INTERACTION OF A FLUORESCENT PROBE WITH ERYTHROCYTE MEMBRANE AND LIPIDS: EFFECTS OF LOCAL ANESTHETICS AND CALCIUM

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1. Introduction

Conformational changes in the surface membrane and intracellular membranes are believed to occur during processes such as ion translocations, electron transport and interactions with drugs. To study such conformational changes a number of fluorochromes, such as 8-anilino-1-naphthalene sulfonic acid (ANS) have become increasingly useful tools. ANS, which exhibits little fluorescence in water, becomes highly fluorescent, with a shift in emission maximum to a lower wavelength, in solvents of low dielectric strength or high viscosity [1], as well as on binding to hydrophobic regions of proteins [2, 3]. Increased fluorescence has also been observed when ANS reacts with mitochondrial [4], erythrocyte [5, 6] and sarcoplasmic reticulum membranes [7], indicating binding of the fluorescent probe to hydrophobic sites in these membranes. Changes in ANS fluorescence observed in mitochondria during active calcium transport, or upon the addition of substrate and oxygen [4, 8], have been interpreted as demonstrating conformational changes in the membranes.

In order to interpret the meaning of the ANS fluorescence signal in biological membranes, it is essential to know the nature of the binding site(s). The recent observation [8] that calcium and the local anesthetic, butacaine, increased fluorescence of mitochondrial membranes treated with ANS, suggested to us that a reaction with membrane lipids might be involved, since it had already been established that local anesthetics, as well as calcium, react with phospholipids [9, 11]. We have, therefore, studied the effects of local anesthetics and Ca^{2+} on the interaction of ANS with erythrocyte membranes as well as with various lipids and proteins.

2. Materials and methods

Bovine erythrocytes were separated from plasma and leukocytes by centrifugation at 5,000 *g* for 20 min. After resuspension in normal saline, the cells were hemolyzed by the addition of 10 volumes of 5 mM EDTA – 10 mM tris-HCL, pH 7.4. The membranes obtained by centrifugation (30 min at 15,000 *g*) were washed 5–7 times with the tris-EDTA solution and 5 times with 10 mM tris-HCl pH 7.4 to obtain hemoglobin-free membranes. The final preparations contained 2–3 mg of membrane protein per ml. Fluorescence was measured with an Hitachi-Perkin Elmer spectrophotofluorometer. Phospholipid dispersons in water were prepared by ultrasonication

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with a "Sonifier" (Heat-Systems Ultrasonics Inc.) at a 60 W setting. ANS was obtained from K & K Labs (Plainview, New York).

3. Results

Bovine erythrocyte membranes suspended in 10 mM tris-HCl buffer, pH 7.4 reacted with ANS to procedure a marked in fluorescence (about 50-fold in fig. 1) and a shift in the emission maximum from 520 nm (ANS in water) to 470 nm. The addition of calcium chloride or butacaine sulfate further increased the fluorescence emission, about 2-fold in each case, without changing the peak emission wavelength (fig. 1). The number of binding sites and the apparent dissociation constant (K_{app}) for the membrane-ANS interaction was determined by titrating solutions of ANS with increasing concentrations of erythrocyte membranes (measured as protein). The erythrocyte membranes contained 29.5 μmoles of ANS binding sites per g protein, with a K_{app} of 30 μM, values comparable to those previously reported in erythrocyte membranes of other species [5, 6]. Butacaine at a concentration of 2 mM increased the number of availabe ANS binding sites a little more than 3-fold (to 110 μmoles of ANS bound/g protein)

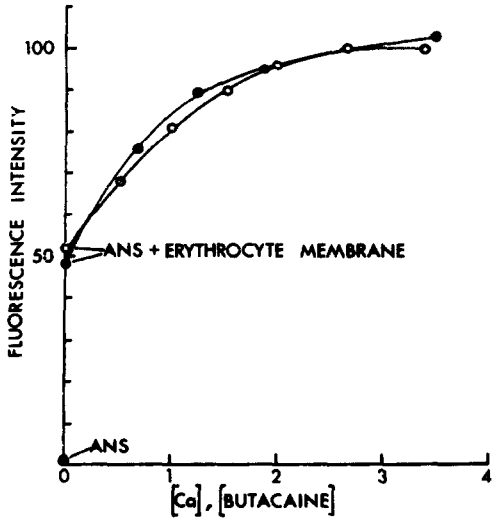

Fig. 1. Titration of bovine erythrocyte ghosts (2.45 mg protein) with calcium (•) or butacaine (○) in the presence of 8.5 μM ANS. Concentrations were expressed in μmoles per ml.

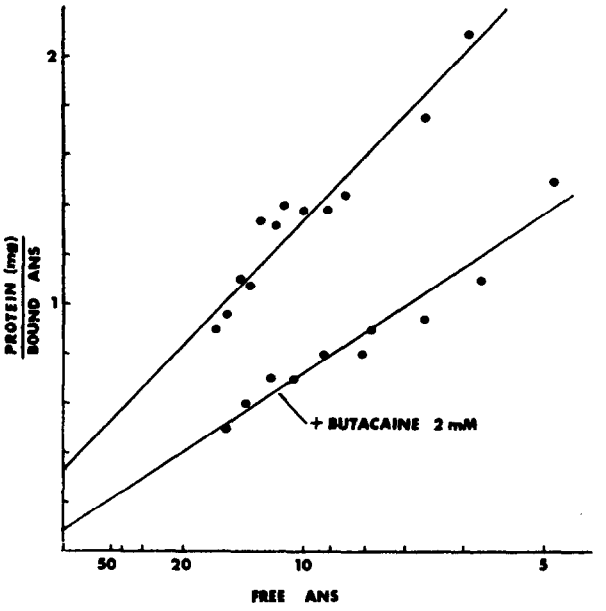

Fig. 2. The binding of ANS to bovine erythrocyte ghosts as a funtion of ANS concentration. The data are plotted according to the equation [15]: $\frac{P_O}{xD_O} = \frac{1}{n}\frac{(1+K_{app})}{(1-x)D_O}$, where P_O and D_O are the total protein and ANS concentrations respectively. x is the fraction of the ANS bound as determined from the ratio of the observed fluorescence to the fluorescence obtained when all dye is bound (fig. 3). n is the number of binding sites and K_{app} is the apparent dissociation constant for binding the dye to the n^{th} sites. The intercept of the line, plotted by the method of least squares, on the ordinate is $1/n$ and the slope of the line is K_{app}/n.

with little change in K_{app} (fig. 2). As was previously found to be the case with Ca^{2+} [6], the relative fluorescence intensity of ANS was not affected by butacaine when all the dye was bound to the erythrocyte membrane (fig. 3).

Of the several proteins studied (lysozyme, ovalbumin, protamine, phosvitin), only bovine serum albumin fluoresced intensely with ANS, but it showed only a slight fall in fluorescence when titrated with Ca^{2+} or butacaine. The other proteins, which fluoresced little if at all with ANS, also failed to exhibit any increase in fluorescence on addition of Ca^{2+} or butacaine. This was notable in the case of phosvitin because of the previously described ability of this protein to form complexes with local anesthetics and Ca^{2+} [10].

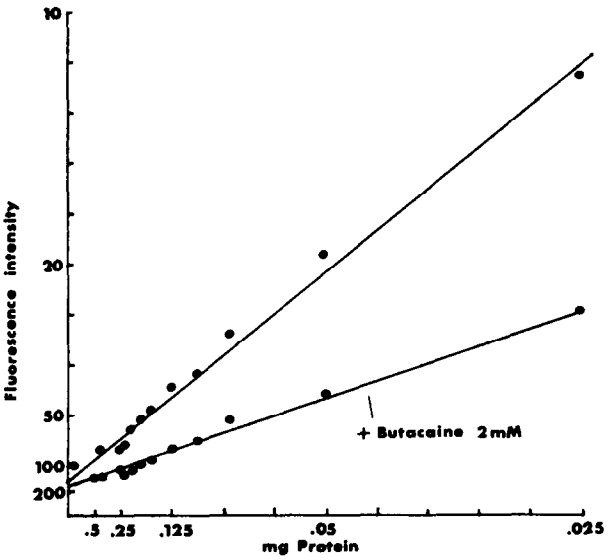

Fig. 3. A reciprocal plot of the binding of ANS to bovine erythrocyte ghosts as a function of protein concentration. The ANS concentration was 8.5 μM, total volume-2.0 ml.

The lipids which were studied varied considerably in their ability to bind ANS with a resultant increase in fluorescence. Acidic phospholipids such as phosphatidylserine, phosphatidyl inositide and cardiolipin showed very little fluorescence with ANS, whereas those phospholipids which form neutral or positively charged micelles, such as lecithin and sphingomyelin [12, 13] had a much greater fluorescence intensity (fig. 4). Gangliosides (*N*-acylneuraminic acid-containing glycosphingolipids) were intermediate in effect, but surprisingly, they showed much greater fluorescence than phosphatidylserine, despite the presence of the strongly acidic neuraminic acid group.

The low fluorescence of ANS-acidic phospholipid mixtures was very markedly enchanced by the addition of butacaine (>50-fold in case of phosphatidylserine) but much less so by Ca^{2+} (fig. 4). ANS-ganglioside fluorescence was increased nearly 10-fold by butacaine but only 2-fold by Ca^{2+}, whereas the high native fluorescence of sphingomyelin with ANS was increased only about 60 to 100% by butacaine or calcium. Sphingomyelin is by far the predominant phospholipid of bovine erythrocyte ghosts [14].

Fluorescence of membrane-ANS [5, 7] or lipid-ANS mixtures [7] has also been shown to be highly

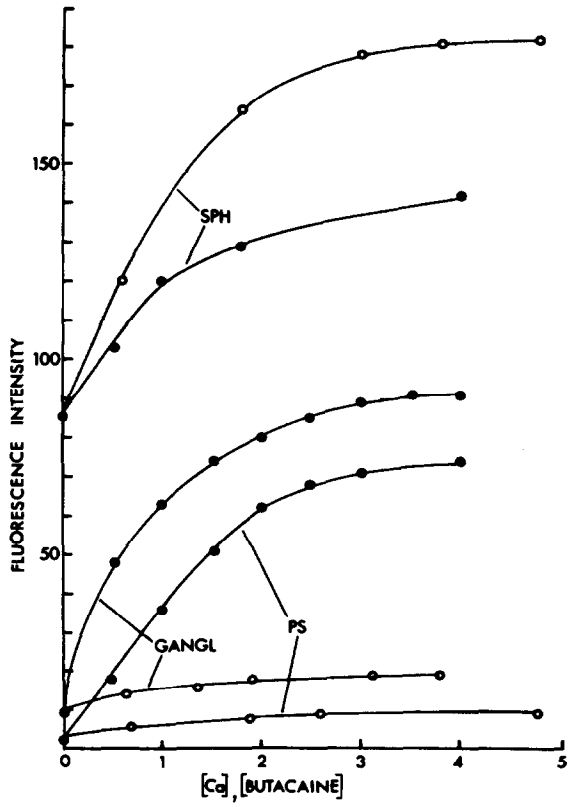

Fig. 4. ANS binding to sphingomyelin (SPH), ganglioside (GANGL) and phosphatidyl serine (PS) as a function of calcium (○) or butacaine (●) concentration (μmoles/ml). Lipid and ANS concentration were 0.5 mg/ml and 8.5 μM respectively.

pH dependent (fig. 5). ANS-bovine erythrocyte membrane fluorescence increased about 15-fold as the pH was lowered from 7.4 to 2, with an inflection point between pH 3.5–4.5. Phosphatidylserine-ANS fluorescence increased 30-fold as $[H^+]$ increased, whereas with ganglioside and sphingomyelin the increases were 7- and 4-fold respectively.

4. Discussion

Generally, the reactivity of lipid micelles with ANS conformed to the observations of Vanderkooi and Martonosi [7], as well as those of Rubalcava et al. [6] on ANS interaction with detergent micelles of differing net charge.

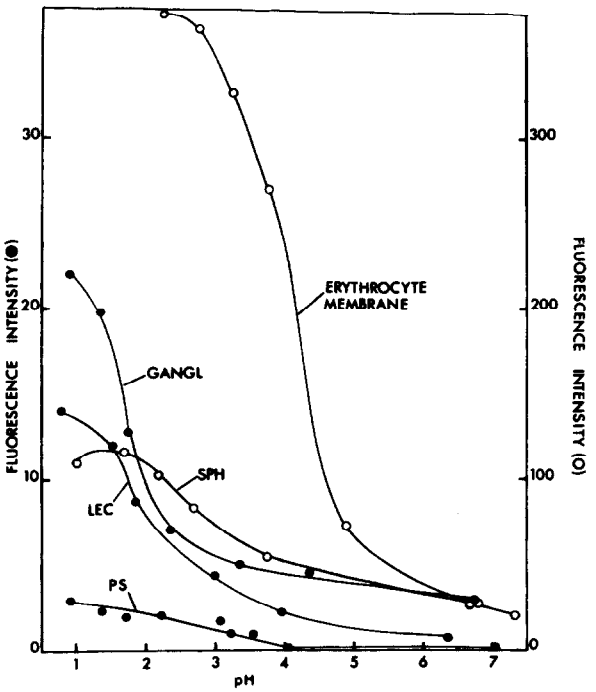

Fig. 5. Effect of pH on ANS binding to erythrocyte membrane (0.119 mg protein/ml) ganglioside (GANGL), sphingomyelin (SPH), egg lecithin (LEC) and phosphatidyl serine (PS). The lipids were at a concentration of 0.05 mg/ml except for ganglioside which was present at 0.1 mg/ml because of its higher molecular weight. ANS concentration was 8.5 μM.

The increase in fluorescence due to addition of calcium or butacaine to ANS-phospholipid combinations may have been the result of reaction with the phosphate group, resulting in a shielding of the anionic charge [6, 9, 10]. The nature of the reaction of butacaine with ganglioside remains unknown.

The influence of pH on lipid-ANS fluorescence may also be explained by the suppression of the ionization of the strongly acidic phosphate group of the phospholipids, the carboxyl group in phosphatidylserine, and the carboxyl group of the neuraminic acid moiety of the ganglioside. This would lead to a net decrease of the surface negativity of the lipid micelles, thereby, allowing greater binding of the anionic ANS molecules. Ionization of the sulfonic acid group of ANS would of course also be suppressed as pH decreases. Presumably the pH titration curve for the erythrocyte membranes may be explained on a similar basis, but identification of the reacting groups is not readily apparent from the *p*K.

Our results support the view that membrane lipids are important sites for the interaction with ANS [7]. Furthermore, the data suggest that the effects of calcium and local anesthetics on the binding of ANS to membrane were due to their ability to react with these lipids as well. It may be possible to elucidate the mechanism by which calcium and local anesthetics affect membrane permeability through detailed analysis of their influence on ANS-membrane interactions.

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