Biochimica et Biophysica Acta, 298 (1973) 8–14

BBA 76216

THE IMPORTANCE OF THE HYDROPHOBIC INTERACTIONS OF LOCAL ANESTHETICS IN THE DISPLACEMENT OF POLYVALENT CATIONS FROM ARTIFICIAL LIPID MEMBRANES

MARTA S. FERNÁNDEZ and JORGE CERBÓN

Departamento de Bioquímica, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apartado Postal 14-740, México 14, D.F. (México)

(Received August 22nd, 1972)

SUMMARY

1. Paramagnetic cations such us europium and praseodymium allow the distinction between the NMR signals arising from internal and external trimethylammonium groups of lecithin liposomes, the peak from the external groups being shifted. This effect seems to depend on the binding of the cation to the phosphate groups of lecithin and on the modification of the magnetic properties of the trimethylammonium protons in the neighborhood.

2. The displacement of Pr^{3+} away from the membrane surface by different local anesthetics was related to the ability of these compounds to interact hydrophobically with the membrane.

3. Tetracaine, which interacts hydrophobically with zwitterionic lecithin, was able to reverse the paramagnetic induced shift very efficiently, as judged by the complete return of the shifted peak to its original position. Procaine, lidocaine and tetramethylammonium chloride, which do not interact hydrophobically with the liposomes, failed to displace the paramagnetic cation.

4. The property of the local anesthetic to bind hydrophobically to the lipid membrane enables the positively charged group of tetracaine to complete favourably with the polyvalent cation for the lecithin phosphates. The capacity of local anesthetics to displace polyvalent cations away from the artificial membrane surface could be directly related to their anesthetic potency.

INTRODUCTION

Local anesthetics are drugs whose action consists in a blockade of nerve conduction and this property seems to be due to the inhibition of the large increase in Na^+ permeability associated with the action potential[1,2]. These compounds can also decrease the Na^+ permeability in the resting state of the membrane[3]. In this respect, they behave like Ca^{2+} (ref. 4) in stabilizing the nerve.

Since Skou[5] demonstrated that there is a correlation between the potency of local anesthetics and their ability to penetrate lipid monolayers, artificial lipid membranes have been widely used as models to study the mechanism of action of these drugs.

Bangham *et al.*[6] found that local anesthetics, behaving as cations, reduce the magnitude of the negative zeta potential of egg lecithin–dicetyl phosphoric acid liquid crystals. This change in potential decreases the diffusion rate of cations out of the vesicles.

Feinstein[7] demonstrated that local anesthetics inhibit the extraction of Ca^{2+} from an aqueous into a chloroform phase containing phospholipids, and correlated this finding with the fact that local anesthetics seem to displace Ca^{2+} from the nerve membrane. He also reported that lecithin interacts weakly with the anesthetics if compared with acidic phospholipids.

Experiments of McLaughlin[8] with phosphatidylserine bilayers indicate that local anesthetics decrease the enhanced conductivity to K^+ produced by nonactin. Ca^{2+} behaves in a similar manner[9]. The effects of Ca^{2+} and local anesthetics are correlated with their ability to decrease the magnitude of the negative surface potential, which in turn results in a lower concentration of monovalent cations in the vicinity of the membrane.

Nuclear magnetic resonance techniques have also been used to investigate the interaction of local anesthetics with membrane lipids. Hauser *et al.*[10] reported that the interaction occurs with acidic phosphatidylserine but not with zwitterionic lecithin, indicating that the interaction is of an electrostatic nature. However, data obtained in our laboratory[11] indicate that some local anesthetics, such as tetracaine and butacaine, are able to react with liposomes which do not carry a net charge (lecithin at pH 5.5), the phenomenon being dependent on the capacity of the anesthetic to interact hydrophobically.

It is also known that local anesthetics are able to displace polyvalent cations from lipid monolayers and that the ability of straight-chain amines to displace Ca^{2+} from phosphatidylinositol films increases with the number or carbon atoms up to C_{12} (ref. 12) (increment in their capacity to interact hydrophobically).

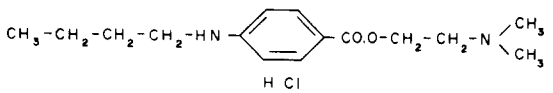
Therefore, the efficiency of local anesthetics in displacing cations from the lipid-water interface, could be related to their capacity to penetrate into the lipid films, so that their basic groups are more effective in neutralizing the negative charge of the phospholipid than if they were acting as simple gegenions.

It has been reported that a paramagnetic cation such as europium induces an upfield shift in the NMR signal arising from protons of the trimethylammonium groups of zwitterionic lecithin facing the external surface of the liposome[13]. Praseodymium can also affect this signal but shifts it downfield. Since some local anesthetics are capable of interacting with lecithin and the NMR results indicate that their positively charged polar ends also face the liposome surface[11], it would be expected that they could displace the polyvalent cations and restore the original position of the shifted NMR signal. The shift of the signal can be followed quantitatively and the effect of different local anesthetics on this shift can be taken as a measure of their capacity to remove the polyvalent cations away from the artificial membrane surface.

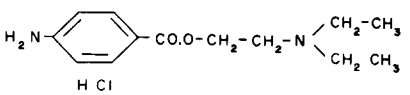
EXPERIMENTAL

All reagents were analytical reagent grade. ^2H_2O was purchased from Sigma Chemical Company. The local anesthetics tetracaine, procaine and lidocaine were

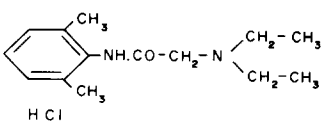
a gift from Hoechst Laboratories (Mexico). The compounds were used in the form of hydrochlorides and their chemical structures are shown in Fig. 1. $PrCl_3$ and $EuCl_3$ were obtained from K and K Laboratories Inc. Egg yolk lecithin from Sigma was purified by column chromatography on alumina as described by Singleton *et al.*[14] and the purity checked by thin-layer chromatography.



TETRACAINE HYDROCHLORIDE



PROCAINE HYDROCHLORIDE



LIDOCAINE HYDROCHLORIDE

Fig. 1. Chemical structures of the local anesthetics used in this work.

The appropriate amount of lecithin dissolved in chloroform–methanol (2:1, v/v) was deposited in a tube and the solvent evaporated with N_2. The residue, taken up in ^2H_2O, was sonicated in an ultrasonic desintegrator (Branson Instruments) for 20 min in an ice-cooled vessel. The pH of the resulting dispersion was always between 5.5 and 6.0. From this preparation equivalent samples were taken to obtain: (1) the spectra of the lecithin dispersion; (2) the spectra of lecithin in the presence of paramagnetic cations; and (3) the spectra showing the effect of the different local anesthetics on the Pr^{3+}-containing sample.

NMR spectra were recorded in a Varian A 60 Mcycles/s system. All spectra were run at 33 °C. Tetramethylsilane was used as external reference and the signal corresponding to H^2HO as internal standard. The experiments were repeated at least 3 times. The results are an average of at least five single scans in each experiment.

RESULTS AND DISCUSSION

As reported by Bystrov *et al.*[13], the addition of $EuCl_3$ to a lecithin dispersion gives rise to splitting of the trimethylammonium signal from the phospholipid, and, upon the addition of $MnCl_2$, the high-field shifted peak (from the external groups) disappears. A comparison was made in our laboratory of the effects of equivalent amounts of $EuCl_3$ and $PrCl_3$ on the lecithin dispersion. Pr^{3+} shifts the signal from

external trimethylammonium groups downfield, while Eu^{3+} induces and upfield displacement. The magnitude of the shift produced by Pr^{3+} was larger (about 7 Hz) than the one caused by Eu^{3+}. Therefore, we chose to work with the former so as to get more accurate measurements of the shift and its subsequent reversal as induced by the anesthetics.

Fig. 2 shows the NMR signal of trimethylammonium groups for a 4% lecithin dispersion in ^2H_2O. If $PrCl_3$ is added to the dispersion, the trimethylammonium peak splits into two components (Fig. 2b): a low-field component (Peak A) and a high-field component (Peak B). The more Pr^{3+} is added, the lower the field towards which Peak A shifts (see Fig. 3). Peak B appears to be shifted upfield only slightly (not more than 3 Hz). As the Pr^{3+} concentration increases up to 20–30 mM the dispersion becomes turbid, and therefore the rest of the experiments were performed at concentrations not higher than 10 mM.

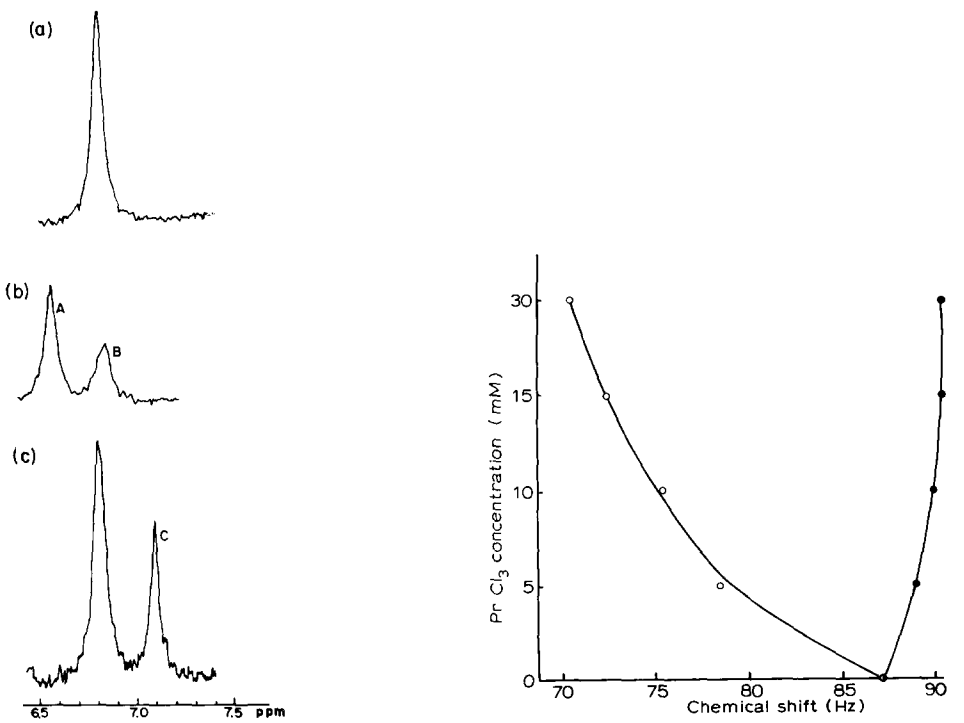


Fig. 2. NMR spectra of a sonicated 4% (w/v) dispersion of egg lecithin in ^2H_2O under different experimental conditions: (a) control: signal from trimethylammonium protons (+87.3 Hz); (b) splitting of the trimethylammonium signal upon the addition of 10 mM $PrCl_3$. Peak A (+75.5 Hz) represents the external groups of the vesicles and Peak B (+90.0 Hz) the internal ones. The addition of 10 mM $MnCl_2$ results in the dissappearance of peak B; (c) reversal of the Pr^{3+}-induced splitting upon the addition of 30 mM tetracaine. The trimethylammonium signal appears as in (a) (+87.0 Hz); Peak C corresponds to the $-N(CH_3)_2$ protons from the tetracaine added. Line positions were determined relative to the H^2HO signal.

Fig. 3. Variation with $PrCl_3$ concentration of the chemical shift of the NMR signal from $-N^+(CH_3)_3$ protons of lecithin 4% in ^2H_2O. ◑, control; ○, external $-N^+(CH_3)_3$ protons; ●, internal $-N^+(CH_3)_3$ protons. Line positions were determined relative to the H^2HO signal.

In order to assign Peaks A and B to each of the two kinds of trimethylammonium groups present (internal and external), $MnCl_2$ was used because of its paramagnetic effects and its inability to penetrate into the liposome[13]. When this salt (10 mM) is added to the lecithin dispersion previously doped with Pr^{3+}, Peak A disappears while Peak B is not affected. Therefore, Peak A should represent the external trimethylammonium groups and Peak B the internal ones.

Measurements of the relative areas under both signals (A and B) allow us to assign a value of $35.7\% \pm 0.7$ to the proportion of lecithin molecules facing the interior of the vesicles; this figure is in good agreement with that reported by Kornberg and McConnell[15] using spin labels (35%).

The mode of action of lanthanide shift reagents has not been definitely established, but their properties have been explained in terms of two possible mechanisms, namely contact or pseudocontact[16]. It is not the purpose of this work to attempt to distinguish between them. However, since it was found that the addition of $PrCl_3$ to a $(CH_3)_4NCl$ solution does not shift the NMR signal from the tetramethylammonium protons, it is possible to suggest that the shift induced in the spectrum of lecithin liposomes must depend on some property of the vesicle surface. The phosphate groups could enhance the binding of the paramagnetic cations to the membrane and hence affect the magnetic properties of the neighbour $-N^+(CH_3)_3$ protons.

As shown previously, tetracaine interacts with lecithin liposomes[11]. To detect the effect of this interaction on the position of the Pr^{3+}-shifted signal, increasing quantities of the local anesthetic were added to the lipid dispersion containing 10 mM $PrCl_3$ (see Fig. 4). As the tetracaine concentration increases, Peak A (representing external $-N^+(CH_3)_3$ protons) shifts upfield until it reaches its original position (+87.0 Hz). The effect of the lanthanide is completely reversed at a tetracaine concentration of 30 mM (Fig. 2c).

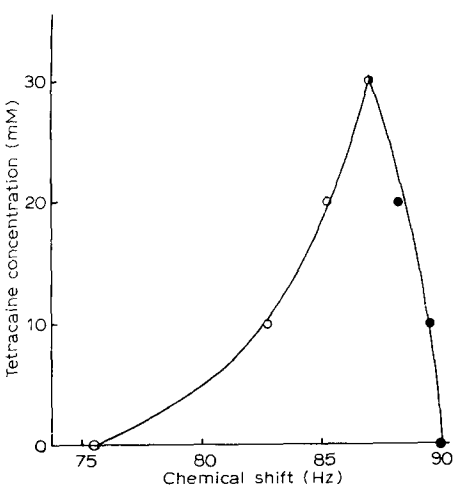


Fig. 4. Reversal of the Pr^{3+}-induced splitting of the NMR signal of $-N^+(CH_3)_3$ protons of lecithin 4% in ^2H_2O, as related to increasing concentration of tetracaine. $PrCl_3$ concentration was kept constant (10 mM). ○, external $-N^+(CH_3)_3$ protons; ●, internal $-N^+(CH_3)_3$ protons; ●, complete reversal of splitting: internal and external $-N^+(CH_3)_3$ appear as a single peak. Line positions were determined relative to the H^2HO signal.

The portion of the spectrum shown in Fig. 2c shows two peaks: the low-field peak (+87.0 Hz) from the $-N^+(CH_3)_3$ protons of lecithin, and the high-field peak (Peak C: +100.5 Hz) from the $-N(CH_3)_2$ protons of the tetracaine added. In this zone, the anesthetic also contributes to the area of the trimethylammonium peak of lecithin (+87.0 Hz) with the signal of its $-CH_2-N(CH_3)_2$ (methylene) protons. This explains why the signal arising from the phospholipid exhibits a larger area. When the contribution from the $-CH_2-N(CH_3)_3$ protons of the local anesthetic is subtracted from the total area, the original integral value of the trimethylammonium peak is obtained.

If the reversal of the splitting induced by tetracaine is due mainly to its cationic dimethylamino moiety, it would be expected that a compound with a very similar polar group would induce a similar reversal. However, upon addition of $(CH_3)_4NCl$ to the Pr^{3+}-containing lecithin dispersion, the splitting of the lecithin trimethylammonium peak is not reversed at all, even at concentrations as high as 100 mM $(CH_3)_4NCl$. Furthermore, neither procaine nor lidocaine at a concentration 60 mM (twice the tetracaine concentration) are able to abolish the lanthanide effect. Therefore, the phenomenon described here does not seem to depend only on the local anesthetics acting merely as gegenions, but, in addition, on the possibility of another kind of interaction.

Further work performed in this laboratory (in preparation) shows that the magnitude of the interaction between local anesthetics and lecithin as detected by NMR is a function of the hydrophobicity of the anesthetic. This can be shown by the broadening of the NMR signals from the benzenic protons of these compounds, in the order: tetracaine $\gg$ procaine $\simeq$ lidocaine.

Lecithin being a zwitterion, it may be considered that its trimethylammonium groups act as relatively fixed counterions of the phosphate[17]. The cations which have a lipophilic chain long enough to interact hydrophobically will compete more efficiently with the trimethylammonium groups for the phospholipid phosphates.

The results presented here suggest that tetracaine can displace Pr^{3+} from the liposome surface because of its capacity of anchoring into the hydrophobic palisade and, in this way, more effectively neutralizing the lecithin phosphates with its cationic dimethylamino groups. The property of penetrating the artificial membrane seems to be highly dependent on the presence of the butyl group in the hydrophobic part of the molecule. Lidocaine and procaine, which do not carry such an alkyl tail (Fig. 1), have very small capacities of interacting in this way.

Preliminary experiments performed with proparacaine (2-diethylaminoethyl-3-aminobenzoate) and butacaine (2-dibutylaminopropyl-3-aminobenzoate) show that these compounds, at a concentration of 30 mM, also reverse the effect of Pr^{3+} on lecithin. In addition, they interact hydrophobically with this phospholipid.

Thus, the displacement of polyvalent cations from liposomes may be taken as indirect evidence of the capacity of a local anesthetic to penetrate into the hydrophobic palisade of phospholipid vesicles.

It has been suggested that the pharmacological effect of local anesthetics depends on their capacity to remove calcium from the nerve membrane[1].

The anesthetic potency is larger for tetracaine than for lidocaine or procaine[6,18] and this is also the order for their ability to displace Pr^{3+}. Since the same pattern of response has been found for the displacement of Ca^{2+} (ref. 12) our results give

further support to the proposal that the process of anesthesia should be related to the latter phenomenon.

ACKNOWLEDGEMENT

The authors are indebted to Dr M. Montal for his review of the manuscript.

REFERENCES

1 Shanes, A. M. (1958) *Pharmacol. Rev.* 10, 59–273
2 Tobias, J. M. (1958) *J. Cell. Comp. Physiol.* 52, 89–105
3 Toman, J. E. P. (1962) in *Neurochemistry* (Elliot, K., Page, I. and Quastel, J., eds) pp. 758–765, Charles C. Thomas, U.S.A.
4 Frankenhauser, B. and Hodgkin, A. L. (1957) *J. Physiol. London* 137, 218–244
5 Skou, J. C. (1954) *Acta Pharmacol. Toxicol.* 10, 281–337
6 Bangham, A. D., Standish, M. M. and Miller, N. (1965) *Nature* 208, 1295–1297
7 Feinstein, M. B. (1964) *J. Gen. Physiol.* 48, 357–374
8 McLaughlin, S.G.A. (1972) *Biophys. Soc. Abstr.*, 181a
9 McLaughlin, S.G.A., Szabo, G. and Eisenman, G. (1971) *J. Gen. Physiol.* 58, 667–687
10 Hauser, H., Penkett, S. A. and Chapman, D. (1969) *Biochim. Biophys. Acta* 183, 466–475
11 Cerbôn, J. (1972) *Biochim. Biophys. Acta*, 290, 51–57
12 Hauser, H. and Dawson, R. M. C. (1968) *Biochem. J.* 109, 909–916
13 Bystrov, V. F., Dubrovina, N. I., Barsukow, L. I. and Bergelson, L. D. (1971) *Chem. Phys Lipids* 6, 343–350
14 Singleton, W. S., Gray, M. S., Brown, M. and White, J. L. (1965) *J. Am. Oil Chem. Soc.* 42, 53–56
15 Kornberg, R. and Mc Connell, H. (1971) *Biochemistry* 10, 1111–1120
16 Horrocks, W. and Sipe, J. P. (1971) *J. Am. Chem. Soc.* 93, 6800–6804
17 Parsegian, V. A. (1967) *Science* 156, 939–942
18 Covino, B. G. (1971) *Anesthesiology* 35, 158–167