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NMR EVIDENCE FOR THE HYDROPHOBIC INTERACTION OF LOCAL ANAESTHETICS

POSSIBLE RELATION TO THEIR POTENCY

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SUMMARY

Local anaesthetics molecules with non-polar residues of variable chain length attached to the polar functional group (protonated quaternary amine) or at the aromatic group at the opposite end of an aminoethyl group, constitute model compounds which, because of their simple and well-differentiated NMR spectra, allows their use in NMR spectroscopy to probe the physical properties of the different regions of lipid interphase (hydrocarbon interior, polar interphase and structured water at its surface). In the present report the use of local anaesthetics containing residues of variable chain length allowed the following observations to be made: (i) The region of the glycerol backbone, as well as that of the first 3–4 C atoms of the fatty acid chains of the phospholipid bilayer, exhibit tight molecular packing. (ii) A hydrophobic tail of 4 C atoms at the non-polar end allows the local anaesthetic tetracaine to interact with the lipid film even in the absence of net negative charge. (iii) The local anaesthetic molecules without such a hydrophobic tail interact first electrostatically and then hydrophobically with charged bilayers and do not interact at all with the uncharged ones. (iv) The differential broadening of the hydrophobic tails at the polar part of the molecules provides evidence for the existence of structured water at the surface of the bilayer and allows one to estimate their magnitude. The (N)CH_3 groups of tetracaine become completely broadened, the CH_3 groups of the *N*-ethylene residues of procaine are less affected and the CH_3 groups of the *N*-butylene residues of butacaine are not affected at all. (v) The relative order of the strength of the hydrophobic interactions of the local anaesthetics tested is: dibucaine > tetracaine > butacaine > procaine and is apparently related to their potency, length of duration of the anaesthetic effect, and to their capacity in displacing Ca^{2+}.

INTRODUCTION

Despite the numerous attempts to elucidate the mode of interaction of local anaesthetics with the nerve membrane, using various types of phospholipid model membranes (monolayers, bilayers, liposomes, *etc.*) and employing techniques ranging

from partition between aqueous to non-aqueous phases to NMR, the question of whether the drugs penetrate into the lipid bilayer, react at the surface, or are adsorbed into the interior still remains unsolved. The results so far obtained had been interpreted in different ways: (a) It has been suggested that the interaction is of an electrostatic nature, since interaction was easily detected with the acidic phosphatidylserine but not with the zwitterionic lecithin (Hauser *et al* [1]). (b) A limited penetration into the hydrophilic head group region has also been proposed (Gershfeld[2] and Feinstein[3]) (c) Another suggestion is the occurrence of primary hydrophobic interaction (Skou[4] an Seeman[5]) Furthermore, there are doubts as to whether the C_4 hydrophobic chain of tetracaine (pantocaine), would be long enough to penetrate the hydrophobic fatty acid region of the phospholipid film (Hauser and Dawson[6]), which could also apply to procaine or lidocaine that have no hydrophobic tail It has also been considered that the aminobenzoate extreme of local anaesthetics behaves as a hydrogen donor and from the X-ray analysis of the crystal structure of procaine, it has been suggested that this may be an important additional aspect of the interaction between local anaesthetics and the phospholipids in the membrane (Sax and Pletcher[7]). The experiments reported in this and forthcoming papers were undertaken in order to elucidate the possible polar and apolar interactions that might occur between different local anaesthetics and phospholipids. The results indicate that the protonated quaternary amine of local anaesthetics is capable of reacting with the phosphate group of phospholipid dispersions, while the aromatic side is inserted between the fatty acid chains of the lipid dispersions. Interactions with purified zwitterionic lecithin were detected for tetracaine but not for procaine.

Butacaine, which has an extra CH_2 between the aromatic non-polar group and the cationic amine and two hydrophobic tails (located, however, at the polar end of the molecule), showed less hydrophobic interaction than tetracaine but larger than procaine On the basis of these results, a discussion is presented on the importance of the hydrophobic interaction of local anaesthetics in their narcotic potency and their effectiveness in displacing Ca^{2+}

A preliminary report of this work has been presented[15]

EXPERIMENTAL

All materials were reagent grade. (99.7 %) ^2H_2O was purchased from Sigma Chemical Company Procaine, tetracaine and butacaine were a gift of Hoechst A G. Laboratories of Mexico Ovolecithin was purchased from Hopkin and Williams Ltd, Fraction III of bovine brain from Sigma, aluminium oxide "suitable for chromatographic absorption" from E. Merck, and DEAE-cellulose from Pharmacia

Lecithin

Lecithin was purified on alumina using chloroform–methanol (9:1, v/v) as eluting solvent according to Singleton *et al.*[8].

Phosphatidylserine

Fraction III of bovine brain (Folch) was fractionated on DEAE-cellulose following the method described by Rouser *et al* [9] Thin-layer chromatography of lecithin and phosphatidylserine on silica gel G, using chloroform–methanol–acetic acid–water (85·20.8 4, by vol) as the solvent, resulted in a single spot being obtained

Preparation of phospholipid dispersions

The lipid dispersions were prepared as previously described (Cerbón[10]). The phospholipid–local anaesthetic mixtures were made up by dissolving the local anaesthetic in the lipid dispersions or by mixing 0.5 ml ultrasonicated phospholipid dispersions with the required amount of local anaesthetic, dissolved in 10 μl of ^2H_2O in order to avoid the possible changes in the size and shape of the liposomes induced by dilution.

NMR studies

The NMR spectrometer was a Varian A60 megacycles/s system. The band positions were taken relative to an external reference of 5 % tetramethylsilane in carbon tetrachloride as zero, or to the ^{2}HHO signal as internal reference. All the spectra were run at 33 °C. Sufficient time was allowed for the tube and its content to come into thermal balance before the measurements were initiated.

Due care was taken to ensure that the radiofrequency power was below the level causing saturation.

Phospholipid mixture

Commercial ovolecithin contained phosphatidylcholine, approx. 78 %; phosphatidylethanolamine, 18 %; and lyso-derivatives, 4 % The fatty acid composition as determined by gas chromatography showed the main components to be C_{16}, $C_{16\ 1}$, C_{18} and $C_{18\cdot 2}$ acids.

RESULTS

Interaction of procaine and egg phospholipids

The NMR spectra of procaine–HCl alone in H_2O, procaine–HCl in 5 % dextran in H_2O and procaine–HCl in 5 % phospholipid mixture are shown in Fig. 1 together with assignments. It can be seen that the NMR spectrum of procaine in 5 % dextran

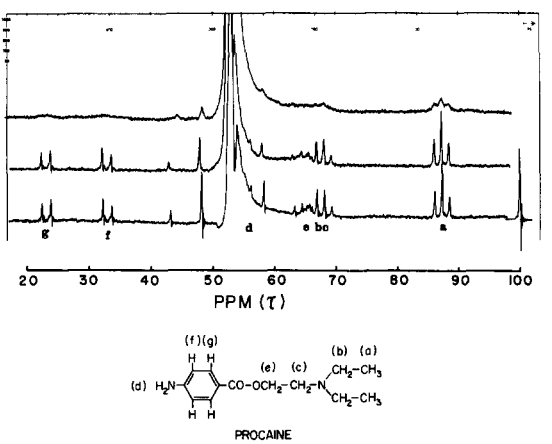

Fig 1. The NMR spectra of procaine–HCl (5 %, w/v) in water, lower line; of procaine–HCl (5 %, w/v) in dextran (5 %, w/v) in water (ηr = 0 037 a u), middle line, and procaine–HCl (5 %, w/v) in sonicated egg-phospholipid dispersion (5 %, w/v) in water (ηr = 0 015 a u), upper line. 33 °C CPS = cycles/S

(middle line) is identical to that obtained for procaine dissolved in bidistilled water (lower line) while the one obtained in a 5 % lipid dispersion (upper line) becomes broadened to such an extent that the aromatic protons (g and f) as well as the protons e, b and c are faintly seen. The CH_3 protons of the ethylene group (protons a) are also broadened but identifiable. At this spectrum amplitude of 4.0 the signals arising from the lipid molecules are undetectable. Dextran was utilized to show the effect of the solution viscosity on the NMR spectra of the local anaesthetic molecule. The ηr of the 5 % lipid dispersion was 0.015 a.u., that of the 5 % dextran 0.037 a u. Increasing the dextran concentration up to 10 % (ηr = 0.100) does not at all affect the spectrum of procaine–HCl. On the other hand, a gradual increase in the lipid concentration to 10 % (ηr = 0 019) leads to a spectrum of the local anaesthetic molecule in which the signals of the aromatic protons (f and g) first disappear and then those of protons b and c, and only the CH_3 (a) protons of the ethylene chain attached to the quaternary amine are faintly seen. All these results indicate that the aromatic protons are more drastically affected than those between the aromatic non-polar side of the molecule and the quaternary amine. The less affected protons are those of the CH_3 groups which suggests that they are located facing the aqueous phase.

Interaction of tetracaine and butacaine with egg phospholipids

If, as suggested by the above-mentioned results, the local anaesthetic molecules orient themselves at the lipid interphase, the protons of the butylene residue of tetracaine–HCl (at the non-polar side of the molecule) would become broadened while the protons of the butylene residues of the butacaine–HCl (at the polar end of the molecule) would remain narrow, or at least not become as broadened as those of the tetracaine–HCl molecule, while interacting with the lipid dispersion. In order to visualize the signals arising from the lipids, ^2H_2O was utilized instead of H_2O in these experiments. It can be seen in Fig. 2 (lower line) that at this spectrum amplitude the protons of the lipid dispersion are detectable and the CH_3N protons of the lecithin (main component of the lipid mixture) are easily identifiable. The middle line shows the interaction of 2 % tetracaine–HCl with a 5 % lipid dispersion It can be seen that all the protons became broadened and only the CH_3–N protons (h) of the polar part of the molecule are less affected but still identifiable. The upper line shows the spectrum of 2 % tetracaine in ^2H_2O. In Fig. 3 are illustrated: lower line, the spectrum of the lipid dispersion; middle line, the spectrum resulting from the interaction of 2 % butacaine with the 5 % lipid dispersion; and upper line, the spectrum of 2 % butacaine–HCl. The protons of the butylene residues at the polar end of the molecule (signals a and c mainly) are not as strongly affected by the interaction as those of the non-polar part of the molecule (signals g, f and e) Hence, there is a clear difference in the degree of broadening observed in the spectrum of the CH_3 protons of the butylene residues depending upon their localization in the local anaesthetic molecules. Those located at the non-polar part of the molecule (tetracaine) are drastically affected while those at the polar part of the molecule (butacaine) remain almost as they appear in aqueous solution

Interaction of local anaesthetics with purified phospholipids

To determine whether a net charge is necessary for the interaction between

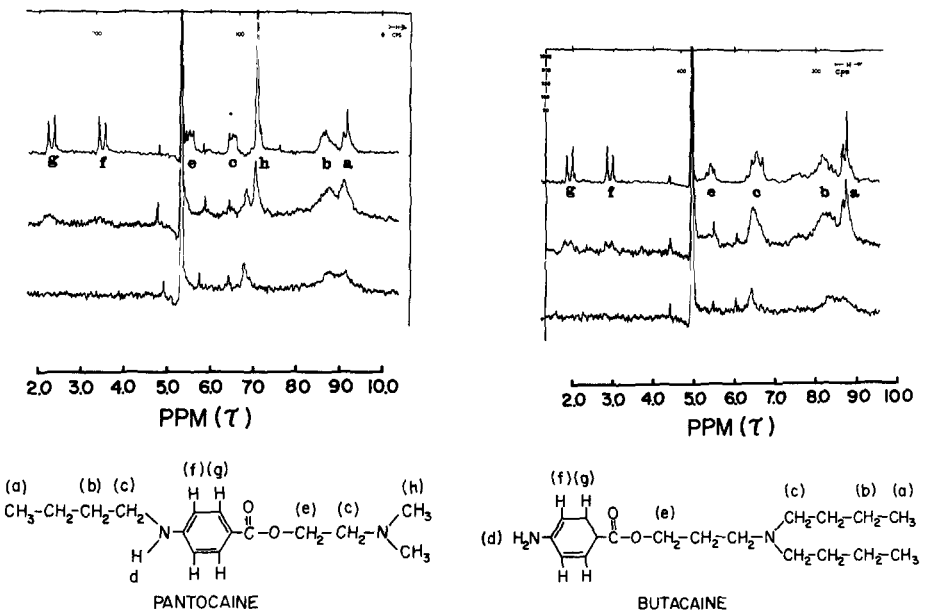

Fig 2 The NMR spectra of pantocaine–HCl (2%, v/v) in ^2H_2O, upper line; of pantocaine–HC (2%, w/v) in sonicated egg-phospholipid dispersion (5%, w/v) in ^2H_2O, middle line, and sonicated egg-phospholipid dispersion (5%, w/v) in ^2H_2O, lower line 33 °C

Fig. 3 The NMR spectra of butacaine–HCl (2%, w/v) in ^2H_2O, upper line; of butacaine–HCl (2%, w/v) in sonicated egg-phospholipids dispersion (5%, w/v) in ^2H_2O, middle line; and sonicated egg-phospholipid dispersion (5%, w/v) in ^2H_2O, lower line 33 °C

local anaesthetics and lipids, the interactions of procaine–HCl and tetracaine–HCl with zwitterionic egg lecithin and brain phosphatidylserine were studied. Aqueous dispersions of phosphatidylserine (2–5%, w/v) were precipitated by both local anaesthetics, while lecithin dispersions (5%, w/v) were not precipitated with 10–25 mM anaesthetic and began to precipitate with concentrations from about 30 mM. The interaction of 10 mM tetracaine–HCl with 5% lecithin dispersion gives rise to a spectrum identical to that obtained by their interaction with the lipid mixture (Fig. 2, middle line), indicating that even in the absence of a net charge, tetracaine–HCl is able to interact with the lipid in a similar way to that observed with the phospholipid mixture. Procaine–HCl, however, does not interact with purified lecithin.

DISCUSSION

Careful examination of the high-resolution spectra obtained from sonicated egg yolk phospholipids *plus* local anaesthetics shows that the addition of the drug up to about 10 mM does not cause a decrease in the intensity of the lipid $(CH_2)_n$ hydrocarbon chain proton resonance. However, addition of the areas does occur. Also, the $N^+(CH_3)_3$ signal of the lecithin seems not to be at all affected, suggesting that these lipid groups have a considerable motional freedom. There is, however, broadening of the protons c and e of the tetracaine, and the equivalent protons in procaine and butacaine, when these local anaesthetics interact with the phospholipid mixture.

Taking into account the electrostatic interaction between the phosphate groups of the lipids and the quaternary amine of the local anaesthetics, these protons (c and e) should be at the same level as those of the glycerol region of the phospholipids (as determined by the use of stereomodels) The aromatic protons g and f that would be located at the level of the first 2–3 chain methylene groups near the glycerol also became highly broadened. The results suggest that these are regions where tight molecular packing occurs. Similar interpretations have been reported by the comparative analysis of the NMR spectra of egg yolk lecithin sonically dispersed in ^2H_2O with that observed with lecithin dissolved in [^{2}H]chloroform[11] and also by the ESR spin label method[12] and deuteron NMR[13]. In these last two techniques, the effect of the paramagnetic group for ESR. and the isotope substitution must produce, however, local perturbations of the molecular environment. One interesting observation was the significant intensity decrement and relatively slight broadening of the $N(CH_3)_2$ signal of the tetracaine, since these groups should be theoretically located at the same level as that of the $N(CH_2)$ groups of the choline, and should be facing the aqueous medium. Based on previous results on the capacity of the lipid interphase to structure water[10–14], it can be assumed that the above-mentioned broadening is being caused by this property of the lipid interphase The CH_3 groups of the butylene residues at the polar part of the butacaine are not at all affected by the interaction with the lipids and will be theoretically located about 2.6 Å above the region mentioned. The results clearly show that the C_4 hydrophobic tail of pantocaine has the capacity to penetrate the lipid barrier even in the absence of a net negative charge. The CH_3 terminal of the butylene residue of tetracaine may reach the level of the 7–8th CH_2 of the fatty acid residues of the phospholipids. Other local anaesthetics, without a hydrophobic residue of such magnitude at the non-polar part of the molecule, require the presence of negative charge in the phospholipids for interaction, at least when the concentration of the local anaesthetic tested is in a range below 10 mM (physiological conditions). Once the local anaesthetics have been attracted by the opposite charge at the surface, they can interact with the upper part of the lipid carbon chain. As a consequence of their small film penetration as compared to tetracaine, they can be washed out more easily and therefore their capacity to displace Ca^{2+} is also lower. 0 10 μmole of tetracaine displaces 50 % of the Ca^{2+} bound to a phosphoinositide film, while 0 75 μmole of procaine are required for a similar displacement. Dibucaine which has not only a butoxy group at the non-polar part of the molecule, but also an extra aromatic ring and therefore should have a stronger hydrophobic interaction, exhibits the greatest capacity in displacing Ca^{2+} (ref. 6). The length of duration of the anaesthetic effect and toxicity may be related to the strength of the hydrophobic interaction, *i.e.* dibucaine > tetracaine > butacaine > procaine. The stronger the hydrophobic interaction, the more difficult the desorption and the more effective the competence of the local anaesthetic *versus* Ca^{2+} becomes.

REFERENCES

1 H Hauser, S A Penkett and D Chapman, *Biochim Biophys Acta*, 183 (1969) 466.
2 N Gershfeld, *J Phys Chem*, 66 (1962) 1923
3 M B Feinstein, *J Gen Physiol*, 48 (1964) 357
4 J C Skou, *Acta Pharmacol Toxicol*, 10 (1954) 325
5 P Seeman and O W Kwant, *Biochim Biophys Acta*, 183 (1969) 512

6 H Hauser and R M. C Dawson, *Biochem J*, 109 (1968) 909.
7 M Sax and J Pletcher, *Science*, 166 (1969) 1546
8 W. S Singleton, M. S Gray, M L. Brown and J L White, *J Am Oil Chem. Soc*, 42 (1965) 53
9 G Rouser, A J Bauman, G Kritchevsky, D. Heller and J S O'Brien, *J Am. Oil Chem. Soc*, 38 (1961) 544
10 J Cerbón, *Biochim Biophys Acta*, 144 (1967) 1.
11 E C Finer, A G Flook and H Hauser, *FEBS Lett*, 18 (1971) 331
12 W L Hubbell and H. M. McConnell, *J Am Chem Soc*, 93 (1971) 314.
13 O Oldfield, D Chapman and W Derbyshire, *FEBS Lett*, 16 (1971) 102.
14 J Cerbón, *Biochim Biophys Acta*, 211 (1970) 389.
15 J Cerbón, *Biophys Soc. Abstr*, 10 (1970) 73a.