FLUOROMETRIC STUDY WITH 1-ANILINO-8-NAPHTHALENE SULPHONATE ON MEMBRANE SURFACE CHARGE CHANGES INDUCED BY INHALATION ANAESTHETICS

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1. Introduction

From in vitro studies on various biomembranes and model membranes, many investigators have suggested that anaesthetics act by fluidizing nerve cell membranes [1–3]. Some of these observations were performed using fluorescent dyes to probe the structure of the hydrophobic regions in the membranes. We have found that inhalation anaesthetics, dose-dependently, increase the fluorescence intensity of several ANS-labeled biomembranes, but decrease the intensity of the neutral phospholipid model membrane. From the analysis of the difference of fluorescence changes among these membranes, we believe that inhalation anaesthetics not only fluidize the membrane but also change the membrane surface charge.

2. Materials and methods

Red blood cell ghosts were prepared essentially as in [4]. Synaptosome and mitochondria were isolated from rabbit brain homogenates by the method in [5]. Finally these membrane specimens were suspended in 0.1 M Tris–HCl buffer (pH 7.4). As a model membrane, dipalmitoyl phosphatidylcholine was sonicated in the same buffer to produce a translucent vesicle suspension.

Abbreviations: ANS, 1-anilino-8-naphthalene sulphonic acid, Na salt; methoxyflurane, 2,2-dichloro-1,1-difluoroethylmethyl ether; enflurane, 2-chloro-1,1,2-trifluoroethyl difluoromethyl ether; halothane, 2-bromo-2-chloro-1,1,1-trifluoroethane; NPN, *N*-phenyl-1-naphthylamine

The membrane suspensions were incubated at 37°C in closed test cuvettes with ANS and various amounts of anaesthetics. The same buffers saturated with anaesthetics were used as stock solutions, and anaesthetic concentrations were calculated from the vapor pressures and solubilities of each anaesthetic to water. The fluorescence measurements were performed using the Union fluorescence spectrometer FS-401 at 37°C. Chloroform, diethylether, methoxyflurane and enflurane were the anaesthetics used in this study. Because halothane, whose bromide strongly quenches ANS fluorescence, was revealed to act in different manner compared with other anaesthetics in a preliminary investigation, it was omitted from this report although it is a most common agent.

3. Results and discussion

Figure 1 shows the typical emission spectra of ANS-labeled membranes in the absence and presence of chloroform. It was recognized that the decrease of fluorescence intensity in the phospholipid model membrane was accompanied by a red-shift of the emission maximum wavelength. Such changes in ANS fluorescence are usually thought to indicate changes in the hydrophobicity or fluidity of ANS binding sites. While in biomembranes, the increases of fluorescence intensity were not accompanied with measurable shifts. In the protein solutions such as bovine serum albumin and β-lactoglobulin, fluorescence changes by anaesthetics were analogous to those in the model membrane but not in the biomembrane. Effects similar to the case using chloroform were

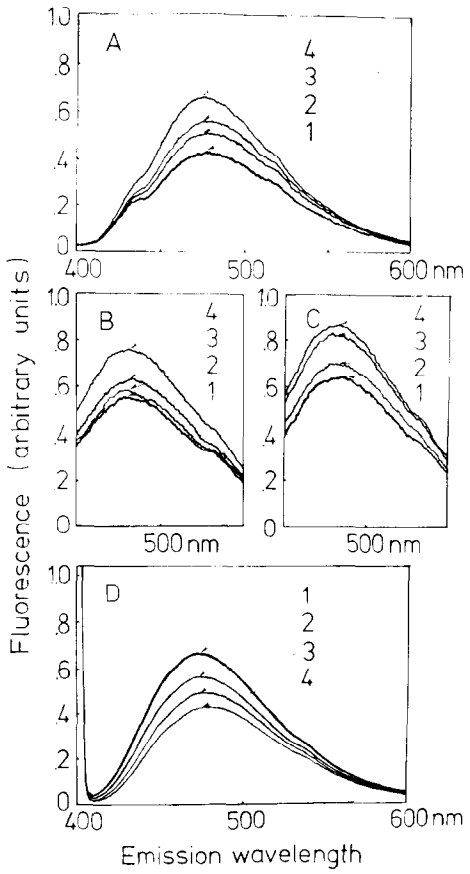

Fig.1. Emission spectra of ANS-labeled membranes. (A) 0.1 mg protein/ml red blood cell ghosts; (B) 0.1 mg protein/ml synaptosome; (C) 0.1 mg protein/ml mitochondria; (D) 1 mM dipalmitoyl phosphatidylcholine bilayer membrane. ANS was 30 μM in all cases. Concentrations of chloroform were zero, 7.2, 14.4 and 21.6 mM in 1, 2, 3 and 4 in each spectrum. Excitation wavelengths were 380 nm for (A), 395 nm for (B) and (C), and 398 nm for (D).

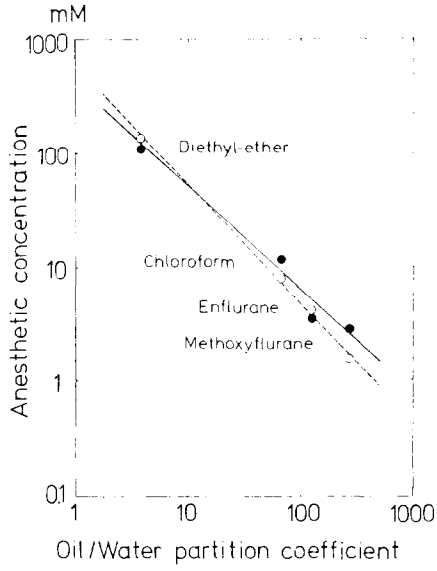

Fig.2. Correlations of logarithms of the oil/water partition coefficients to logarithms of the effective doses of anaesthetics for 20% increase of fluorescence intensity on red blood cell ghosts (o- - -o- - -o) and to logarithms of the effective doses of anaesthetics for 20% deccrease of fluorescence intensity on dipalmitoyl phosphatidylcholine bilayer membranes (•—•—•). Correlation coefficients were 0.998 on red blood cell ghosts and 0.986 on phospholipid membranes.

observed in the presence of other anaesthetics on both the biomembranes and the model membrane, and dose–response curves were obtained. As seen in fig.2, there were good correlations between the oil/water partition coefficients and the effective doses of anaesthetics to produce 20% increase of fluorescence intensity on red blood cell ghosts or to produce 20% decrease on the model membrane. This means that anaesthetics act upon the hydrophobic regions in both the biomembrane and the model membrane.

Changes in fluorescence intensity could be due to the changes in the quantum yield of the ANS molecules bound to the membranes or to the changes in ANS binding to the membranes. From the double reciprocal plot of fluorescence intensity to membrane concentration at a fixed concentration of ANS [6], it was distinguished that in red blood cell ghosts, anaesthetics caused no changes in the quantum yields but increased ANS binding to the membrane, but on the other hand, they changed the quantum yields in the model membrane.

Using the equations in [7], the dissociation constant, K_d, and the number of binding sites, n, for ANS to red blood cell ghosts in the absence and presence of anaesthetics were calculated:

$$\frac{1}{F} = \frac{1}{F_{max}} + \frac{K_d}{F_{max}} \times \frac{1}{[ANS]} \qquad (1)$$

where F is the fluorescence intensity and F_{max} is the

maximal fluorescence when [ANS] is infinitely large. At $[ANS]_{added} \gg$ [membrane], $[ANS] \approx [ANS]_{added}$. Then K_d can be calculated from a plot of $1/F$ to $1/[ANS]_{added}$. As for n, under conditions of $n \times [membrane]_{added} \gg$ [ANS–membrane]:

$$\frac{[ANS]_{added}}{F} = \frac{1}{\psi} + \frac{K_d}{\psi\, n\, [membrane]_{added}} \qquad (2)$$

where [ANS–membrane] is the concentration of the ANS–membrane complexes and $\psi = F/[ANS\text{-}membrane]$. With the value of K_d obtained from eq. (1), n can be calculated from a plot of $[ANS]_{added}/F$ against $1/[membrane]_{added}$. The results from these calculations are summarized in fig.3. The values of n increased with anaesthetic concentrations and the magnitude of the increases were comparatively greater than that of K_d.

Further measurements were performed using NPN which has an analogous structure to ANS but no negative charges. In the presence of anaesthetics, the fluorescence of NPN was dose-dependently quenched

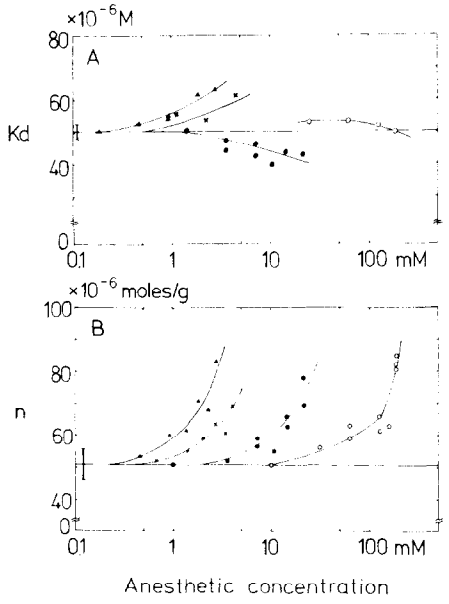

Fig.3. Changes of (A) K_d and (B) n for ANS to red blood cell ghosts with concentrations of methoxyflurane (▲—▲—▲), enflurane (×—×—×), chloroform (●—●—●) and diethylether (○—○—○). Control values were: $K_d = (50.4 \pm 2.4) \times 10^{-6}$ M; $n = (50.8 \pm 4.8) \times 10^{-6}$ mol/g.

in red blood cell ghosts. These observations may be explained by assuming that the binding of charged dyes to non-charged membranes (e.g., ANS to model membrane) and the binding of non-charged dyes to charged membranes (e.g., NPN to ghost), depend upon the same properties, such as fluidity or hydrophobicity, while, in the binding of charged dyes to charged membranes (e.g., ANS to ghost), the charges on both sides are determinant. The interpretations of electrostatic interaction of ANS to membranes are supported by our own data that ANS-ghost fluorescence was enhanced by lowering the pH or by increasing the ionic strength, and by other reports [8] that ANS bindings to various membranes were increased by cations, local anaesthetics and low pH, since all these factors should lead to decrease the net negative charge of the membrane surface.

Equation (1) is formally analogous to a normalized Lineweaver-Burk plot [7]. Our data showed that anaesthetics bind to red blood cell ghosts non-competitively with ANS, in contrast with competitive binding to the model membrane. We assume that in biomembranes, hydrophobic binding of anaesthetics causes changes in the conformation of membrane proteins and/or in the orderliness of lipids which give rise to the decrease of net negative charges (or the increase of net positive charges) of the membrane surface. If so, the non-competitive binding of anaesthetics with ANS to red blood cell ghosts suggests that ANS, an amphiphatic molecule, will bind to rather hydrophilic regions in charged membranes, while in the neutral model membrane, ANS will behave hydrophobically, similar to anaesthetics.

It is difficult to say, at this stage, which is essential to the mechanism of anaesthesia, membrane surface charge or fluidity. However, the former, at least, can affect ion fluxes directly even if the magnitude of changes is very small. This may be the reason why in many reports, concentrations several times higher than clinically used anaesthetics were required to obtain measurable changes in membrane fluidity.

Acknowledgements

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