J. Pharm. Pharmacol. 1982, 34: 359–363
Received October 5, 1981

0022–3573/060359–05 $02.50/0


# Interaction of local anaesthetics with model phospholipid membranes. The effect of pH and phospholipid composition studied by quenching of an intramembrane fluorescent probe

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The interaction of local anaesthetics, tetracaine and procaine, with model phospholipid membranes has been examined by measurement of drug-induced quenching of the fluorescence of a membrane incorporated probe, 12-(9-anthroyl)stearic acid. The pH dependence of quenching curves obtained for neutral phosphatidylcholine and acidic phosphatidylserine bilayers indicates that, contrary to previous suggestions, both charged and uncharged forms of amine local anaesthetics may be incorporated into lipid membranes.

Local anaesthetics block the action potential of nerve axons by preventing the transient increases in permeability of sodium and potassium ions to the axonal membrane (Richtie & Greengard 1961). Even though the molecular mechanism of local anaesthetics action is not fully understood attention has been drawn to the correlation between the anaesthetic potency of the drugs and their ability to interact with membrane phospholipids (Skou 1954; Feinstein 1964; Papahadjopoulos 1970; Papahadjopoulos 1972; Lee 1976; Vilallonga & Phillips 1979).

The question whether it is the uncharged or charged form of amine local anaesthetics that is incorporated into lipid membranes has been raised. There are claims that only neutral forms are incorporated (Ueda et al 1977) whereas other results suggest that both charged and uncharged species may be incorporated into the bilayers (Lee 1977, 1978). We have attempted to verify these hypotheses by studying the pH dependence of the anaesthetic – phospholipid membrane interaction.

Partitioning of amine local anaesthetics, tetracaine and procaine, into phospholipid bilayers was followed by measuring fluorescence quenching of the intramembrane probe, 12-(9-anthroyl)stearic acid. A similar method has been used previously by Koblin et al (1975) for studying the penetration of anaesthetics into red blood cell membranes. The fluorescence quenching technique has been also successfully used for studying membrane action of pesticides (Lakowicz et al 1977: Lakowicz & Hogan 1977) and antidepressive drugs (Römer & Bickel 1979).

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## MATERIALS AND METHODS

Chromatographically pure egg yolk phosphatidylcholine was prepared by the method of Singleton et al (1965). Highly purified bovine brain phosphatidylserine, procaine hydrochloride, tetracaine hydrochloride and 12-(9-anthroyl)stearic acid were obtained Sigma and used without further purification.

For preparation of fluorescence labelled phospholipid vesicles, chloroform solutions of appropriate phospholipid and 12-(9-anthroyl)stearic acid were mixed at a lipid – probe molar ratio of 100:1. The solvent was evaporated under argon and buffer (50 mM Tris–acetic acid, 100 mM NaCl, 0·1 mM EDTA) was added to the dried lipid to establish a concentration of 5 mg of phospholipid $ml^{-1}$ of buffer. The lipid suspension was sonicated for 20 min in an ice bath using an MSE sonicator. After sonication, titanium particles and larger lipid aggregates were removed by centrifugation at 40 000 *g* for 20 min. Clear supernatants were diluted with the buffer to a final concentration of 0·25 mg lipid $ml^{-1}$. Phospholipid concentrations were determined by phosphate assay (Chen et al 1965).

To study the effect of anaesthetics on fluorescence spectra of membrane-incorporated 12-(9-anthroyl)stearic acid, microlitre quantities of concentrated aqueous solution of appropriate drug were added to 2·5 ml vesicle samples. After each addition

the suspension was mixed and left to equilibrate for 5 min before fluorescence measurements. After this time no further changes in fluorescence intensities were detected.

All fluorescence measurements were at room temperature (20 °C) using a Jobin Yvon JY3 spectrofluorimeter. It was verified that neither unlabelled phospholipid vesicles nor drugs at concentrations used exhibit any detectable fluorescence.

## RESULTS AND DISCUSSION

The fluorescence spectrum of phosphatidylcholine vesicles labelled with 12-(9-anthroyl)stearic acid is shown in Fig. 1. In the presence of tetracaine and procaine, fluorescence intensity decreases without significant changes in the shape of the emission spectrum. The lack of drastic alterations in the spectrum indicates that the interaction of amine anaesthetics with the fluorescence probe does not lead to the formation of a new fluorescence species. On the other hand, the observed decrease in emission intensity shows that both drugs may effectively quench the fluorescence of 12-(9-anthroyl)stearic acid.

In general, fluorescence quenching may occur as a static or dynamic process. Both types of quenching result in identical dependence of fluorescence intensity on quencher concentration (Lakowicz & Hogan 1977):

$$\frac{F_0}{F} = 1 + Kc$$

where F and $F_0$ are fluorescence intensities corresponding to the presence and absence of quencher, c is the concentration of quencher, and K refers to dynamic or static quenching constants. The identification of the quenching type and an accurate description of the quenching process would require fluorescence lifetimes measurements with the use of special equipment. Nevertheless, fluorescence intensity determinations also allow study, at least qualitatively, of some aspects of drug – membrane interactions. As discussed by Koblin et al (1975) and Thulborn & Sawyer (1978) it is obvious that for a membrane-incorporated probe, regardless of the quenching mechanisms, the decrease in fluorescence intensity will be dependent on the effective concentration of the quencher in the vicinity of the fluorophore rather than on its bulk concentration in aqueous medium. Therefore, in view of the location of the anthroyl moiety in the hydrocarbon region of the lipid bilayer (Podo & Blasie 1977; Thulborn & Sawyer 1978; Thulborn et al 1979) fluorescence quenching of 12-(9-anthroyl)stearic acid can be directly related to the ability of the drug to be incorporated into the bilayer structure of phospholipid vesicles.

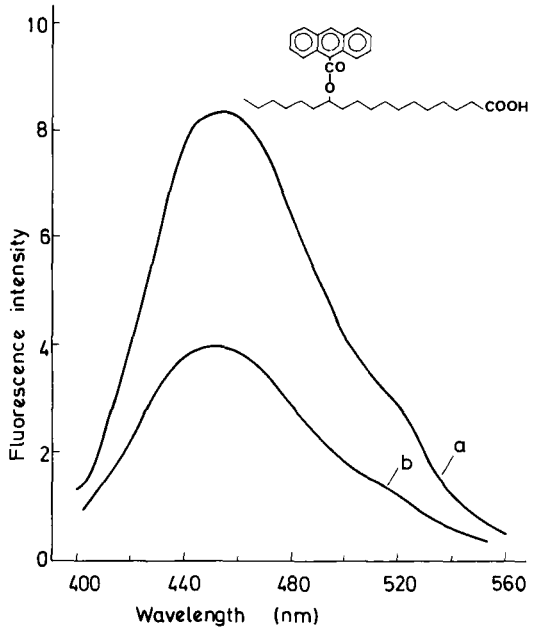


FIG. 1. Fluorescence emission spectrum of 12-(9-anthroyl)stearic acid-labelled phosphatidylcholine vesicles. Excitation wavelength 385 nm, pH 7·2. (a) No drug. (b) Tetracaine added, 1·3 mM.

Fig. 2 illustrates the dependence of fluorescence intensity of 12-(9-anthroyl)stearic acid labelled phosphatidylcholine membranes on the concentration of tetracaine and procaine added to the vesicle suspension. Both drugs quench the fluorescence of the membrane-incorporated probe, although the quenching is much more effective with tetracaine. This is in agreement with the previous data showing higher membrane affinity of tetracaine, compared with procaine (Papahadjopoulos 1972). It also correlates well with the anaesthetic potencies of these drugs (Strichartz 1976).

For both anaesthetics investigated, the depression of fluorescence intensity exhibits a strong pH dependence. The quenching is much more effective in stronger alkaline medium. This is indicative of a different contribution of charged and uncharged forms of the drugs to the fluorescence quenching.

One explanation that could account for the increasing fluorescence quenching with increasing pH value is that only the neutral form of anaesthetics binds to the membrane, as asserted by Ueda et al

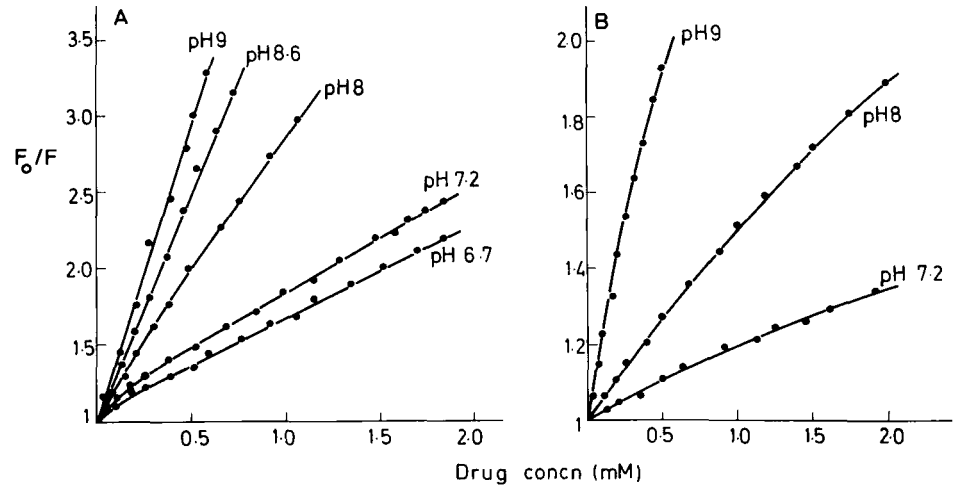


FIG. 2. Fluorescence quenching of 12-(9-anthroyl)stearic acid-labelled phosphatidylcholine vesicles by local anaesthetics, at different pH values. (A) Tetracaine. (B) Procaine.

(1977). Alternatively on the basis of the model proposed by Lee (1977, 1978), both charged and uncharged forms of amine anaesthetics may be incorporated into the bilayer. Apparently, the higher binding ability of the drugs to neutral phosphatidylcholine membranes observed at greater alkalinity should be attributed to the build-up of a positive charge on the liposomes. This surface charge, caused by the binding of positively-charged anaesthetics, would limit further incorporation of the drug.

The present results seem to contradict the hypothesis that only the uncharged forms of amine local anaesthetics are capable of being incorporated into the bilayer structure of phosphatidylcholine membranes. This is based on two observations. Firstly, the quenching curves exhibit negative deviations from linearity which are marked at low pH values and which gradually decrease with increasing alkalinity. This may be explained on the assumption that both charged and neutral forms of anaesthetics bind to the membrane. The initial rapid binding of the protonated form results in a net positive charge on the bilayer, resisting further binding of the drug and thus leading to negative deviations from linearity

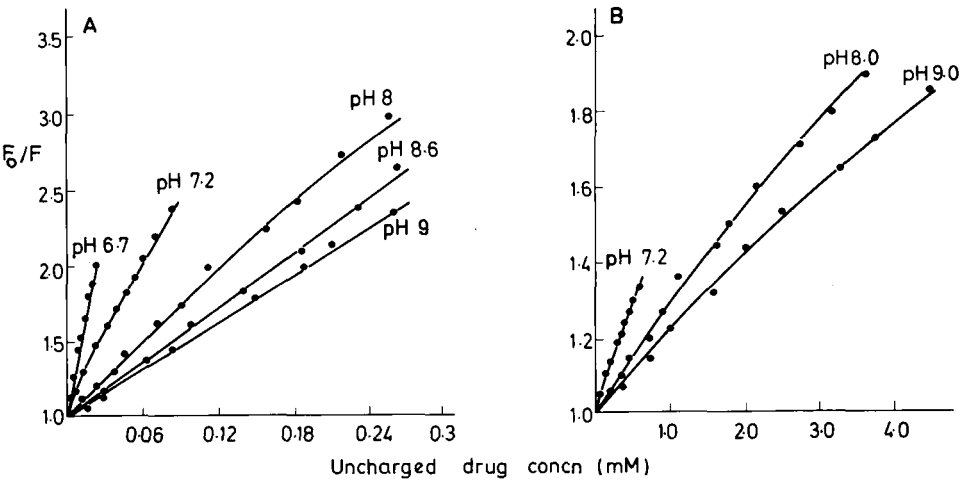


FIG. 3. The values of fluorescence quenching $F_0/F$ of 12-(9-anthroyl)stearic acid-labelled phosphatidylcholine vesicles of Fig. 2 replotted against the concentration of uncharged form of anaesthetics. If only unchanged molecules penetrated the bilayer, all curves obtained at different pH values should fall on the same line.

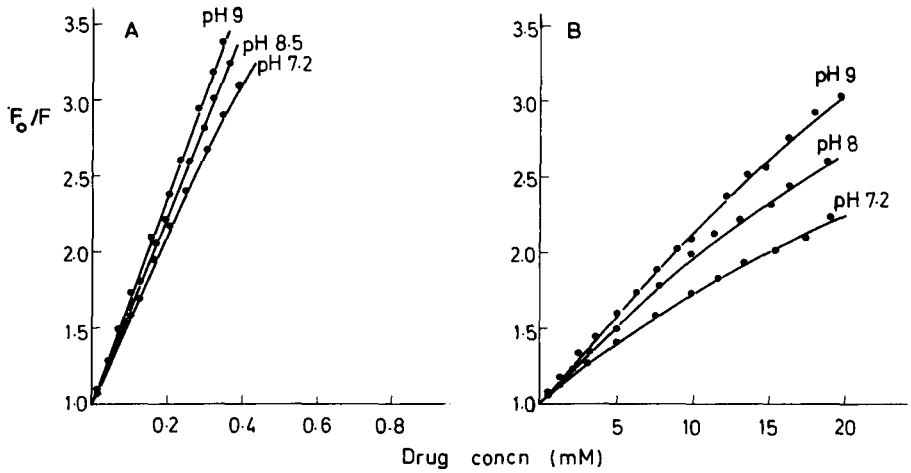


FIG. 4. Fluorescence quenching of 12-(9-anthroyl)stearic acid labelled phosphatidylserine vesicles by local anaesthetics, at different pH values. (A) Tetracaine; (B) Procaine.

in the quenching curves. Such a characteristic of $F_0/F$ vs drug concentration plots at different pH values would be difficult to explain if only neutral forms of anaesthetics interact with the membrane. Secondly, to estimate the contribution of charged and uncharged forms to the quenching of intramembrane fluorescent probe, the concentrations of each form were calculated using the Henderson-Hasselbach equation*

$$\log \frac{[A_{charged}]}{[A_{uncharged}]} = pH - pK_{\alpha}.$$

In Fig. 3 the fluorescence intensity ratio $F_0/F$ has been plotted against the calculated concentration of the uncharged forms. If the membrane-bound anaesthetics were composed solely of the uncharged species, all curves at different pH should be superimposable. It can be seen from Fig. 3 that this assumption is not fulfilled and therefore it may be concluded that the membrane incorporated molecules include both the charged and the uncharged species.

The interaction of local anaesthetics with lipid membranes was also studied using phosphatidylserine vesicles. The negative charge of phosphatidylserine should, at least partly, neutralize the effects previously attributed to the build-up of positive charge on originally uncharged phosphatidylcholine membranes. It can be seen (Fig. 4a) that the quenching curves obtained for tetracaine are indeed almost identical at different pH values of the medium. For procaine, some small pH dependence still exists (Fig. 4b). However, it is much less pronounced than that observed for the interaction of procaine with phosphatidylcholine membranes.

These data provide further and more direct evidence that both charged and uncharged forms of amine anaesthetics participate in partitioning into lipid bilayers. Moreover, they indicate that the partitioning of tetracaine into negatively charged membranes is roughly equally effective for both forms of the drug whereas with the less hydrophobic drug, procaine, it is still more effective for its uncharged form.

The ability of amine local anaesthetics to quench fluorescence of membrane-incorporated 12-(9-anthroyl)stearic acid may also provide a useful method for studying some other aspects of local anaesthetic – membrane interactions.

*Acknowledgements*

The authors wish to express their sincere gratitude to Dr J. Mikucki, Academy of Medicine, Lodz, for making available the MSE sonicator. This work was supported by Research Grant R.III.13.

* The $pK_{\alpha}$ values of the drugs are 8·5 for tetracaine and 9·0 for procaine (Richtie & Greengard 1961).

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