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EFFECT OF LIPID COMPOSITION CHANGES ON CARBOCYANINE DYE FLUORESCENT RESPONSE

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SUMMARY: Egg yolk phosphatidyl choline liposomes containing variable amounts of phosphatidyl ethanolamine, phosphatidyl inositol or phosphatidyl serine demonstrated important variations in the fluorescence of 3.3' dipropylthiodicarbocyanine. When the membrane contained no cholesterol, fluorescence was not correlated with membrane fluidity as measured by diphenyl hexatriene polarization. Increasing cholesterol concentration in valinomycin containing liposome membranes decreased the potassium induced apparent membrane potential and prevented sorption of dye to the membrane. Discontinuity in the apparent potential occurred at 30 mol % cholesterol but could not be correlated with changes in microviscosity. These results indicate that great care should be taken when correlating rapid variations of fluorescence to changes in membrane potential. We propose that changes in phospholipid metabolism could well explain fluorescent changes when monitoring the fluorescence of cyanine dye molecules sorbed to biological membranes.

During the last decade numerous groups have focused on the use of fluorescent dyes (1-8) to study plasma membrane potential of living cells because of difficulties in using microelectrodes. The intensity of the fluorescence of several cyanine dyes has been correlated with the membrane potential in a number of different cell preparations (1-13). Among these dyes, 3.3' dipropylthiodicarbocyanine [diS-C_3-(5)] is one of the most commonly used (5,8-10,12-13).

Abbreviations used: EYPC, Egg yolk phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; Chol, cholesterol; diS-C_3-(5), 3.3' dipropylthiocarbocyanine; diS-C_2-(5), 3.3' diethylthiocarbocyanine; DPH, 1.6 diphenyl-1.3.5 hexatriene; FMLP, N-formyl-methionyl-leucyl-phenylalanine; PMN, polymorphonuclear neutrophils.

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Recently membrane potential changes have been monitored in polymorphonuclear leukocytes (PMN) recording fluorescence of diS-C_3-(5) and diO-C_5-(3) before and after stimulating the cells with the chemotactic peptide formyl-Met-Leu-Phe (FMLP) (10-13). FMLP has been reported to induce rapid depolarization of the membrane (< 20 sec) with an apparent positive potential as judged by the maximum change in fluorescence intensity (12). It has also been reported that stimulation of PMN by FMLP induces rapid (< 20 sec) and large (approx. 10 %) variations in the composition of the different categories of plasma membrane lipids studied (14-15).

Although attention has been given to the study of the interaction of cyanine dyes with liposomes or bilayer membranes (16-18), very little is known about the effect of phospholipid turnover and fast changes in phospholipid composition on the penetration of fluorescent dyes in the membrane or their absorption at the membrane interface. The penetration and (or) sorption of the dye to the membrane have been reported to be responsible for variations in diS-C_3-(5) fluorescence (2-4).

The purpose of this study was to demonstrate how the fluorescence changes of diS-C_3-(5) and diS-C_2-(5) in phosphatidyl choline liposome membranes may be modulated by variable amounts of phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol or cholesterol.

The effect of increasing amounts of cholesterol on the apparent potential of liposomes containing valinomycin and high concentration of potassium, was also investigated.

MATERIALS AND METHODS

Liposome membranes were prepared as previously described (19). Briefly, they were prepared from egg yolk phosphatidyl choline (EYPC) or mixtures of EYPC and variable amounts of phosphatidyl serine (PS), egg yolk phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) or cholesterol (chol). Dry films evaporated under nitrogen from chloroform solutions were allowed to swell by vigorous vortexing at 35°C in an aqueous phase which consisted of a Tris-HCl Buffer (20 mmol/l pH 7.4) containing NaCl (120 mmol/l) or KCl (120 mmol/l). EYPC, PS, PE and chol were from Sigma Chemical Co. (St.louis, Mo). PI is from Koch light (Colinbrook England) and Valinomycin is from Calbiochem (San Diego,

Ca). The cyanine dye diS-C_3-(5)I and diS-C_2-(5)I were kindly supplied by Dr. Alan Waggoner. Stock solutions of dye ($.4.10^{-3}$ mol/l) were made in ethanol and kept in the dark at -10°C. This solution was diluted 200 fold in the buffer prior each experiment. The fluorescence intensity of diS-C_3-(5) was recorded with a SLM 4800 spectrofluorometer at 670 nm under excitation light at 622 nm. 220 μl of liposomes suspension (1 mg PL/ml) were injected in the cuvette containing 2 ml of buffer with diS-C_3-(5) (or diS-C_2-(5)).

The fluorescence polarization of liposomes labelled with 1.6 diphenyl 1.3.5 hexatriene (Sigma Chem. Co.) 1 ml °/°° lipid was measured between 20° and 45°C with an SLM 4800 (SLM Instruments Inc., Urbana, Ill.) equipped with Glan-Thompson polarizer or with an Elscint MV_{1a} microviscosimeter (Elscint Ltd., Haifa, Israel).

RESULTS AND DISCUSSION

Fig. 1 illustrates typical results of 3 series of experiments designed to investigate the influence of increasing concentrations of three lipids, namely PS (fig.1a), PE (fig.1b) and PI (fig.1c), on the process of quenching of cyanine dye fluorescence in EYPC liposomes. Only lipids bearing a net negative charge were found to sorb the dye in a dose dependent manner.

Fig. 2 illustrates the effect of increasing concentrations of cholesterol in EYPC liposomes on the evolution of fluorescence of diS-C_3-(5). It is evident that cholesterol inhibits the sorption and quenching of the probe in the membrane.

Fig.3a shows the variation of fluorescence depolarization of DPH at 25 and 37°C indicating that the microviscosity of the membrane increases in a monotonous manner as the cholesterol content augments. The fluorescence plateau values of diS-C_3 in EYPC: chol liposomes containing valinomycin are given in figure 3b as a function of the cholesterol content of liposomes. Vesicles had either the same buffer composition inside and outside (NaCl or KCl 120 mmol/l - curves I and II) or contained high K^+ inside to develop a potential across the membrane (KCl 120 mmol/l inside and 12 mmol/l outside - curve III). The apparent membrane potential as calculated by the Nernst equation is given in fig. 3c. The potential clearly vanishes as cholesterol concentration is increased in the liposomes ($>$ 30 mol %).

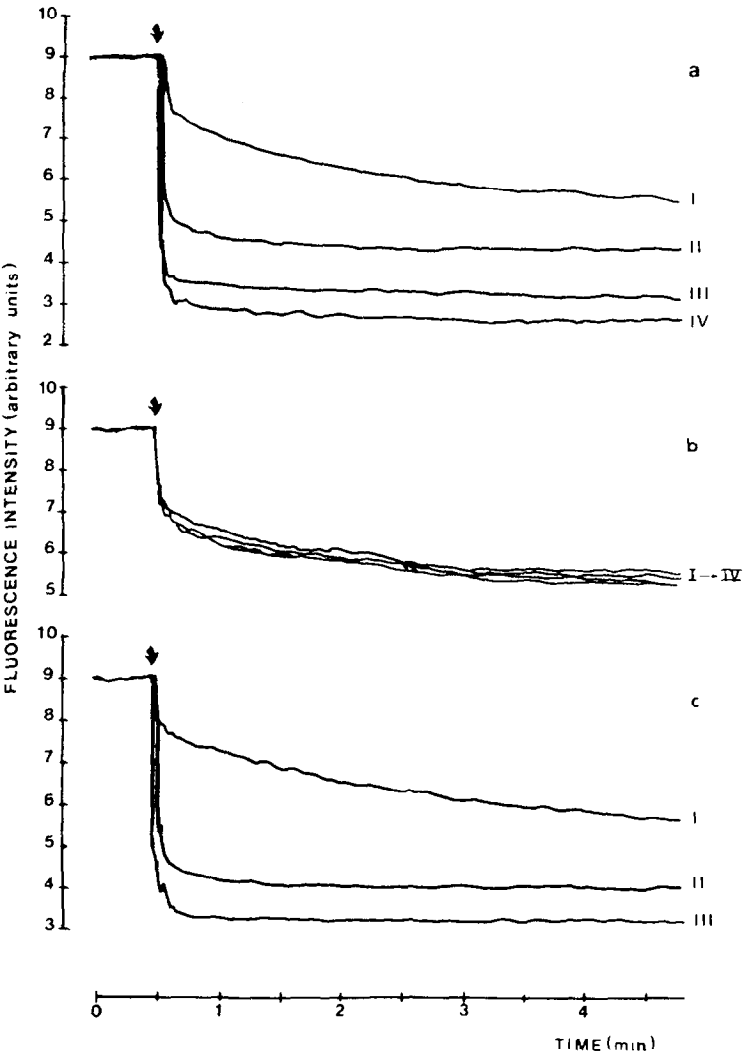

Fig.1a,b,c: effect of increasing concentrations of PS (a), PE (b) and PI (c) on the fluorescence of diS-C_3-(5) in EYPC liposomes as function of time. Arrow indicates time of injection of the phospholipid vesicles. Concentrations of PS, PE and PI were 10 % molar (curves II), 20 % molar (curves III) and 30 % molar (curves IV) as compared to control experiments (curves I: pure EYPC liposomes). Curves are the mean of 2 or 3 experiments.

The present results demonstrate that changes in phospholipids may substantially affect fluorescence quenching of the cyanine dye generally used to measure the membrane potential of vesicles and cells. A number of experiments repeated with 3,3' diethylthiodicarbocyanine iodide (diS-C_2-(5)I) showed essentially the same results. Negatively charged phospholipids (PS, PI) augment the quenching of the dye in the membrane. Because the dye mole-

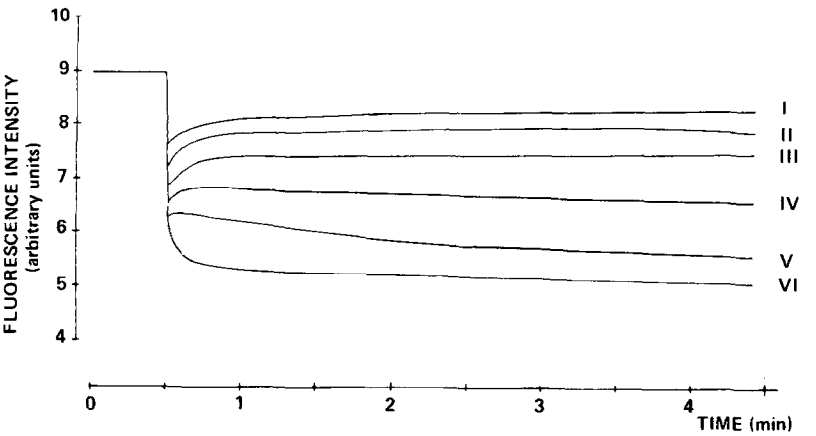

Fig.2: effect of increasing the concentration of cholesterol in EYPC liposomes on the fluorescence of diS-C_3-(5) as a function of time. Curves I to IV refer to liposomes containing 50, 40, 30, 20, 10 and 0 mol % cholesterol respectively. Curves are the mean of two experiments.

cule carries a net positive charge, our findings indicate that negatively charged phospholipids augment the sorption of the dye to membranes. This also explains the lack of effect of a neutral phospholipid (PE).

Increasing cholesterol content diminishes the amount of sorbed dye. This is probably due to specific interactions of cholesterol with EYPC (20) and also in part to membrane viscosity. The effect of different lipids studied on the intensity of fluorescence of diS-C_3-(5) suggests that changes in fluorescence monitored in biological membranes are caused by alterations in phospholipid turnover or PI cycle (21-22) rather than by eventually associated changes in membrane potential. This hypothesis is further strengthened by the fact that the first component of the response observed in PMN as triggered by FMLP, is not modified when substituting the major external ions concentrations by choline or K^+ (11-12).

This indicates that one must be careful when characterizing changes in fluorescence as changes in membrane potential Moreover, phospholipid metabolism of PMN exposed to the chemotactic peptide FMLP is activated and large variations (approx. 10 %) in specific phospholipids are shown within 15 seconds (14-15, 23). If these phospholipid changes are localized in the plasma membrane, they would certainly affect the sorption-desorption mechanism of the dye. The rapid decrease in PI,

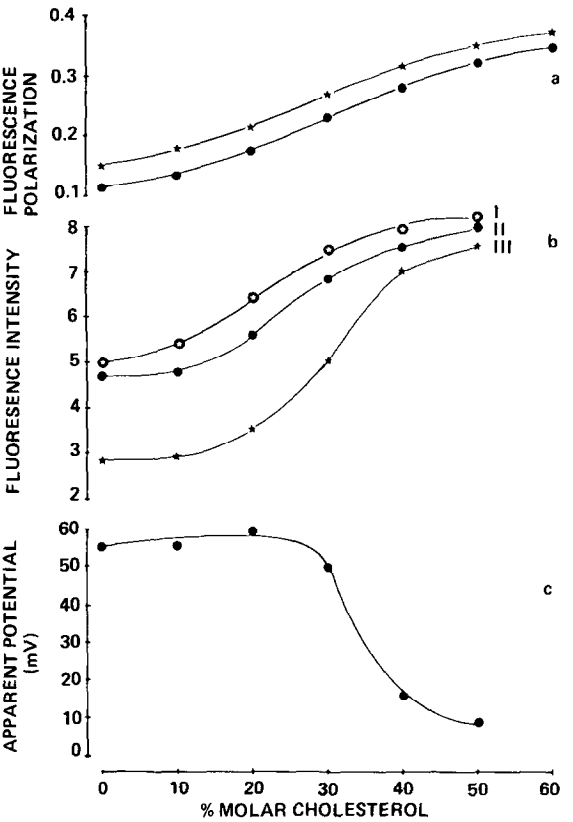

Fig.3a,b,c,: evolution of the fluorescence polarization of DPH (a), of the fluorescence intensity plateau value of diS-C_3-(5) (b), and of the apparent membrane potential (c) as the molar fraction of cholesterol increases in EYPC liposomes.
a) Polarization values at 25°C (upper curve) and 37°C (lower curve)
b) Fluorescence intensity of vesicles containing valinomycin with the same buffer composition inside and outside (NaCl or KCl 120 mmol/l; curves I and II respectively) or with high K^+ inside and low K^+ outside (curve III; KCl 120 mmol/l inside and NaCl 108 mmol/l, KCl 12 mmol/l outside).
c) Apparent membrane potential calculated from the fluorescence intensities relative to the value calculated by the Nernst equation for pure EYPC-valinomycin membranes.

largely exceeding the increase in phosphatidic acid, could explain the fast recovery of fluorescence because of dequenching of the probe.

As a matter of fact, it should be noted that the PI content returns to normal after 60-120 sec (14) and that these temporal changes parallels the effect of FMLP on fluorescence reported by different authors (11-13).

The slow decrease in fluorescence (quenching) below baseline levels observed after 60-120 sec, considered by some

to reflect hyperpolarization of the cell, could in fact be due to an increase of total negative phospholipids immobilizing increased amounts of the probe.

It should be noted that our experiments have been conducted with large multilamellar vesicles (MLV) to avoid the effect of surface curvature on sorption of the dye reported with unilamellar vesicles (17). The final lipid concentration is 0.1 mg/ml. If one considers that MLV may be formed by 15-25 concentric bilayers, the external surface of liposomes at this lipid concentration represents the plasma membrane surface of 2.10^7 cells/ml. This is close to the number of cells generally used in these experiments (10-13).

Finally, it could be argued that cholesterol also hinders valinomycin movements. As a matter of fact, it has been already reported that membrane fluidity modulates ion movements (24) in a manner which does not parallel the membrane viscosity (25). However, for the movement of monovalent cations (e.g. ^{22}Na), discontinuity could not be demonstrated with increasing viscosity (increasing content of cholesterol) as reported here when measuring fluorescence.

In conclusion, we believe great care should be taken when studying membrane potential changes with fluorescent probes. Rapid variations in carbocyanine dye fluorescence induced by ligands in some cell types might well reflect alterations in the composition of membrane lipids rather than changes in the electrical transmembrane potential.

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