DPPC vesicles

Up to concentrations of 0.1 M, ethanol and diethyl ether have negligible effects on the chemical shifts of the ^{1}H-NMR signals obtained from the vesicles as shown in Fig. 1. Chloroform, however, causes a marked upfield shift of the outer headgroup signal 'O' towards signal 'I'. The change is approximately linear with concentration and is 3% at 100 mM $CHCl_3$.

All three compounds decrease the line width of the headgroup and acyl chain signals. Again chloroform has the most marked effect. The widths at half-height ($\nu_{1/2}$) of the acyl chain signal (H) were measured in Hz over a range of temperatures spanning the phase transition temperature T_c, in the absence (control) and in the presence of the three compounds under study. The measurements of $\nu_{1/2}$ were converted to spin-spin relaxation

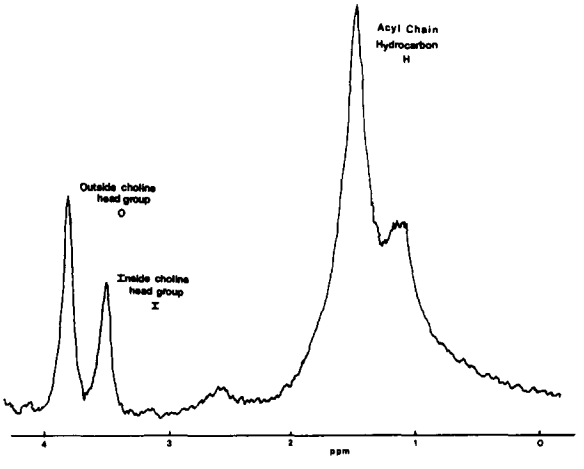

Fig. 1. The 60-MHz ^{1}H-NMR spectrum of dipalmitoylphosphatidylcholine vesicles at 60°C in the presence of 5 mM Pr^{3+}, showing signals from the extravesicular choline headgroups (O), the intravesicular choline headgroups (I) and the lipid acyl chain signals (H). The chemical shifts are shown with reference to external TMS.

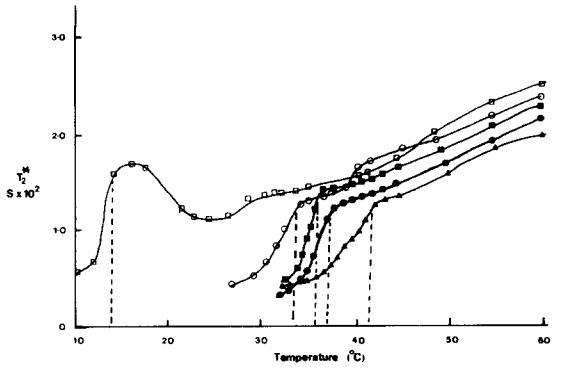

Fig. 2. The temperature dependence of the spin-spin relaxation time (T_2^*) for the acyl chain signal (H in Fig. 1) from dipalmitoylphosphatidylcholine vesicles, ▲———▲, Control. Vesicles in the presence of: ●———●, 86 mM ethanol; ■———■, 86 mM diethyl ether; ○———○, 25 mM chloroform; □———□, 86 mM chloroform. Dotted lines indicate the onset of the liquid-crystal to gel phase transition.

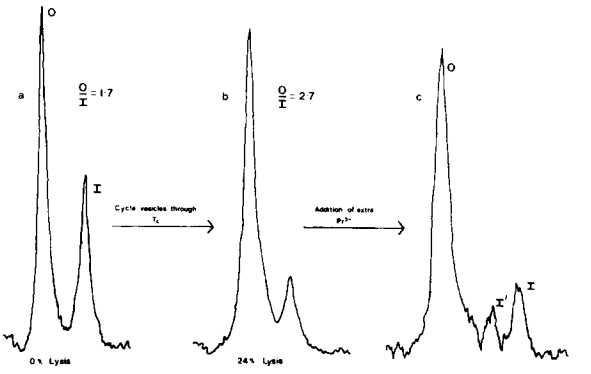

Fig. 3. Effect on the ^{1}H-NMR headgroup signals from dipalmitoylphosphatidylcholine vesicles in the presence of 5 mM extravesicular Pr^{3+} (O extravesicular headgroup signal, I intravesicular headgroup signal) of cycling the vesicles through the phase transition. (a) signals from the vesicles at 60°C before cycling; (b) signals from vesicles at 60°C after six sets of three cycles through T_c (60°C-30°C-60°C) with time intervals as shown in Fig. 4 (control); (c) signals from the vesicles as in (b) except that the extravesicular concentration of Pr^{3+} has been increased to 20 mM.

times, T_2^* using the relationship $T_2^* = 1/\pi\nu_{1/2}$. The effect of the compounds on T_2^* and on the phase transition temperature can be seen in Fig. 2. In each case the T_2^* values are increased by the presence of all three compounds at temperatures above the phase transition. The temperature range over which liquid-crystalline and gel phases co-exist is known to be greater for small unilamellar vesicles than larger bilayer structures [28]. For the pure DPPC vesicles T_c can be seen to extend from about 41°C to 35°C. The onset of the liquid-crystal to gel phase transition (indicated by the dotted lines in Fig. 2) is significantly lowered by all three compounds, with chloroform having the greatest effect on a molar basis. It was important to determine the extent of the range of T_c in the presence of various concentrations of the compounds studied since we wished to ensure that the temperature range used in subsequent lysis experiments were sufficient to span the gel to liquid-crystal phase change even in the presence of the added ethanol or anaesthetics.

2. Effects on vesicular lysis

On cycling through the phase transition, channels are formed in the vesicles which allow rapid equilibration of the Pr^{3+} ions across the bilayer. However, only a fraction of the vesicles lyse during each cycle. This process is demonstrated in Fig. 3 where a sample of DPPC vesicles (5 mM extravesicular Pr^{3+}) has been cycled three times through T_c (60°C-30°C-60°C) over 60 min and the process repeated at the time intervals shown in Fig. 4. The effect on the ratio of the signals O/I is clearly seen (1.7 → 2.7). Addition of extra Pr^{3+} (20 mM) to the sample after lysis reveals the signal I' from the inside headgroup signal of the lysed vesicles (as shown in Fig. 3) while the remaining signal I arises from the unlysed vesicles. This also demonstrates that vesicular integrity is maintained during the lysis procedure. Thus from the change in O/I ratio the % lysed vesicles is readily calculated.

Fig. 4 shows a plot of the % lysis against time, when a series of cycles through T_c are used separated by incubation periods at 60°C. In each case the vesicles are seen to be impermeable to Pr^{3+} ions during the incubation periods at 60°C but on passing through T_c (three cyles in one hour) the % lysis increases. Fig. 4 also plainly shows the effect of ethanol and diethyl ether is to increase the lysis, while that of chloroform is to decrease the % of the vesicles which lyse. The temperature range used for the cycles through T_c were such as to ensure passage into the gel and liquid-crystalline phases as judged by the effects of the three compounds on the range of T_c as shown in Fig. 2.

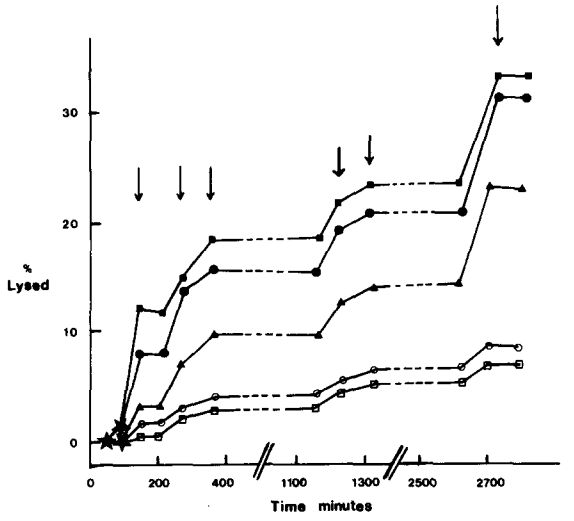

Fig. 4. The effect of ethanol, diethyl ether and chloroform on lysis of dipalmitoylphosphatidylcholine vesicles caused by cycling the vesicles through the phase transition temperature. ★ Indicates spectra taken during initial incubation period. Arrows ↓ indicate the times at which spectra were recorded at 60°C after three cycles through T_c. Other points indicate the times at which spectra were recorded at 60°C after an incubation period at 60°C, ▲———▲, control. Vesicles in the presence of: □———□, 86 mM chloroform; ○———○, 25 mM chloroform; ●———●, 86 mM ethanol; ■———■, 86 mM diethyl ether.

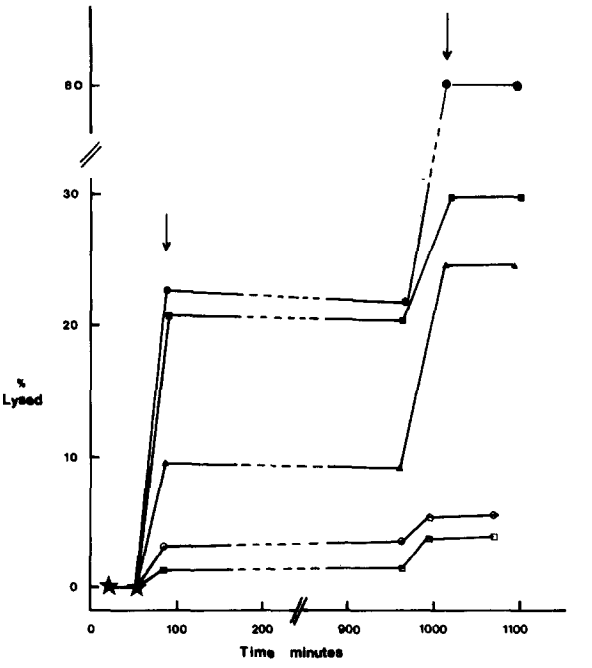

Fig. 5. The effect of ethanol, diethyl ether and chloroform on lysis of dipalmitoylphosphatidylcholine vesicles caused by cycling the vesicles, in the presence of 0.1 mM Triton X-100, through the phase transition. ★ Indicates spectra taken during initial incubation period. Arrows ↓ indicate the times at which spectra were recorded after one cycle through T_c. Other points indicate the times at which spectra were recorded after incubation at 60°C, ▲———▲, control. Vesicles in the presence of: □———□, 86 mM chloroform; ○———○, 25 mM chloroform; ■———■, 86 mM diethyl ether; ●———●, 86 mM ethanol.

During the lytic processes the peak width of the acyl chain signal (H) was monitored. Variations of only a few Hz were observed, indicating that no appreciable fusion of vesicles occurred over the duration of the experiment.

3. Vesicular lysis and fusion in the presence of Triton X-100

Similar experiments were carried out using vesicles in the presence of 0.1 mM Triton X-100. At this concentration Triton does not increase the diffusion permeability of the vesicles to Pr^{3+} [10]. The effect of this concentration of Triton X-100, however, greatly increases the lysis of the vesicles at the phase transition. Thus only one cycle through T_c over 20 min was used between each measurement of the *O/I* ratio and incubation period at 60°C. Fig. 5 shows the % lysis obtained for the control (Triton X-100 only) and in the presence of ethanol, diethyl ether and chloroform. The results are similar to those in Fig. 4 with chloroform strongly inhibiting the lysis even in the presence of Triton X-100.

The spectra recorded during these experiments showed a broadening of the acyl chain signal (H) and the signal width was measured in order to estimate the degree of fusion of the vesicles occurring. Increase in this signal width results from the decrease in tumbling rates of the larger vesicles produced by fusion, with a consequent loss of averaging of the dipolar relaxation which contributes to the linewidth. Prestegard and co-workers [16,17] have shown that the linewdith increases linearly with vesicle radius and that for complete 1 : 1 vesicle fusion (during which the radius of all the vesicles will increase by $\sqrt{2}$) the linewidth of the methylene acyl chain signal (H) increases by a factor 1.57. Our observed increases in the value of

$\nu_{1/2}$ for the acyl chain signals corresponding to the results shown at the end of the experiment in Fig. 5 were: control (17 to 24 Hz); diethyl ether (15 to 25 Hz); ethanol (18 to 28 Hz) and chloroform at 86 mM (14.4 to 17.4 Hz). The values of the % fusion calculated using Prestegard's factor 1.57 from the linewidth increases are, then: control 75%, diethyl ether 100%, ethanol 100%, chlorofrm 35%. Thus chloroform has the effect of inhibiting both the lysis and fusion promoted by the presence of Triton X-100.

It should be noted that this limited 1 : 1 fusion, in which the radius increases by $\sqrt{2}$, will have only a small effect on the O/I ratio. It can readily be calculated that when vesicles of membrane thickness 4 nm increase in radius from 14 nm to $14\sqrt{2}$ nm the O/I signal will decrease from 1.70 to 1.57. Thus the 75% fusion produced in the control sample of vesicles would cause a decrease in the O/I ratio from 1.7 to about 1.6. In contrast a 75% lysis of the vesicles would increase the O/I ratio from 1.7 to 9.8. The changes in O/I ratio used to calculate the % lysis in Fig. 5 are then almost entirely due to the lytic effect of Triton at T_c and are hardly affected by the limited fusion occurring.

4. Effect of ethanol and general anaesthetics on channels formed by Alamethicin 30 and on carrier-mediated transport by A23187

As we have shown previously and outlined in Materials and Methods, the time-dependent changes in the headgroup signals of the vesicles can be used to monitor transmembrane transport of Pr^{3+} by carrier [11–13] and pore-forming ionophores [13]. Fig. 6 illustrates the observed downfield movement of the intravesicular headgroup signal I during the transport of Pr^{3+} ions from outside to inside the vesicles by Alamethicin 30 in the presence of 5 mM $CHCl_3$. The uniform downfield movement of signal I indicates that equal amounts of Pr^{3+} are transported into all vesicles. These shifts of signal I are converted into intravesicular concentrations of Pr^{3+} using a calibration graph [12]. Data obtained as in Fig. 6 can then be converted into the time-dependence of the transport of Pr^{3+} into the vesicles.

Plots of the intravesicular concentration of Pr^{3+} against time are shown in Fig. 7 which illustrates the data obtained using 20 μg Alamethicin 30 per 1 ml of vesicular solution (10 mg DPPC) and shows the effect of various concentrations of

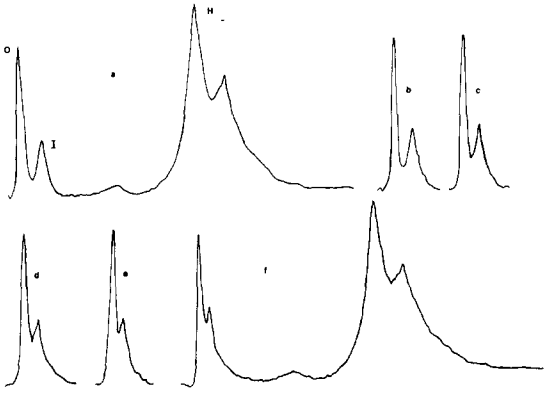

Fig. 6. Time-dependent downfield shifts of the intravesicular headgroup signal (I) from phosphatidylcholine vesicles at 50°C in the presence of 5 mM extravesicular Pr^{3+}, 5 mM chloroform and Alamethicin 30 (20 μg per 10 mg DPPC). The spectra show the result of transport of the Pr^{3+} from outside to inside the vesicles at the following intervals after the addition of the 5 mM Pr^{3+}: (a) 3.0 min; (b) 7.0 min; (c) 13.25 min; (d) 24.0 min; (e) 35.5 min; (f) 46.0 min. The full spectrum is given in (a) and (f) to indicate the lack of broadening of the acyl chain signal H during the transport of Pr^{3+}.

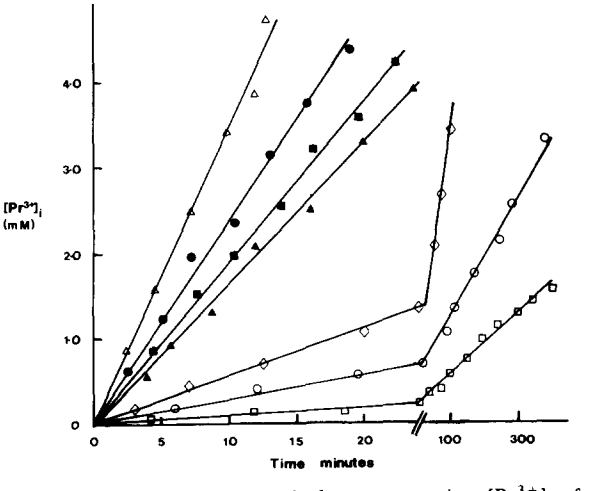

Fig. 7. Increase in the intravesicular concentration, $[Pr^{3+}]_i$ of Pr^{3+} as a function of time at 50°C using 20 μg Alamethicin 30 per 1 ml of vesicles (10 mg DPPC) and an extravesicular concentration of 5 mM Pr^{3+}. ▲———▲, control. Vesicles in the presence of: □———□, 86 mM chloroform; ○———○, 25 mM chloroform; ◇———◇, 5 mM chloroform; ■———■, 86 mM diethyl ether; ●———●, 86 mM ethanol; △———△, 172 mM ethanol.

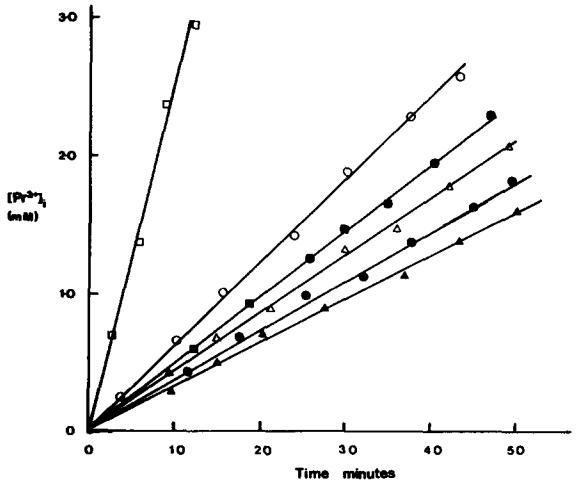

Fig. 8. Increase in the intravesicular concentration, $[Pr^{3+}]_i$ of Pr^{3+} as a function of time at 50°C using 10 μg A23187 per 1 ml of vesicles (10 mg DPPC) and 5 mM extravesicular Pr^{3+}. ▲———▲, control. Vesicles in the presence of: ●———●, 86 mM ethanol; △———△, 172 mM ethanol; ■———■, 86 mM diethyl ether; ○———○, 25 mM chloroform; □———□, 86 mM chloroform.

ethanol, diethyl ether and chloroform on the channel-mediated transport. The plots are seen to be linear and the slopes therefore give the rates of transport of Pr^{3+} across the vesicular bilayer. Chloroform strongly inhibits the rate of transport, the effect being marked down to the lowest concentration studied, 5 mM. Also the action of ethanol and diethyl ether is similar to their effects on vesicle lysis in that channels are again stabilised to allow increase in transport of the Pr^{3+}.

As a comparative experiment using a carrier-type ionophore, the effects of these same compounds on A23187-mediated transport was measured. The results are shown in Fig. 8. It is significant that chloroform increases the transport rate, in contrast to the channel-mediated mechanisms. The effect of ethanol and diethyl ether is, however, again to stimulate transport rates. Similar experiments were also performed with the carrier ionophore ionomycin (results not shown) and again all three compounds increased the transport rate.

Discussion

Our observation that chloroform decreases the linewidth of the lipid signals agrees with previous studies by Shieh et al. [29] and Vanderkooi and co-workers [7] and the implication is that the anaesthetic fluidises the bilayer. The lowering of the phase transition temperature has also been previously observed for all three compounds [7] and other fluorinated ether anaesthetics [30] and the effect is ascribed to a disordering of the acyl chains. The observed upfield shift of the outer headgroup signal *O* implies that there is sufficient chloroform in the bilayer-water boundary region to influence the binding of the Pr^{3+} to the phosphate sites on the outer headgroups of the vesicles. A similar deduction was made by Koehler et al. [31] who observed that Gd^{3+} caused changes in the linewidth of the ^{19}F-NMR signal from halothane only in the presence of phosphatidylcholine liposomes. These experiments indicate that anaesthetic and lanthanide ion coexist at the membrane surface.

The mechanism by which the permeability of lipid bilayers is increased at the phase transition is still unresolved. Permeable boundary layers between coexisting gel and fluid domains or an increase in the lateral compressibility of the bilayer have both been proposed and discussed [32]. Observations using planar lipid bilayers show large conductance changes via transient single channels indicating that a few defects of quite extended size occur at T_c [33,34]. Our previous observations [8,10] and the results reported here confirm this since in DPPC vesicles the channels which open at T_c are sufficiently large to allow equilibration of the 5 mM Pr^{3+} across the phospholipid bilayer. We have described this process as vesicular lysis, but it should be noted that as demonstrated in Fig. 3, the vesicular membrane integrity is not lost and impermeability is restored on raising the temperature above T_c.

Our observation that ethanol and diethyl ether both increase the extent of lysis at T_c, while chloroform decreases it, may result from a change in the activation energy for the opening of the channels (resulting in more or less channels being opened per passage through T_c) or from channels being stabilised or extended by the ethanol and diethyl ether and destabilised by chloroform. A number of authors have implicated the effect of water penetration and interaction into the lipid bilayer in modifying the energy barrier to ionic

permeability [22,23]. Also in the case of gel areas dispersed in fluid, the boundary effect will induce increased water penetration into the acyl chain region of the bilayer [35]. A reasonable hypothesis consistent with our observations would therefore be that the effects of the three compounds on vesicular lysis is mediated by their action on the structure or properties of the water which is associated with the channels.

Both our preliminary studies using vesicles [10] and work elsewhere with planar membranes [14] suggest that at low concentrations near its critical micelle concentration (approx. 0.25 mM), Triton X-100 stabilises channels formed in lipid bilayers. The results reported above confirms this (compare the control vesicle lysis in Figs. 4 and 5, noting the much smaller number of cycles through T_c used in the presence of Triton). The effect of the three compounds studied is even more marked for Triton-stabilised channels (Fig. 5) than for Triton-free channels (Fig. 4). Again the striking feature is increased lysis by the oxygen-containing compounds and decreased lysis by chloroform.

Triton X-100, like all molecules containing polyoxyethylene groups including poly(ethylene glycol), is strongly hydrated in solution. The channels which are stabilised by Triton will be lined by this amphipathic molecule, so introducing an additional water content to the channel. The strong effect of ethanol, diethyl ether and chloroform on these channels again implies that their interaction with the channel water is significant in determining their action on lytic channels. The fusongenic action of Triton X-100 [10,15] is probably mediated by inverted micelles of Triton and lipid being formed between the fusing bilayers, if the general mechanism of fusion suggested by Cullis and de Kruijff [36] is applicable in this case. The inverted micelles will contain water and our suggestion is that the observed effects of ethanol and diethyl ether in promoting fusion, and chloroform in inhibiting it, can be mediated by their effects on the inverted micelle water or the interaction of the water with the phospholipid headgroups.

The polypeptide, channel forming, ionophore Alamethicin 30 has been extensively studied by conductivity measurements using planar lipid bilayers, and the work recently reviewed [37]. Following the observation that this ionophore could also transport lanthanide ions across planar membranes [38] we used the NMR method described above to examine Alamethicin 30 mediated transport of Pr^{3+} across vesicular membranes [13]. Detailed structural information based on X-ray analysis and model building of Alamethicin is now available [24]. These studies indicate that in the channel formed by the aggregation of four or more monomers hydrogen bonding is extensive, especially at the annulus formed by Gln 7 residues. The Gln 7 side-chain amide protons will donate a hydrogen bond to water forming a ring of hydrogen-bonded water, the structure of which effectively controls the size of ion channel. Changes in this hydrogen-bonded water provide an explanation of the observation that the higher conductance substates of the Alamethicin channels are only moderately sensitive to cation radius [39] which implies that the current increases are due to changes in channel cross-section rather than increase in the number of parallel open channels. Given that halogenated hydrocarbon anaesthetics such as chloroform have been shown by infra-red methods [40] to perturb hydrogen bonds in membranes, the hydrogen bonded water content of the Alamethicin channels must be regarded as a likely target for the observed effects of ethanol and the two general anaesthetics, as indicated in Fig. 7. In contrast to the channel-mediated transport, when A23187 or Ionomycin are used, all three compounds, alcohol, ether and chloroform, increase the rate of transport as seen in Fig. 8.

In these initial experiments we were not rigorously attempting to explore the action of these compounds at clinical levels of concentration for general anaesthesia. These latter concentrations are difficult to obtain experimentally but quoted values are of the order 50 mM ethanol, 15 mM diethyl ether and 1 mM chloroform [19,41]. Seeman indicates that concentrations required for nerve block (as in local anaesthesia) are ten times these values [41]. For ethanol we have therefore used physiological relevant concentrations. The lowest values of chloroform studied were 5 mM for Alamethicin transport. The large effect on the transport rate observed indicates that inhibition will be expected down to physiological concentrations for this type of anaesthetic.

Taken together the similarities in the effects of

the three compounds on each channel system imply a common element in the mechanism. Since for each mechanism of channel formation studied, the importance of water structure and function in the channels is strongly indicated, it is difficult to avoid the implication that the effect of the compounds on channel water is the unifying feature of the results.

It would clearly be premature to claim to have demonstrated a possible novel mechanism of general anaesthesia on the basis of a small range of compounds and model systems. However, the lipid-based theories of general anaesthesia [18] have recently been strongly and effectively criticised [2,19] and direct protein-anaesthetic action is seen to be unlikely on structural grounds [19]. Our results suggest a modification of the protein hypothesis which would be worthy of further investigation, namely that an important locus of action of the general anaesthetics could be their effect on water structure and function in the ion channels at synapses in the central nervous system. We are aware that water structure was previously proposed as a mechanism of anaesthesia by Pauling [42] and Miller [43], but these theories were based on a direct effect of the hydrophilic anaesthetic on Ice I clathrate structures. We are proposing a more general interaction of the type put forward by Eyring and co-workers [44] which would include a variable effect on hydrogen bonding depending on the chemical nature of the anaesthetic. A recent theoretical model of ion channels which accounts well for the electrical properties of Na^+ and K^+ channels, is based on a hydrogen-bonded ordered water channel at a protein site spanning the membrane [45,46]. Experiments such as those described here begin to provide specific tests for this theory.

Richards [2] has pointed out that it is very unlikely that all general anaesthetics act at a single type of site. Clearly, further experiments on a wide range of anaesthetics, systems of membrane channels and fusion sites will be required to reinforce the hypothesis that effects on water structure and properties are important in the mechanism of anaesthesia.

Acknowledgement

The support of the Sir Halley Stewart Trust in part of this work is gratefully acknowledged, as also the efficiency of Mrs. M. Morris in preparing the manuscript.

References

1 Bowman, W.C. and Rand, M.J. (1980) Textbook of Pharmacology, 2nd Edn., Blackwell, Chapter 7
2 Richards, D.C. (1980) Mechanisms of General Anaestheisa, in Topical Reviews in Anaesthesia, Vol. 1 (Norman, J. and Whitwam, J.G., eds.), pp. 1–84, J. Wright and Sons, Bristol
3 Barker, J.L. (1975) Brain Res. 92, 35–55
4 Hendry, B.M., Urban, B.W. and Haydon, D.A. (1978) Biochim. Biophys. Acta 513, 106–116
5 Dluzewski, A.R. and Halsey, M.J. (1981) Br. J. Anaesth. 53, 184P
6 Pope, C.G., Urban, B.W. and Haydon, D.A. (1982) Biochim. Biophys. Acta 688, 279–283
7 Vanderkooi, J.M., Landberg, R., Selick, H. and McDonald, G.G. (1977) Biochim. Biophys. Acta 464, 1–16
8 Hill, M.W. (1975) Biochem. Soc. Trans. 3, 149–152
9 Hunt, G.R.A. and Tipping, L.R.H. (1978) Biochim. Biophys. Acta 507, 242–261
10 Hunt, G.R.A. (1980) FEBS Lett. 119, 132–136
11 Hunt, G.R.A. (1975) FEBS Lett. 58, 194–196
12 Hunt, G.R.A., Tipping, L.R.H. and Belmont, M.R. (1978) Biophys. Chem. 8, 341–355
13 Hunt, G.R.A. and Jones, I.C. (1982) Biosci. Rep. 2, 921–928
14 Schlieper, P. and De Robertis, E. (1977) Arch. Biochem. Biophys. 184, 204–208
15 Alonso, A., Villena, A. and Goni, F.M. (1981) FEBS Lett. 123, 200–204
16 Gent, M.P.N. and Prestegard, J.H. (1974) Biochemistry 13, 4027–4033
17 Liao, M.-J. and Prestegard, J.H. (1980) Biochim. Biophys. Acta 599, 81–94
18 Janoff, A.S. and Miller, K.W. (1982) A Critical Assessment of the Lipid Theories of General Anaesthesia in Biological Membranes, Vol. 4 (Chapman, D., ed.), pp. 417–476, Academic Press, New York
19 Franks, N.P. and Lieb, W.R. (1982) Nature 300, 487–493
20 Fink, B.R. (ed.) (1980) Molecular Mechanisms of Anaesthesia (Progress in Anaesthesiology, Vol. 2), Raven Press, New York
21 Kenehisa, M.I. and Tsong, T.Y. (1978) J. Am. Chem. Soc. 100, 424–432
22 Griffith, O.H., Dehlinger, P.J. and Van, S.P. (1974) J. Membrane Biol. 15, 159–192
23 Israelachvili, J.N., Marcelja, S. and Horn, R.G. (1980) Q. Rev. Biophys. 13, 121–200
24 Fox, R.O. and Richards, F.M. (1982) Nature 300, 325–330
25 Hunt, G.R.A. and Tipping, L.R.H. (1980) J. Inorg. Biochem. 12, 17–36
26 Bergelson, L.D. (1978) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 9, pp. 275–335, Plenum Press, New York
27 Hutton, W.C., Yeagle, P.L. and Martin, R.B. (1977) Chem. Phys. Lipids 19, 255–265
28 Lee, A.G. (1975) Prog. Biophys. Mol. Biol. 29, 3–56

29 Shieh, D.D., Ueda, I., Lim, H.-C. and Eyring, H. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3999–4002
30 Koehler, K.A., Jain, M.K., Stone, E.E., Fossel, E.T. and Koehler, L.S. (1978) Biochim. Biophys. Acta 510, 177–185
31 Koehler, L.S., Fossel, E.T. and Koehler, K.A. (1977) Biochemistry 16, 3700–3707
32 Marcelja, S. and Wolfe, J. (1979) Biochim. Biophys. Acta 557, 24–31
33 Antonov, V.F., Petrov, V.V., Molnar, A.A., Prevoditelev, D.A. and Ivanov, A.S. (1980) Nature 283, 585–586
34 Boheim, G., Hanke, W. and Eibl, H. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3402–3407
35 Fettiplace, R. and Haydon, H. (1980) Physiol. Rev. 60, 510–550
36 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
37 Lattore, R. and Alvarez, O. (1981) Physiol. Rev. 61, 77–150
38 Gögelein, H., De Smedt, H., Van Driessche, W. and Borghgraef, R. (1981) Biochim. Biophys. Acta 640, 185–194
39 Eisenberg, M., Kleinberg, M.E. and Shaper, J.H. (1977) Ann. N.Y. Acad. Sci. 303, 281–291
40 Sandorfy, C. (1980) in Molecular Mechanisms of Anaesthesia, Progress in Anaesthesiology, Vol. 2 (Fink, B.R., ed.), pp. 353–359, Raven Press, New York
41 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655
42 Pauling, L. (1961) Science 134, 15–21
43 Miller, S.L. (1961) Proc. Natl. Acad. Sci. U.S.A. 47, 1515–1524
44 Kamaya, H., Ueda, I. and Eyring, H. (1980) in Molecular Mechanisms of Anaesthesia, Progress in Anaesthesiology, Vol. 2 (Fink, B.R., ed.), pp. 429–433, Raven Press, New York
45 Edmonds, D.T. (1980) Proc. R. Soc. Lond. B211, 51–62
46 Edmonds, D.T. (1981) Trends Biochem. Sci. 6, 92–94