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EFFECT OF ANESTHETICS ON THE INTERACTION OF A FLUORESCENT PROBE WITH HUMAN ERYTHROCYTE MEMBRANES

PAUL A. KRAMER and TING-KAI LI

Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Ind. 47907, and the Department of Medicine, Indiana University School of Medicine, Indianapolis, Ind. 46202, U.S.A.

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Abstract—The fluorescent probe, 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), was used to study the interaction of various anesthetics with human erythrocyte membranes. The enhancement of the fluorescence of membrane-bound TNS produced by the drugs was compared with that produced by Ca^{2+}. Titrations were performed to determine whether the observed modifications were due to changes in binding, quantum yield, or both. Chlorpromazine (0·1 mM) and promazine (0·1 mM) decreased by 60 per cent the fluorescence of bound TNS (quantum yield), whereas $CaCl_2$ (1·0 mM) had no effect. However, both phenothiazines increased the number of TNS binding sites 7-fold, as compared to only a 2-fold increase for $CaCl_2$. Promazine and $CaCl_2$ produced small increases (< 30 per cent) in the affinity (K_{app}) of TNS for the membranes, whereas chlorpromazine doubled K_{app}. Results showed that while TNS is useful in the qualitative assessment of the membrane expansion and charge-shielding effects of certain drugs capable of hydrophobic interaction with membranes, its fluorescence is insensitive to other agents such as diethyl ether, procaine and *n*-butanol.

The "critical volume" hypothesis for the mechanism of anesthetic action states that anesthesia occurs when the volume of hydrophobic regions of membranes reaches a certain critical value by the absorption of drug molecules [1]. Anesthetic action appears to be mediated by an increase in the disorder of lipid molecules and an expansion of the lipid portion of the membrane [2]. Studies on the effect of anesthetics on the freedom of motion of lipids in erythrocytes, synaptosomes, myelin and vagus nerve membranes [3, 4] and on the cation permeability of artificially prepared phospholipid vesicles [5] are all indicative of a site of interaction within the hydrophobic regions of the lipid component of the membrane. Seeman *et al.* [6] have shown the interaction of the local anesthetic, chlorpromazine, with erythrocyte membranes to be hydrophobic in nature and to result in a membrane volume expansion of 2–6 per cent [7, 8]. Further elucidation of the sites and modes of interaction of anesthetics with membranes is thus of considerable interest.

Fluorescent dyes have been used recently as spectroscopic probes of the hydrophobic regions of membranes [9]. 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) is such a probe that binds non-specifically to membranes and whose fluorescence is greatly enhanced by interaction at sites of low dielectric constant. It thus seemed reasonable to suppose that increased lipid bilayer fluidity, volume expansion and perhaps protein conformational changes that occur when chlorpromazine and other anesthetic agents interact with erythrocyte membranes might produce alterations in the fluorescence of TNS. Preliminary evidence in support of such an effect has been presented by Keeler and Sharma [10], who found that promazine produced enhanced fluorescence emission from 1-anilinonaphthalene-8-sulfonate (ANS) in the presence of rabbit erythrocyte membranes. The present study was undertaken to verify by this technique past observations of membrane expansion by chlorpromazine, to determine the relative contribution of quantum yield and binding parameters in the fluorescent enhancements produced by the phenothiazines, and to assess the applicability of fluorescent probe techniques to the study of drug–membrane interactions in general.

MATERIALS AND METHODS

Materials

Chlorpromazine HCl and trimeprazine tartrate were gifts from Smith, Kline & French Laboratories. Methapyrilene HCl was obtained from Abbott Laboratories, lidocaine HCl monohydrate from Astra Pharmaceutical Products, Inc., promazine HCl from Wyeth Laboratories, histamine from Schwartz/Mann and procaine HCl from Mallinckrodt. 2-*p*-Toluidinylnaphthalene-6-sulfonate was obtained as the potassium salt from

Sigma Chemical Co. and was used without further purification. All other chemicals were analytical grade reagents. Water was deionized and doubly distilled from an all-glass system.

Preparation of membranes

Human erythrocyte membranes were prepared from fresh blood collected over EDTA (1 mg/ml). All of the blood used in this investigation was obtained from one of the authors (P. A. K.) in order to minimize the variability in membrane preparations. Blood was washed three times with 8 vol. normal saline, and the "buffy coat" was removed. The cells were collected by centrifugation, at 3020 *g* for 5 min in a Sorvall model RC2-B refrigerated centrifuge, after each wash. The cells were then lysed at 4° for 30 min in 8 vol. of 15 mM Tris–HCl buffer, pH 7·5. This concentration of buffer was employed because it has recently been shown that the general permeability and integrity of erythrocyte membranes are impaired when the membranes are stored in solutions which are below 15 mosM [11]. Membranes were collected by centrifugation at 22,000 *g* for 15 min and washed five times with 8 vol. of 15 mM Tris–HCl buffer, pH 7·5. After the final wash and centrifugation, the membranes were faintly pink in color.

Membranes were stored in 15 mM Tris at 4° before use, but never for more than 12 hr. When studies were performed in 5 mM Tris–HCl buffer, the membrane suspension was dialyzed against 30 vol. of this buffer for 1 hr immediately before use. Membranes were never stored in 5 mM Tris. The protein content of the membrane suspension was determined by the method of Lowry *et al.* [12], using bovine serum albumin as the standard.

Fluorescence titrations

Titrations of fixed amounts of membranes with TNS were performed in a total volume of 3·0 ml at 25°. The membrane suspension and the drugs to be studied were added to a fluorescence cuvette and thoroughly stirred. The background fluorescence of this mixture in the absence of TNS was negligible. Additions of microliter aliquots of methanol (MeOH) produced negligible changes in fluorescence under the conditions of this study. Titration of 0·1 mM chlorpromazine or 1 mM $CaCl_2$ with TNS in the absence of membrane also produced negligible fluorescence. Microliter aliquots of a 2×10^{-3} M TNS solution in methanol were then added, and the fluorescence emission was recorded after 3 min with an Aminco–Bowman spectrophotofluorometer equipped with a Hewlett–Packard model 7035B x-y recorder. Three min of equilibration time was allowed between TNS additions. An excitation wavelength of 366 nm and an emission wavelength of 450 nm, determined as maximal for our instrument, were used throughout this study. All measurements in the presence of drug were referenced to a control cuvette run identically in the absence of drug.

The fluorescence of a fixed concentration of TNS, when all the probe molecules were bound (F_{max}), was determined by measuring the fluorescence emission of a solution of TNS (or TNS plus drug) at different membrane concentrations and extrapolating a plot of 1/relative fluorescence vs 1/membrane concentration to the y-axis. F_{max} was determined at found different TNS concentrations ranging from $1{\cdot}5 \times 10^{-5}$ to $1{\cdot}0 \times 10^{-4}$ M and was found to be linearly related to TNS concentration.

Determination of parameters for binding of TNS to membranes

The titrations described above were used to determine the fraction of probe molecules bound at various probe concentrations in the presence and absence of drugs. This value was taken to be the ratio of the observed fluorescence at a given membrane concentration to that at infinite membrane concentration (F_{max}). The data were then plotted according to the equation developed by Klotz [13] to describe the binding of dye molecules to independent sites on proteins:

$$P_0/xD_0 = 1/n + [K_{app}\, n(1 - x)D_0]^{-1} \qquad (1)$$

where P_0 is the total protein concentration, D_0 is the total dye concentration, x is the fraction of the dye bound, n is the number of independent binding sites, and K_{app} is the average apparent affinity constant for the binding of the dye. For individual membrane preparations, the data obtained fit this equation extremely well. The degree of variation from preparation to preparation is illustrated in Fig. 2.

RESULTS

A number of "membrane-active" agents, including several anesthetics, were examined for their effect on TNS fluorescence in the presence of human erythrocyte membranes. Each agent was added to a suspension of erythrocyte ghosts in 5 mM Tris–HCl buffer, pH 7·5, and the suspension titrated with a 2×10^{-3} M solution of TNS in methanol. Figure 1 shows typical titration curves in the presence of $CaCl_2$ and chlorpromazine. The plateau value for the hyperbolic relationship observed between fluorescence and TNS concentration was taken as a relative measure of the maximal fluorescence of a ternary mixture of TNS, erythrocyte membrane and "drug" for that concentration of membrane and "drug" examined. Mixtures of TNS and 5 mM Tris buffer alone exhibited negligible fluorescence as did also mixtures of TNS and membranes when suspended in distilled water. Table 1 lists the relative plateau fluorescence values obtained for Tris, Ca^{2+}, chlorpromazine and the other agents examined. A wide variation in the enhancement of TNS fluorescence was observed. Inorganic cations such as Ca^{2+} and Al^{3+} produced large increases in TNS emission, while such potent anesthetics as diethyl ether and lidocaine had little or no effect. Tris–HCl and NaCl were only weakly effective, and concentrations of these monovalent cations nearly 100 times greater than that of calcium were required to produce the same degree

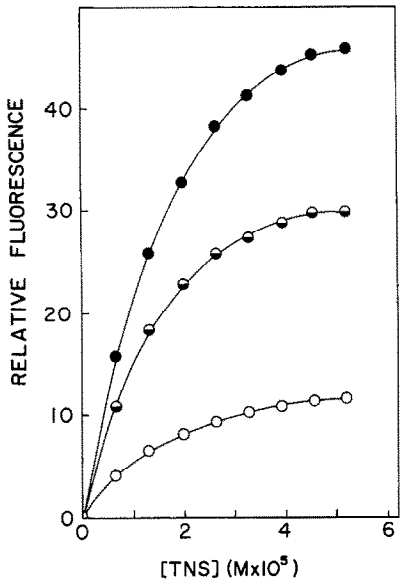

Fig. 1. Titration of human erythrocyte membrane suspensions with 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS). Microliter quantities of a 2×10^{-3} M solution of TNS in methanol were added to 3 ml membrane suspension (0·12 mg protein/ml in 5 mM Tris–HCl buffer, pH 7·5) in a fluorescence cuvette with thorough stirring. Samples were equilibrated for 3 min before reading. Key: ○ buffer alone; ◒ 0·1 mM chlorpromazine; and ● 1 mM $CaCl_2$. T = 25°; excitation wavelength = 366 nm; emission wavelength = 450 nm; and methanol = 0·33 to 3·3%.

of enhancement. Chlorpromazine at a concentration of 0·1 mM produced a large enhancement of TNS fluorescence in the presence of membranes suspended in 5 mM Tris–HCl buffer. Increasing the ionic strength with NaCl swamped out this effect, and the fluorescence in isotonic Tris–HCl buffer was increased less than 10 per cent by the addition of 0·1 mM chlorpromazine, presumably because the high Na^+ concentration had already increased the number of TNS binding sites that would otherwise have been produced by the addition of the drug.

The fluorescence enhancements could have been produced by either increasing the number of TNS binding sites or by increasing the quantum yield of TNS in the presence of these agents. Because of the large effects produced by $CaCl_2$ and chlorpromazine on TNS fluorescence and the documented effects of both of these agents on erythrocytes [7, 14], the mechanism of enhancement by these compounds was investigated further.

The titrations with TNS described above were plotted according to the equation developed by Klotz [13] for evaluating the binding of dye molecules to proteins which assumes no interactions between sites (Fig. 2). Table 2 lists the average values of K_{app} and n obtained from such plots for the binding of TNS to human erythrocyte ghosts in the presence of 5 mM Tris–HCl,

Table 1. Maximum fluorescence for a fixed concentration of erythrocyte membranes titrated with TNS in the presence of various drugs*

Drug		Relative maximum emission at 450 nm
Tris–HCl	(5 mM)	1·0
NaCl	(1 mM)	1·0
$BaCl_2$	(1 mM)	3·1
$CaCl_2$	(1 mM)	4·0
$AlCl_3$	(1 mM)	8·9
Tris–HCl	(25 mM)	2·0
NaCl	(25 mM)	2·1
n-Butanol	(100 mM)	1·0
Diethyl ether	(100 mM)	0·96
Methapyrilene	(1 mM)	1·1
Lidocaine	(1 mM)	1·2
Procaine	(10 mM)	1·5
Chlorpromazine	(0·1 mM)	2·9
Chlorpromazine	(0·1 mM) + $CaCl_2$ (1 mM)	4·3

* Five mM Tris–HCl buffer (pH 7·5); MeOH = 0·33 to 3·3%; protein concentration = 0·12 mg/ml; and excitation wavelength = 366 nm.

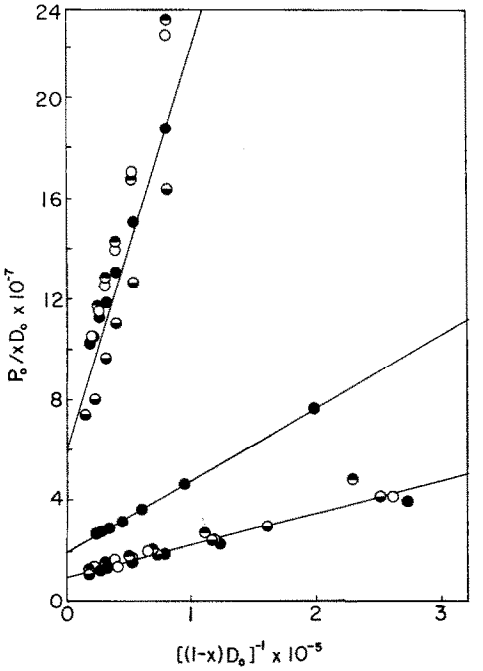

Fig. 2. Binding of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) to human erythrocyte membranes. Data of Fig. 1 and replicates were plotted according to method of Klotz (equation 1) [13]. Top: Tris–HCl buffer alone; middle: 1 mM $CaCl_2$; and bottom: 0·1 mM chlorpromazine. Symbols represent different experiments. Conditions are the same as in Fig. 1.

Table 2. Effect of various drugs on the binding of TNS to human erythrocyte membranes*

Additions to erythrocyte membranes in 5 mM Tris–HCl buffer (pH 7·5)		K_{app} ($M^{-1} \times 10^{-4}$)	n (moles/mg $\times 10^8$)
None		3·7 ± 0·9 (6)†	1·7 ± 0·3 (6)
$CaCl_2$	(1 mM)	4·8 ± 1·0 (3)	3·8 ± 0·9 (3)
Chlorpromazine	(0·1 mM)	6·6 ± 0·4 (5)	11·2 ± 2·2 (6)
Promazine	(0·25 mM)	4·6‡	13·0‡

* Conditions are the same as in Fig. 1; T = 25°.
† Values given for K_{app} and n are the mean ± 1 S. D. for the number of experiments indicated in the brackets.
‡ Average value from two determinations.

pH 7·5, and the effects of adding chlorpromazine, promazine and calcium. It can be seen that both the affinity of TNS for the membrane (K_{app}) and the number of TNS binding sites on the membrane (n) increase in the presence of these agents. Chlorpromazine produces greater effects than $CaCl_2$ and at a much lower concentration. Promazine although somewhat less effective than chlorpromazine, behaves similarly. The results obtained with $CaCl_2$ compare favorably with those reported by Rubalcava *et al.* [9] for the binding of ANS to hemoglobin-free rabbit erythrocyte membranes: in 20 mosM Tris–HCl, pH 7·4, the affinity constant they reported was $2·3 \times 10^4\ M^{-1}$ and n was $1·52 \times 10^{-8}$ moles/mg. When 1 mM $CaCl_2$ was added, the affinity constant increased 60 per cent to $3·7 \times 10^4\ M^{-1}$, while n increased 270 per cent to $4·1 \times 10^{-8}$ moles/mg. Our observations were a 30 per cent increase in the affinity constant and a 220 per cent increase in n with the addition of 1 mM $CaCl_2$.

In the course of compiling data for the Klotz plots, information was obtained on the fluorescence of a fixed concentration of TNS under conditions where all of the dye molecules were bound. This parameter, F_{max}, is a relative measure of the average quantum yield of the bound molecules. Table 3 lists the values of F_{max} obtained. Diethyl ether had no effect of F_{max}, showing that its failure to enhance TNS fluorescence was not due to an alteration in the number of TNS binding sites which was exactly compensated by a change in F_{max}. Chlorpromazine and promazine decreased the F_{max}, while Ca^{2+} had no effect at the concentrations employed. The 300 per cent net increase in TNS plateau fluorescence observed when 0·1 mM chlorpromazine was added to human erythrocyte ghosts is thus due to the 700 per cent increase in the number of TNS binding sites occupied which more than compensate for the 60 per cent decrease in F_{max}.

Table 3. Effect of additions on the fluorescence of a fixed concentration of totally bound TNS molecules*

Additions to erythrocyte membranes in 5 mM Tris–HCl buffer (pH 7·5)	Ratio $\frac{F_{max}\ (Additive)}{F_{max}\ (Tris)}$
Chlorpromazine (0·1 mM)	0·39 ± 0·07† (6)
$CaCl_2$ (0·25 mM)	1·0‡
$CaCl_2$ (1 mM)	1·0 ± 0·1 (3)
Promazine (0·1 mM)	0·44 ± 0·06 (3)
Diethyl ether (112 mM)	1·0‡

* Protein concentration = 0·15 to 1·3 mg/ml; TNS concentration = $2·47 \times 10^{-5}$ M; T = 25°; and MeOH = 1·25%.
† Standard deviation.
‡ Average value from two determinations.

Attempts to determine the affinity of chlorpromazine for the membrane by measuring the dependence of the TNS fluorescence enhancement on chlorpromazine concentrations were not successful. A linear relationship between relative plateau fluorescence and drug concentration was observed for anesthetic concentrations between 0·05 and 5 mM rather than the hyperbolic relationship expected if a single type of chlorpromazine binding site were involved. This result is in accord with a report by Kwant and Seeman [7] that chlorpromazine binding involves multiple sites at concentrations above 2×10^{-5} M. Lower concentrations were not investigated because the fluorescence enhancements produced were too small to be quantified.

DISCUSSION

The observation that TNS fluoresence is greatly enhanced in the presence of phenothiazines is in accord with the results of Keeler and Sharma [10], who found enhanced emission of membrane-bound ANS in the presence of promazine. As shown by our data, phenothiazines alter not only the binding parameters n and K_{app} for the interaction of TNS with membranes, but also the F_{max} of the membrane-bound TNS. If membrane expansion and increased fluidity of hydrophobic regions of the membrane were the only bases for these effects, one would expect diethyl ether, n-butanol and other anesthetic compounds to produce similar effects on TNS fluorescence. No such effect was evident in the present study (Table 1).

The enhancement of TNS fluorescence produced by chlorpromazine could be due to cationic shielding of anionic membrane constituents such as phospholipid phosphates, which would facilitate the penetration of the anionic TNS probe into the hydrophobic regions of the membrane [9]. This hypothesis is supported by the large enhancements observed with such inorganic cations as Ca^{2+} and Al^{3+}. The order of effectiveness,

$Al^{3+} > Ca^{2+} > Na^{+}$, also previously reported by Gomperts *et al.* [15], would seem to implicate a highly polarizable anion, such as phosphate, as the shielded moiety, but carboxyl groups of proteins might also be considered because of the observation of Kwant and Seeman [16] that Ca^{2+} bound to erythrocyte membranes could not be extracted with chloroform methanol. Further support for a shielding effect is provided by our observation that the enhancement of TNS fluorescence produced by chlorpromazine was abolished by raising the ionic strength of the medium to isotonicity with sodium chloride.

An organic cation such as chlorpromazine might also be capable of interacting with hydrophobic regions of membranes. Fernandez and Cerbon [17] have found that such molecules will more effectively compete with polyvalent cations for phospholipid phosphate groups. Kwant and Seeman [7] have shown the interaction of chlorpromazine with erythrocyte membranes to be hydrophobic. Presumably these forces can enhance the binding of organic cations and magnify their charge-shielding effects on TNS fluorescence. Such an effect is manifested in our study by the larger increases in both K_{app} and n produced by chlorpromazine relative to those produced by $CaCl_2$. Thus, chlorpromazine is more effective in enhancing TNS binding than would be expected from ionic effects alone. Moreover, chlorpromazine also differed markedly from Ca^{2+} in that it reduced the F_{max} of membrane-bound TNS, whereas Ca^{2+} had no effect on this parameter.

By use of procaine and its analogs, several authors have concluded that there is a requirement for both a positive charge and hydrophobicity in the enhancement of ANS fluorescence. Procaine itself has been shown by Fernandez and Cerbon [17] to be incapable of hydrophobic interaction with liposomal membranes, and our results show that on a molar basis it produces only a small fraction of the fluorescence enhancement observed for chlorpromazine at pH values where it is cationic. Feinstein *et al.* [18] have found that butacaine enhanced ANS fluorescence in bovine erythrocytes, but it has a hydrocarbon tail presumably capable of hydrophobically interacting with membrane lipids. Koblin *et al.* [19] have shown that 2-(*N*,*N*-diethylamino)-ethyl-*p*-alkoxylbenzoate analogs of procaine enhance ANS fluorescence in bovine erythrocytes in a manner related to the length of their hydrophobic tail.

The ability of chloropromazine to increase the number of available TNS binding sites (n) by 7-fold over that for Tris–HCl would seem to provide confirmatory evidence for the membrane expansion effects of chlorpromazine reported by Seeman *et al.* [6]. Unlike the sites made available by Ca^{2+}, these sites have a lower average quantum yield for fluorescence (F_{max}) than that observed in buffer alone or in buffer containing Ca^{2+}. One explanation for this phenomenon might be that the sites made available by chlorpromazine are less hydrophobic than those present in the unexpanded membrane. However, the likelihood that such change in the dielectric constant at the sites of TNS binding is responsible for the lower quantum yield is reduced by the observation of Feinstein *et al.* [18] that the quantum yield of membrane-bound ANS is not affected by the cation butacaine. The results of Koblin *et al.* [19] have suggested that quenching may be responsible for the lowered F_{max} in the presence of chlorpromazine. They found that *p*-alkoxylbenzoate anesthetics labeled with nitroxide radicals were capable of quenching ANS fluorescence in the presence of bovine erythrocytes and, since the quenching process involved an interaction distance of only 4–6 Å, they concluded that the local anesthetic must be in very close proximity to the dye on the membrane. If a similar proximity relationship exists for chlorpromazine and TNS, the quenching of TNS fluorescence by chlorpromazine may account for the observed decrease in F_{max}. Promazine produced effects qualitatively similar to those of chlorpromazine, also reducing F_{max} and increasing n.

Several authors have reported that phenothiazine anesthetics and Ca^{2+} compete for membrane-binding sites. Kwant and Seeman [16] found that bound Ca^{2+} is released by chlorpromazine in human erythrocyte membrane systems with two molecules of anesthetic being required to displace each Ca^{2+}. Keeler and Sharma [10] reported that promazine decreased the Ca^{2+}-enhanced ANS fluorescence in rabbit erythrocytes, suggesting a possible competition between the two agents. In our study, the enhanced TNS fluorescence emission produced by the addition of $CaCl_2$ (1·0 mM) was not measurably affected by the addition of chlorpromazine (0·1 mM). The enhancement was not decreased by the phenothiazine as in the case of ANS and promazine, nor was it increased by an amount equal to the chlorpromazine enhancement. In a mixed system where both species contribute to the fluorescence enhancement and produce similar values of F_{max} and n for TNS binding, we were not able to determine using our fluorescent probe methods whether or not competition was occurring with the human erythrocytes. The presence or absence of alterations in the observed fluorescence enhancements is not in itself indicative of competition unless accompanied by a comparison of F_{max} and n values in the mixed system relative to those in the presence of the individual components. In our case, differences in these values were not large enough to allow us to draw a valid conclusion.

Several conclusions can be drawn concerning the usefulness of TNS for assessing membrane effects of anesthetics. The quantum yield of this anionic probe is insensitive to both ether and *n*-butanol which expand the membrane and to Ca^{2+} which contracts it, presumably because the hydrophobic environment of the bound TNS molecules is not altered by these agents. Furthermore, TNS fluorescence is only very slightly altered by organic cationic anesthetic agents such as procaine and lidocaine which do not interact strongly with the hydrophobic regions of membranes [17].

Apparently, substantial enhancement requires the ability to hydrophobically interact with the membrane at or near locations involved in TNS binding so that charge shielding can occur. The phenothiazines have such a capability, and the TNS probe is sensitive to this interaction with the erythrocyte membranes. Furthermore, the results suggest that anionic probes might be most useful for studying the membrane effects of cationic drugs, cationic probes for anionic drugs and neutral probes for neutral drugs. Studies are currently being conducted to further examine this hypothesis.

The present study also shows that a decrease in Ca^{2+}-enhanced TNS fluorescence in the presence of a drug is not necessarily indicative of a displacement of Ca^{2+} from the membrane by the drug. The drug could be altering the quantum yield of TNS fluorescence rather than the number of binding sites for TNS or Ca^{2+}. It becomes clear that any observed effect on fluorescence that seems to be due to competition for binding sites on the membrane must be analyzed so as to determine effects on both binding and quantum yield.

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