Pressure-Induced Exclusion of a Local Anesthetic from Model and Nerve Membranes†

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ABSTRACT: Because it is well established that the anesthetic state can be reversed by pressure, a number of molecular theories that have been proposed for the mechanism of action of both local and general anesthetics can be tested by varying the pressure. Using Fourier transform infrared spectroscopy, we report here the first direct observation of the expulsion from lipid bilayers of a local anesthetic, tetracaine, by pressure. Moreover, we establish for the first time that this phenomenon is common to both model membranes and to myelinated and unmyelinated nerve membranes, vindicating the utility of model membrane systems. A distinctive feature of this behavior in model systems is that, in saturated phosphatidylcholines at high pH, expulsion only occurs in the presence of cholesterol, whose ordering effect on the acyl chains evidently assists pressure in squeezing the anesthetic out of the bilayer. This pressure-induced phenomenon may provide insight into the molecular mechanisms underlying the antagonistic effect of pressure against anesthesia.

The molecular mechanism of anesthesia is poorly understood and will remain so until the target sites for anesthetic action in the membrane have been determined (Franks & Lieb, 1982). An important clue in elucidating the molecular mechanism of anesthetic action and in discriminating between lipid and protein as the primary site of anesthetic action is the well-established antagonistic effect of hydrostatic pressure against anesthesia in vivo (Lever et al., 1971; Wann & Macdonald, 1980). This phenomenon has been attributed to the effects of pressure on membrane fluidity (Trudell et al., 1973) or membrane-bound proteins (Jaenicke, 1983), although the possibility that anesthetics are simply expelled from the membrane by pressure, or squeezed away from their target sites (Franks & Lieb, 1982) (be they lipid or protein), has been suggested but never directly confirmed (Trudell et al., 1973; Seelig, 1987).

A particularly convenient way of monitoring changes in the structural and dynamic properties of lipid bilayers induced by pressure is Fourier transform infrared (FT-IR)[1] spectroscopy (Wong, 1987a). The recent development of high-pressure optical cells suitable for aqueous media (Wong, 1987b; Wong et al., 1985) has spurred much of the recent high-pressure vibrational spectroscopic studies of model membrane systems (Wong, 1987a). The use of these cells, which require small sample sizes, enables a straightforward extension of the technique from model to biological systems, as we now describe. In order to see if pressure can induce the expulsion of a local anesthetic, tetracaine, from phosphatidylcholine bilayers in the presence and absence of cholesterol and from nerve membranes, we have monitored the carbonyl stretching infrared band of tetracaine as a function of pressure from 0.001 (1 atm) to 25 kbar. Since the frequency and shape of an ester carbonyl stretching band are sensitive to its environment, this band can be used to probe environmental changes (Mushayakarara et al., 1986). The results indicate that the local anesthetic is expelled by pressure from both model and nerve membranes. For model membrane systems, they also demonstrate that low pH or cholesterol assists pressure in squeezing the anesthetic out of the bilayer.

Experimental Procedures

To prepare lipid dispersions for study by FT-IR, 100 mg of lipid was hydrated with 0.1 mL of a borate–phosphate–citrate buffer (Kelusky & Smith, 1984) made with D_2O and containing about 10 mg of tetracaine. To ensure complete equilibration of the anesthetic in the lipid bilayers, the dispersions were subjected to at least five freeze–thaw cycles (Kelusky & Smith, 1984). Small amounts (typically <0.01 mg) of the homogeneous dispersions resulting from these freeze–thaw cycles were then placed at room temperature, together with powdered α-quartz, in a 0.37 mm diameter hole in a 0.23 mm thick stainless steel gasket mounted on a diamond anvil cell, as described previously (Wong et al., 1985). Prior to the infrared experiments, nerves were soaked for 2 h in a physiological solution (pH 7.4) (Pézolet & Georgescauld, 1985) made with D_2O in the presence and in the absence of the local anesthetic (1% tetracaine). Infrared spectra were measured at 28 °C on a Bomem Model DA3.02 Fourier transform spectrophotometer with a liquid nitrogen cooled mercury cadmium telluride detector. The infrared beam was condensed by a sodium chloride lens system onto the pinhole of the diamond anvil cell (Mao et al., 1982/1983). For each spectrum, typically 512 scans were coadded, at a spectral resolution of 4 cm^{-1} (corresponding to a total measuring time per spectrum of about 10 min). Pressures were determined from the 695-cm^{-1} phonon band of α-quartz, by using data reduction methods described previously (Wong et al., 1985; Siminovitch et al., 1987).

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[1] Abbreviations: FT-IR, Fourier transform infrared; TTC, tetracaine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; ^2H NMR, deuterium nuclear magnetic resonance.

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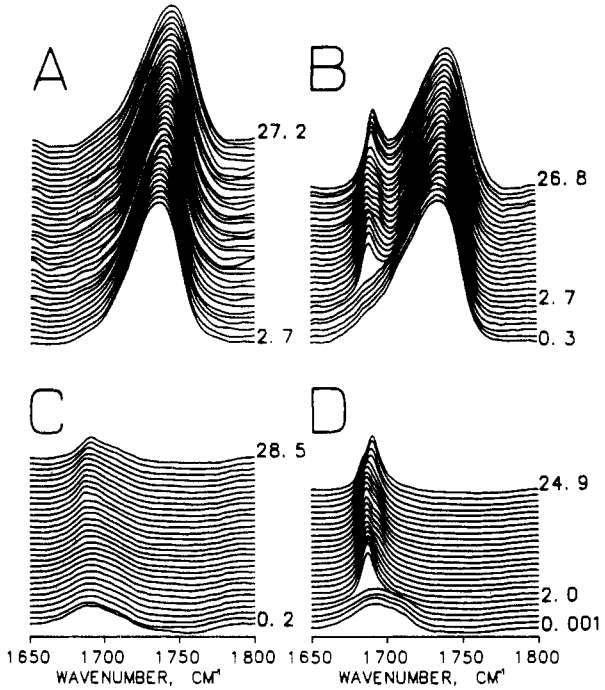

FIGURE 1: Stacked contour plots of infrared spectra of 1,2-dimyristoylphosphatidylcholine (DMPC) and 1,2-di-*O*-hexadecylphosphatidylcholine (DHPC) in the C=O stretching region as a function of hydrostatic pressure at high pH (pH 9.5). Note that the abrupt appearance of the well-defined tetracaine (TTC) C=O band at approximately 1685 cm^{-1} in the spectra of the DMPC/TTC and DHPC/TTC systems occurs only in the presence of cholesterol (right column). To the right of each contour plot, the lowest and highest pressures in kilobars are indicated and, when appropriate, the pressure of the contour that first reflects the bandwidth change of the tetracaine C=O band. (A) DMPC + TTC; (B) DMPC + cholesterol + TTC; (C) DHPC + TTC; (D) DHPC + cholesterol + TTC.

Results and Discussion

Figure 1 shows the effect of a physiological concentration of cholesterol on the pressure dependence of the infrared spectra of an ester-linked (DMPC) and an ether-linked (DHPC) phospholipid. In both lipid systems, the local anesthetic tetracaine was partitioned into the lipid bilayers in the uncharged form (pH 9.5). The partition coefficient K_p, which is the ratio of the anesthetic concentration in the lipid phase to that in the buffer (expressed in grams of anesthetic per gram of each phase), is about 600 for uncharged tetracaine in phosphatidylcholine bilayers (Boulanger et al., 1980). Previous ^2H nuclear magnetic resonance (NMR) studies of tetracaine in egg phosphatidylcholine bilayers have suggested that the uncharged form at high pH penetrates more deeply into the bilayer than the charged form at low pH (Boulanger et al., 1981). This model is entirely consistent with the pressure dependence of the DMPC infrared spectra shown in Figure 1A. In the spectral region between 1650 and 1800 cm^{-1}, and over the pressure range from 0.001 to 25 kbar, the spectra of this system are dominated by the relatively intense ester C=O stretching mode band at 1735 cm^{-1} (Mushayakarara et al., 1986). The shoulder on the low-frequency side of the lipid C=O band is due to the very broad C=O band of tetracaine in the disordered environment of the lipid bilayer (vide infra), a spectral feature that undergoes no apparent change with increasing pressure. Thus, except for the slight increase in the lipid C=O band frequency with increasing pressure, there are no pressure-induced changes in this spectral region, indicating that, in the absence of cholesterol, the relatively deep penetration of tetracaine into the bilayer at high pH cannot be modified by pressures up to 25 kbar. Note that just the presence of gel phase lipid [pressure induced (Wong, 1987a)] is not sufficient to exclude the anesthetic.

^2H NMR studies of systems containing cholesterol suggest that, even at high pH, local anesthetics may be "squeezed" into a hydrophobic site closer to the lipid–water interface and that the partition coefficient is reduced compared to that obtained for the pure lipid (110 vs. 600) (M. Auger, H. C. Jarrell, and I. C. P. Smith, unpublished results). If tetracaine is closer to the interface in the presence of cholesterol, could pressure expel the anesthetic completely out of the bilayer, taking its C=O group from a disordered hydrophobic to an isotropic aqueous environment?. To address this question, DMPC/TTC containing 30 mol % cholesterol was examined as a function of pressure. The FT-IR spectra of this system are shown in Figure 1B. By comparison of the stacked contour plots of parts A and B of Figure 1, it is readily apparent that a striking change occurs at 2.7 kbar in the spectra of the system containing cholesterol. The broad band at 1696 cm^{-1}, which we have attributed to the tetracaine C=O group, undergoes an abrupt decrease in bandwidth. To demonstrate that this C=O band arises from tetracaine, we have examined the DHPC/TTC system under the same conditions as those used for the DMPC/TTC system. With the spectral region between 1650 and 1800 cm^{-1} now free of the intense C=O band of the ester-linked DMPC (Siminovitch et al., 1987), it is evident from the spectra of the DHPC/TTC system shown in parts C and D of Figure 1 that the band at 1696 cm^{-1} must originate from tetracaine. Note in particular that, without cholesterol (Figure 1C), this band at approximately 1696 cm^{-1} remains very broad, whereas with cholesterol (Figure 1D), a sudden decrease in bandwidth occurs at 2.0 kbar, similar to that observed in the DMPC/TTC system. Not only do these DHPC/TTC spectra (Figure 1C, D) prove that the band at 1696 cm^{-1} is due only to the carbonyl group of tetracaine but also, by showing that the influence of cholesterol on the pressure behavior of this band is very similar in both ester- and ether-linked lipids, they rule out any specific role of the lipid ester carbonyl groups. The dramatic change in the bandwidth of the tetracaine C=O band at a particular critical pressure in the lipid/tetracaine systems containing cholesterol is due to the complete exclusion of the anesthetic from the lipid bilayer, since above this critical exclusion pressure, the shape of the tetracaine band and its response to increasing pressure duplicate those observed from pure tetracaine in solution, as shown in Figure 2. Note also that the tetracaine C=O band frequency in solution (or, equivalently, above the critical exclusion pressure) is about 1685 cm^{-1}, whereas in the lipid bilayer, or in a similar hydrocarbon environment such as hexane, this frequency is about 1696 cm^{-1}.

For tetracaine partitioned into DMPC bilayers in its charged form ($K_p \approx 22$ at pH 5.5), the application of pressure eventually does expel the anesthetic even without cholesterol, as shown in Figure 3A. Previous ^2H NMR studies suggest that the charged form of tetracaine at low pH is located near the lipid–water interface (Boulanger et al., 1981). In the presence of cholesterol (Figure 3B), the anesthetic ($K_p \approx 8$) is squeezed even closer to the lipid–water interface, and therefore the critical pressure required for this expulsion is significantly lower. To summarize, Figures 1 and 3 demonstrate that low pH or cholesterol will assist pressure in squeezing the anesthetic out of the bilayer, since in either case the anesthetic molecule is closer to the lipid–water interface.

It is nearly a century since Regnard (1887) made the first observations of the effects of high hydrostatic pressure on the excitability of nerve, but only recently has there been a con-

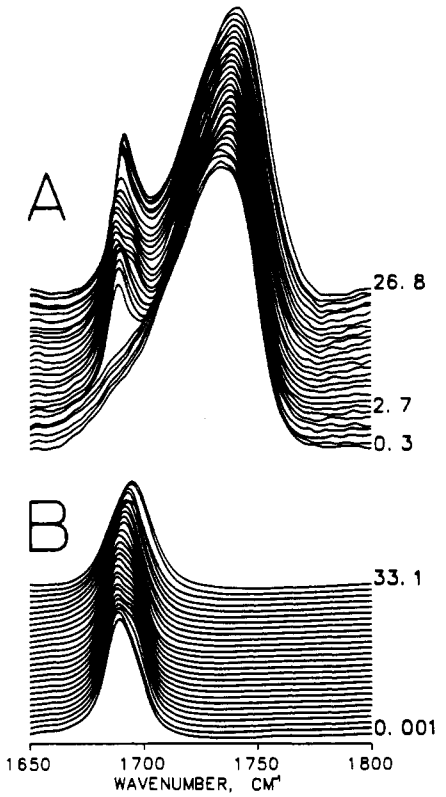

FIGURE 2: A comparison of the pressure-dependent behavior of the C=O band of tetracaine in lipid bilayers and in solution: (A) DMPC + cholesterol + TTC, pH 9.5; (B) TTC in D_2O, pH 9.5.

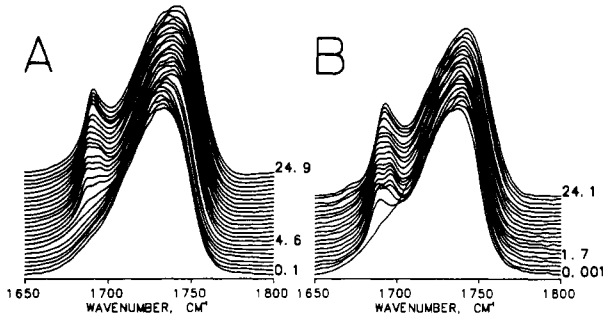

FIGURE 3: Stacked contour plots of infrared spectra of DMPC/TTC in the C=O stretching region as a function of hydrostatic pressure at low pH (pH 5.5): (A) without cholesterol; (B) with cholesterol (30 mol %).

certed attempt to understand these effects at a molecular level (Wann & Macdonald, 1980). One of the most puzzling aspects of barotropic phenomena in excitable cells is the well-established reversal by pressure of local (Halsey & Wardley-Smith, 1975; Kendig & Cohen, 1977) or general (Roth, 1979) anesthesia. The expulsion of local anesthetics by pressure from model membrane systems described above would assume a much larger significance if the same phenomenon were observed in nerve membranes. We have performed the first infrared experiments on myelinated (frog sciatic) and unmyelinated (lobster) nerves as a function of pressure, and we show here that the application of hydrostatic pressure also leads to an expulsion of the anesthetic from the lipid bilayers of these nerve membranes. The pressure dependence of the infrared spectra of frog sciatic nerve in the absence and in the presence of tetracaine is compared in parts A and B of Figure 4, respectively. Similar to the model systems, the tetracaine C=O band at low pressures is very broad (Figure 4B) but undergoes an abrupt reduction in bandwidth at a critical pressure of approximately 5 kbar. Since these changes mimic

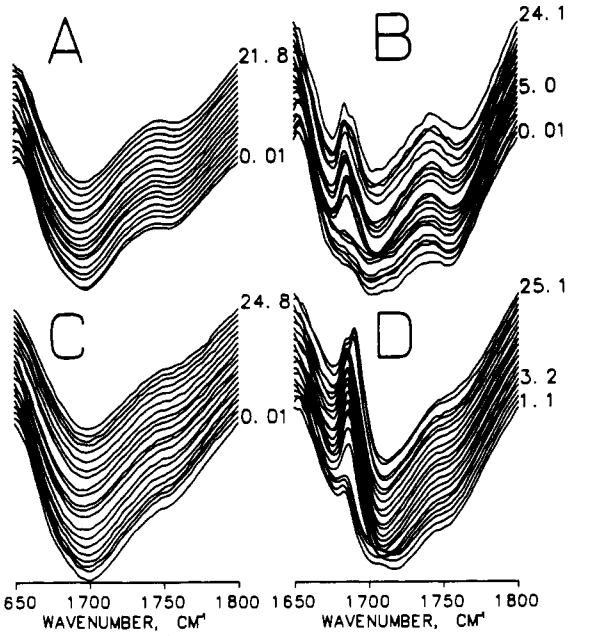

FIGURE 4: Pressure dependence of infrared spectra in the C=O stretching region of nerves at physiological pH (pH 7.4) in the absence (left column) and in the presence (right column) of tetracaine: (A) frog sciatic nerve; (B) frog sciatic nerve + TTC; (C) lobster nerve; (D) lobster nerve + TTC.

those observed in the model systems, we can conclude that tetracaine, partitioned into the nerve membrane at atmospheric pressure and located close to the lipid–water interface, is expelled by pressure. Since the myelin membrane is approximately 75% lipid (Norton, 1984; Morell, 1984), a large proportion of which (about 40 mol %) is cholesterol (Norton, 1981), it is not unreasonable to suggest that, in this system, cholesterol may also play a role in assisting pressure to squeeze out the anesthetic. As shown in parts C and D of Figure 4, over the same pressure range, the pressure behavior of the C=O band of tetracaine partitioned into lobster nerve membranes is similar to that observed in either the frog sciatic nerve spectra (Figure 4A, B) or those of the model systems (Figure 1). Thus, even though the lobster nerves have a lower proportion of membrane (Pézolet & Georgescauld, 1985), it is evident from a comparison of parts C and D of Figure 4 that tetracaine does partition into the nerve, in an environment close to the lipid–water interface, and that pressure will also expel the anesthetic from this membrane. Moreover, for both model and nerve membranes, the pressure-induced exclusion of tetracaine was found to be reversible. In this study, we have simply demonstrated that pressure can exclude anesthetics from membranes. The fact that the exclusion pressure can be as much as an order of magnitude larger than that required in vivo for pressure reversal of anesthesia may indicate that this phenomenon requires only a pressure-induced change in anesthetic location within the membrane, and not complete exclusion. Further experimental work on this problem using high-pressure FT-IR is now underway in our laboratory.

CONCLUSIONS

In model systems, it is clear that both pH and the presence of cholesterol play important roles in determining the ability of pressure to exclude the anesthetic, but in the heterogeneous environment found in the lipid bilayers of nerve membranes, other factors may predominate. Nevertheless, the unity of the pressure-induced phenomenon observed in both model and biological membranes demonstrated in this study is significant and indicates that further experiments using this novel high-

pressure vibrational spectroscopic technique on nerve membranes may lead to a better understanding of barotropic phenomena in these tissues, and of the mechanism of anesthesia.

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REFERENCES

Boulanger, Y., Schreier, S., Leitch, L. C., & Smith, I. C. P. (1980) *Can. J. Biochem. 58*, 986-995.
Boulanger, Y., Schreier, S., & Smith, I. C. P. (1981) *Biochemistry 20*, 6824-6830.
Franks, N. P., & Lieb, W. R. (1982) *Nature (London) 300*, 487-493.
Halsey, M. J., & Wardley-Smith, B. (1975) *Nature (London) 257*, 811-813.
Jaenicke, R. (1983) *Naturwissenschaften 70*, 332-341.
Kelusky, E. C., & Smith, I. C. P. (1984) *Can. J. Biochem. Cell Biol. 62*, 178-184.
Kendig, J. J., & Cohen, E. N. (1977) *Anesthesiology 47*, 6-10.
Lever, M. J., Miller, K. W., Paton, W. D. M., & Smith, E. B. (1971) *Nature (London) 231*, 368-371.
Mao, H. K., Bell, P. M., Xu, J., & Wong, P. T. T. (1982/1983) *Year Book—Carnegie Inst. Wash. No. 82*, 419-421.
Morell, P. (1984) *Myelin*, Plenum, New York.
Mushayakarara, E. C., Wong, P. T. T., & Mantsch, H. H. (1986) *Biochim. Biophys. Acta 857*, 259-264.
Norton, W. T. (1981) *Adv. Neurol. 31*, 93-121.
Norton, W. T. (1984) *Adv. Neurochem. 5*, 47-75.
Pézolet, M., & Georgescauld, D. (1985) *Biophys. J. 47*, 367-372.
Regnard, P. (1887) *C. R. Seances Soc. Biol. Ses Fil. 39*, 406-409.
Roth, S. H. (1979) *Annu. Rev. Pharmacol. Toxicol. 19*, 159-178.
Seelig, A. (1987) *Biochim. Biophys. Acta 899*, 196-204.
Siminovitch, D. J., Wong, P. T. T., & Mantsch, H. H. (1987) *Biophys. J. 51*, 465-473.
Trudell, J. R., Hubbell, W. L., Cohen, E. N., & Kendig, J. J. (1973) *Anesthesiology 38*, 207-211.
Wann, K. T., & Macdonald, A. G. (1980) *Comp. Biochem. Physiol. A 66A*, 1-12.
Wong, P. T. T. (1987a) in *High Pressure Chemistry and Biochemistry* (van Eldik, R., & Jonas, J., Eds.) pp 381-400, D. Reidel, Dordrecht, The Netherlands.
Wong, P. T. T. (1987b) *Vib. Spectra Struct. 16*, 357-445.
Wong, P. T. T., Moffatt, D. J., & Baudais, F. L. (1985) *Appl. Spectrosc. 39*, 733-735.

Articles

Complete cDNA Sequence of Human Complement C1s and Close Physical Linkage of the Homologous Genes C1s and C1r

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ABSTRACT: Overlapping molecular clones encoding the complement subcomponent C1s were isolated from a human liver cDNA library. The nucleotide sequence reconstructed from these clones spans about 85% of the length of the liver C1s messenger RNAs, which occur in three distinct size classes around 3 kilobases in length. Comparisons with the sequence of C1r, the other enzymatic subcomponent of C1, reveal 40% amino acid identity and conservation of all the cysteine residues. Beside the serine protease domain, the following sequence motifs, previously described in C1r, were also found in C1s: (a) two repeats of the type found in the Ba fragment of complement factor B and in several other complement but also noncomplement proteins, (b) a cysteine-rich segment homologous to the repeats of epidermal growth factor precursor, and (c) a duplicated segment found only in C1r and C1s. Differences in each of these structural motifs provide significant clues for the interpretation of the functional divergence of these interacting serine protease zymogens. Hybridizations of C1r and C1s probes to restriction endonuclease fragments of genomic DNA demonstrate close physical linkage of the corresponding genes. The implications of this finding are discussed with respect to the evolution of *C1r* and *C1s* after their origin by tandem gene duplication and to the previously observed combined hereditary deficiencies of C1r and C1s.

The complement C1 subcomponents C1r and C1s represent a distinct class of serine protease zymogens because of their ability to form a calcium-dependent tetrameric complex (C1s-C1r-C1r-C1s), which interacts with the nonenzymatic subcomponent C1q to yield the ordered C1 structure (Colomb et al., 1984). The activation of C1 is a tightly controlled process triggered by the binding of C1q to immune complexes or to certain nonimmune activators. A fundamental feature of this process is the ability of C1r to autoactivate. As a result, the C1s proenzyme is converted to its active form, which in turn triggers the classical pathway of complement by virtue

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