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Potential-sensitive membrane association of a fluorescent dye

G. Andrew Woolley, Moira K. Kapral and Charles M. Deber

Research Institute, Hospital for Sick Children, Toronto M5G 1X8 and *Department of Biochemistry, University of Toronto, Toronto, M5S 1A8, Ontario, Canada*

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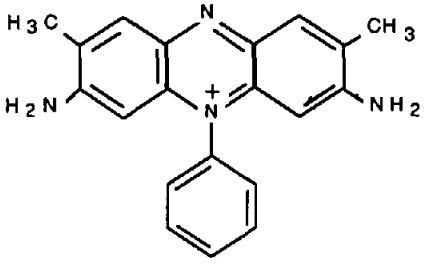
Unilamellar phosphatidylcholine/cholesterol (5:1, w/w) vesicles and the fluorescent dye safranine O mixed in appropriate ratios produced a membrane potential-dependent enhancement of dye fluorescence. The fluorescence enhancement was shown to be dependent on the sign and magnitude of valinomycin-induced potassium diffusion potentials. The enhancement and a blue-shifted maximum (both of which also occur in ethanol vs aqueous solution) provided evidence that the enhanced fluorescence arises from an additional population of safranine O molecules which become associated with a hydrophobic region of the vesicular membrane. Consistent with this interpretation, the polarization of safranine O fluorescence was also found to increase in a potential-dependent manner. A time-dependent decay of the fluorescence enhancement – presumably due to decay of the membrane potential – was attributed to non-specific ion leakage at valinomycin concentrations above 3 μM.

Safranine O; Fluorescence; Membrane potential; Valinomycin; Phospholipid vesicle

1. INTRODUCTION

Safranine O (scheme 1) is a potential-sensitive fluorescent dye suitable for spectroscopic detection of a membrane potential in cellular and vesicular membrane systems. Extensive use of this dye has been made, particularly in measuring mitochondrial membrane potentials [1,2] as well as potentials across other cell membrane preparations ([3], review [4]). Studies of its behavior have been reported in well-defined lipid vesicles prepared by an extrusion technique [5], and in cardiolipin-containing vesicles [6]. In general, most authors have reported quenching of safranine O fluorescence in response to a membrane potential (negative inside), or changes in absorbance, both of which were attributed either to precipitation of the dye (at high concentrations) or stacking of dye molecules at internal sites [2,5]. However, using relatively lower concentrations of safranine O than most previous workers in systems comprised of phosphatidylcholine/cholesterol (PC/Chol) vesicles ~300 Å in diameter, we have observed significant increases in safranine O fluorescence which are sensitive to both the sign and magnitude of the membrane potential. The molecular events underlying this fluorescence enhancement



Scheme 1. Structure of safranine O.

Correspondence address: C.M. Deber, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada

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phenomenon – originally noted in a study of active transport in membrane vesicles from *E. coli* [7] – and its time-dependent decay, are discussed herein.

2. MATERIALS AND METHODS

2.1. *Preparation of unilamellar vesicles*

Egg PC (Avanti Polar Lipids, Birmingham, AL) and Chol (Sigma) in appropriately weighed ratios (normally 5:1 PC/Chol, w/w) were mixed and dissolved in chloroform. Evaporation of solvent under nitrogen left a lipid film which was further dried for 1 h under vacuum. An aliquot of the solution to be included in the vesicles' inner compartment was added to produce a final lipid concentration of ~15 mg/ml (PC). Typically, this solution was 150 mM KCl, 5 mM Hepes, 1 mM EDTA (pH 7.0) except where the major cation or anion was changed as noted. This suspension was vortex-mixed for 1 min and then sonicated in a Branson bath sonicator at room temperature under nitrogen until the solution became clear. These procedures yielded essentially unilamellar vesicles (300 Å diameter) as judged by negative staining electron microscopy [8]. Samples of this preparation (0.5 ml) were then centrifuged through 3 ml Sephadex G-50 (medium) (Pharmacia, Uppsala) gel-filtration columns twice in succession according to Neal and Florini [9]. The columns had been pre-incubated with the appropriate external solution (e.g. 149 mM NaCl, 1 mM KCl, 5 mM Hepes, 1 mM EDTA; pH 7.0). Lipid concentration was then determined by phosphate assay [10] for all preparations. Vesicles were further diluted with external buffer to the desired concentrations in a final volume of 3 ml. Safranine O (Sigma, St. Louis, MO) was added typically as 20 μl of a 0.15 mM solution in water. Valinomycin (Fluka, Switzerland) was added typically as 20 μl of a 1 mg/ml solution in DMSO. Fluorescence was measured on a Spex Instruments fluorolog 2 spectrofluorometer with excitation at 522 nm and emission at 581 nm except where noted.

Polarization measurements were made with an Aminco fluorimeter and are the averages of three experiments. Polarization was calculated from the formula

$$P = (I_{VV} - I_{VH})/(I_{VV} + I_{VH})$$

where the subscripts (V, vertical; H, horizontal) refer to the excitation and emission polarizers, respectively. A scrambler was employed between the emission polarizer and the emission monochromator to eliminate the need to correct for differences in the efficiency of detection of vertically and horizontally polarized light.

3. RESULTS

Safranine O in the presence of PC/cholesterol (5:1, w/w) vesicles gave a steady baseline fluorescence which was higher than that observed in the absence of vesicles. A plot of safranine O fluorescence vs vesicle concentration (not shown) suggests that this initial fluorescence increase is due to low-affinity binding ($K_a \sim 5 \times 10^2$ M^{-1}) of the dye to the lipid vesicles. Addition of the K^+-specific ionophore valinomycin to vesicles with a K^+ gradient (150 mM KCl inside, 1 mM KCl outside) (fig.1a) caused a rapid rise in fluorescence followed by a slow return to baseline over the course of several minutes. Samples prepared in exactly the same way but without a K^+ gradient (150 mM KCl inside and outside) showed no change in fluorescence when valinomycin was added (fig.1b). Control experiments demonstrated that DMSO added in the amounts used to add valinomycin had no effect. The direction of the K^+ gradient was critical, however; vesicles sonicated in NaCl solution and eluted through columns in KCl buffers (which produces a 'negative outside' potential when valinomycin is added) showed no changes in fluorescence. Furthermore, as anticipated from the known K^+ selectivity of valinomycin, an Na^+ gradient of the same sign and magnitude (as for K^+ in fig.1a) produced by sonicating vesicles in NaCl solution and passing them through a choline chloride column also did not induce the fluorescence increase.

The valinomycin-induced fluorescence increase was found to be sensitive to the magnitude of the K^+ gradient. If it is assumed that valinomycin renders the vesicle membrane much more permeable to K^+ than to any other ions [11], the initial membrane potential can be calculated from the Nernst equation:

$$\Delta\psi = RT/zF \ln ([K^+]_{out}/[K^+]_{in}) \quad (1)$$

By varying the external K^+ concentration, $[K^+]_{out}$, we generated a series of membrane potentials as

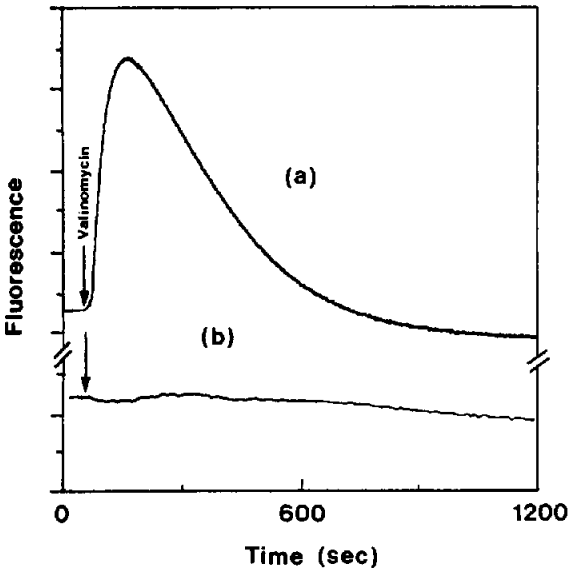

Fig.1. Potential-induced fluorescence enhancement of safranine O (1 μM) in the presence of unilamellar PC/Chol (5:1, w/w) vesicles. (a) Valinomycin (20 μl of 1 mg/ml solution in DMSO) added at time = 0 to PC/Chol vesicles (1 mg/ml) containing 150 mM KCl inside, suspended in external buffer containing 1 mM KCl. (b) Same as (a), but with 150 mM KCl both inside and outside vesicles. See section 2 for further details. Fluorescence of safranine O measured at 581 nm. Chart recording was halted during valinomycin additions.

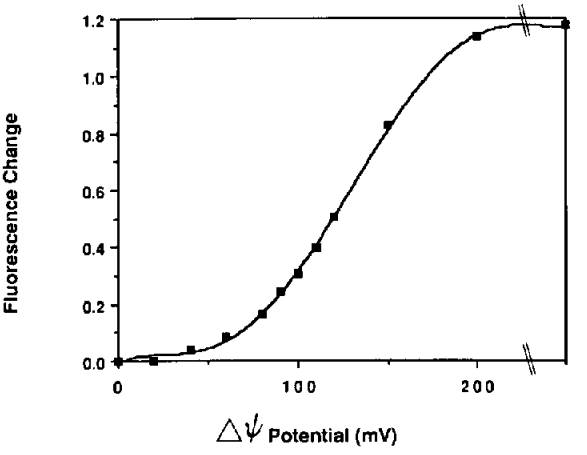

Fig.2. Intensity observed experimentally for potential-induced safranine O fluorescence vs membrane potential calculated using $[K^+]_{out}$, $[K^+]_{in}$ and the Nernst equation (see text and eqn 1).

shown in fig.2, where the magnitude of the observed fluorescence increase (taken at the maximum in each curve) is plotted vs calculated potential (eqn 1). The overall shape of the curve is sigmoidal but the region between 80 and 150 mV can be approximated by a straight line. The shape of this curve corresponds exactly to what would be predicted if the free energy of binding of the dye is proportional to the transmembrane potential (eqn 2):

$$\Delta G_{assoc} = k(\Delta\psi) + \Delta G_0 \quad (2)$$

where ΔG_0 is the intrinsic binding energy in the absence of a potential and k is a proportionality constant. (We note that $\Delta G_{assoc} = RT\ln K_{assoc}$, where K_{assoc} is given by $[D]_b/[D]_f[L]$. In this expression, $[D]_b$ is the concentration of bound dye, $[D]_f$ the concentration of free dye, and [L] is the concentration of unoccupied binding sites. One can thus calculate K_{assoc} as a function of $\Delta\psi$ by combining eqns 1 and 2. Since the total dye concentration is known, the relative amounts of bound and free dye can be calculated at a given potential.) A plateau is reached when either all the dye is bound or all binding sites are saturated.

That the potential-induced increase in fluorescence is due to accumulation of safranine O molecules into a non-polar region of the vesicle membrane is supported by two further observations. First, as shown in fig.3, safranine O fluorescence in the less polar medium ethanol is much more intense than in water. Secondly, measurements of the valinomycin-induced fluorescence increase over baseline at various wavelengths, shown in fig.4, demonstrate that the fluorescence emission maximum wavelength of membrane-associated dye is blue-shifted. The wavelength of maximum fluorescence of the dye is similarly blue-shifted ~20 nm in ethanol vs water (fig.3).

We considered the possibility that the observed fluorescence enhancement is due to changes in quantum yield of (the low-affinity) bound dye rather than to changes in the extent of safranine O binding per se. However, this is unlikely to be the case since (i) the observed changes in fluorescence are relatively large (up to 100%) while most known effects of membrane potential on quantum yield are much smaller [12]; (ii) at higher dye concentrations (10 μM), we noted (by fast separation of

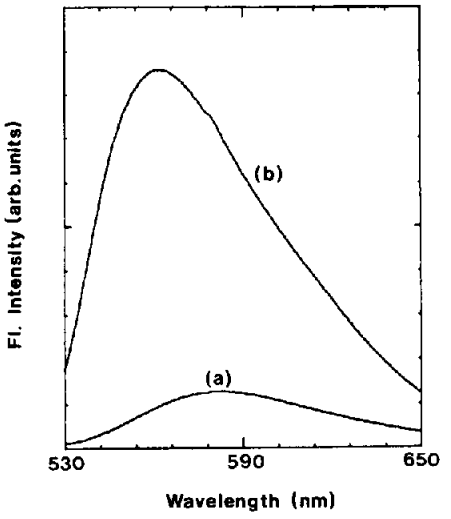

Fig.3. Fluorescence spectrum of safranine O (1 μM) (a) in KCl buffer and (b) in ethanol. Excitation at 522 nm.

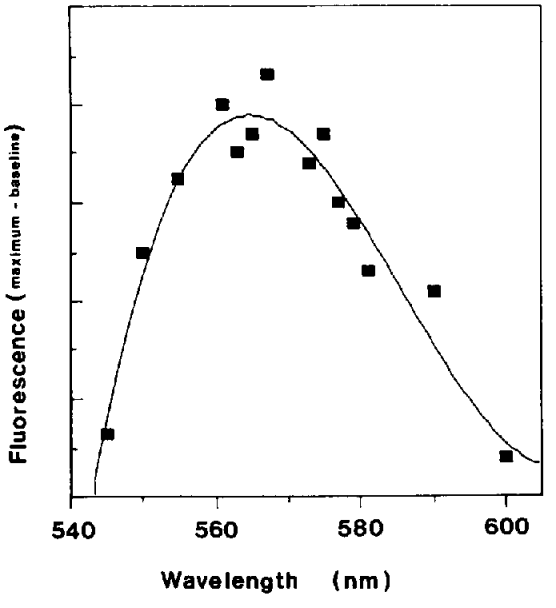

Fig.4. Wavelength dependence of safranine O potential-induced fluorescence intensity in the presence of PC/Chol vesicles. Intensity is measured as the maximum fluorescence minus the baseline at each wavelength. Experimental conditions are as given in fig.1a, but emission wavelength was varied as indicated.

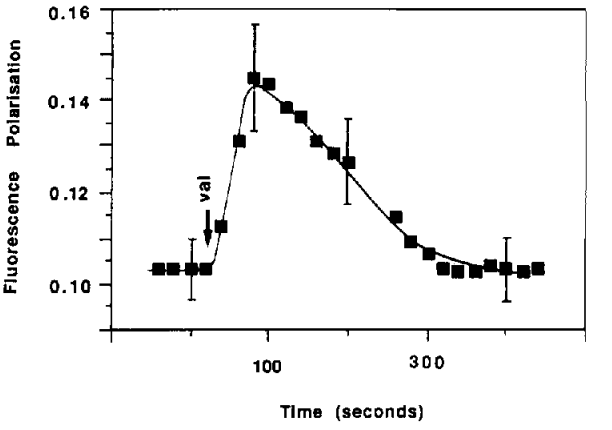

Fig.5. Conditions are as given in fig.1a except that the sample was excited with vertically polarized light. Emission was measured with the polarizer either vertically or horizontally. Polarization was calculated as outlined in section 2. Results are the average of three experiments.

vesicles from the surrounding solution using mini gel-filtration columns) that the amount of vesicle-associated dye is qualitatively greater in those vesicles with an ion gradient, indicating the intrinsic capacity of the vesicles to take up additional dye in the concentration range 1–10 μM; and (iii) safranine O fluorescence polarization was found to increase significantly when valinomycin was added to vesicles having a transmembrane potential (fig.5). Also, the time course observed in polarization experiments was the same as with total fluorescence. This latter behaviour is consistent

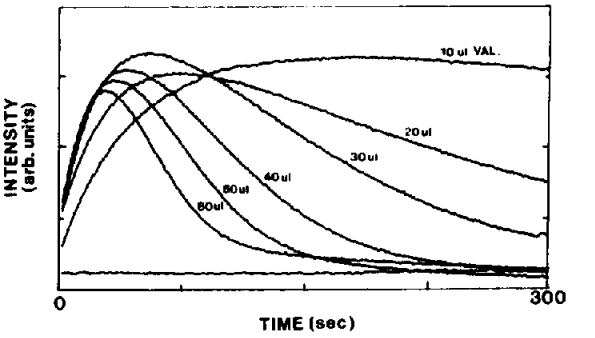

Fig.6. Potential-induced fluorescence enhancement of safranine O in the presence of PC/Chol vesicles. The volume of valinomycin solution (1 mg/ml in DMSO) added (at time = 0) was varied as shown from 10 μl to 80 μl. Experimental conditions are as given in fig.1a.

with increased binding of safranine O to vesicles and consequent slowing of the rotational motion of the dye. A study by Waggoner et al. [13] suggested that a similar type of partitioning is responsible for the potential-dependent changes in fluorescence of a cyanine dye (diS-C2-(5)).

The decay in fluorescence is facilitated by valinomycin in a concentration-dependent manner as can be seen in fig.6. (Importantly, valinomycin added as a 10 μl aliquot to this system produces a relatively stable enhanced fluorescence; fig.6a.) Yet this effect does not depend on the presence of safranine O; timed addition of the dye after addition of valinomycin (not shown) demonstrated that the fluorescence decays in the same manner in the absence of the dye.

4. DISCUSSION

The increases in fluorescence observed in the present experiments appear firstly to be due to our use of one to three orders of magnitude lower concentration of safranine O (e.g. see [5]), since in our system, the formation of dimers or multimers (stacking) which decrease fluorescence should be minimal. Stacking has been shown to occur as the dye concentration approaches 1 mM [14], whereas we have used a dye concentration of 1 μM in the present experiments. Given the intrinsic K_a of 5×10^{-2} M^{-1} (vide infra), the initial bound dye concentration is calculated as ~0.18 μM. We also noted that fluorescence intensity displays a nearly linear dependence on dye concentration in this range (not shown), further suggesting that safranine O self-association does not occur. At high concentrations, binding of safranine O to lipid may already be saturated so that the specific effects of changes in membrane potential on dye fluorescence would accordingly be smaller. In the present system, we therefore conclude that creation of the membrane potential enhances dye fluorescence essentially by inducing further partitioning (additional to the 'low-affinity' initial binding) of the dye into the membrane.

While the mechanism of the fluorescence decay remains to be elucidated fully, we showed that specific transport of other ions by valinomycin probably does not occur. Thus, Na^+ can be replaced by (larger) choline$^+$ in the external solution with little effect on decay rates. In addition, Cl^- can be replaced by (less permanent) SO_4^{2-} without effect. Safranine O fluorescence is insensitive to pH per se, so that small pH changes which might occur in our weakly buffered system would also not affect fluorescence directly. Since the initial height of the potential-dependent fluorescence enhancement remains essentially constant over a range of valinomycin concentrations (fig.6), it is unlikely that increasing amounts of intramembranous valinomycin interfere or compete with safranine O binding. Also, since all concentrations of valinomycin cause fluorescence decay to the same baseline, formation of a valinomycin-safranine O complex of decreased fluorescence is unlikely. Thus, the source of the potential decay appears to be non-specific ion leakage due to membrane disruption (and hence, potential decay) caused by increasing concentrations of valinomycin (i.e. greater than a bulk concentration of 3 μM as in fig.6, 10 μl curve).

The presence of a variety of lipids (as well as proteins) in biological membranes could affect the number and affinity of binding sites for dyes such as safranine O. Further, the fluorescence yield of the dye may vary at different binding sites (e.g. some sites may promote dimerization of safranine O and thus fluorescence quenching). Nevertheless, the present work reaffirms the property of small molecules to be 'driven' into membrane microenvironments in response to a membrane potential. Similar phenomena may affect the binding in vivo of lipophilic/cationic molecules – such as neurotransmitters and neuropeptides – to cellular membranes.

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