Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Spector, A. A., & Yorek, M. A. (1985) *J. Lipid Res. 26*, 1015–1035.
Stubbs, C. D., & Smith, A. D. (1984) *Biochim. Biophys. Acta 779*, 89–137.
Stubbs, C. D., Kouyama, T., Kinosita, K., Jr., & Ikegami, A. (1981) *Biochemistry 20*, 4257–4262.
Szamel, M., Goppelt-Strübe, M., Bersler, W., Wiesmuller, K. H., & Resch, K. (1987) *Biochim. Biophys. Acta 899*, 247–257.
Viret, J., & Leterrier, F. (1976) *Biochim. Biophys. Acta 436*, 811–824.

Role of Membrane Lipids in the Interaction of Daunomycin with Plasma Membranes from Tumor Cells: Implications in Drug-Resistance Phenomena†

Pablo V. Escriba, Antonio V. Ferrer-Montiel, Jose A. Ferragut, and Jose M. Gonzalez-Ros*
Department of Neurochemistry, School of Medicine, University of Alicante, 03080 Alicante, Spain
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ABSTRACT: Equilibrium binding studies on the interaction between the anthracycline daunomycin and plasma membrane fractions from daunomycin-sensitive and -resistant murine leukemia P-388 cells are presented. Drug binding constants (K_S) are 15 000 and 9800 M^{-1} for plasma membranes from drug-sensitive and drug-resistant cells, respectively. Drug binding to the membranes is not affected by either (i) thermal denaturation of membrane proteins or (ii) proteolytic treatment with trypsin, thus suggesting that the protein components of the membranes do not have a major role in determining the observed drug binding. Also, fluorescence resonance energy transfer between tryptophan and daunomycin in the membranes indicates that interaction of protein components with the drug should not be responsible for the observed differences in drug binding exhibited by plasma membranes from drug-sensitive and -resistant cells. Plasma membranes from drug-sensitive cells contain more phosphatidylserine and slightly less cholesterol than membranes from drug-resistant cells. Differences in the content of the acidic phospholipid between the two plasma membranes seem to produce a different ionic environment at membrane surface domains, as indicated by titration of a membrane-incorporated, pH-sensitive fluorescence probe. The possible role of membrane lipids in modulating drug binding to the membranes was tested in equilibrium binding studies using model lipid vesicles made from phosphatidylcholine, phosphatidylserine, and cholesterol in different proportions. The presence of phosphatidylserine greatly increases both the affinity and the stoichiometry of daunomycin binding to model lipid vesicles. The similarity between the effects of phosphatidylserine and other negatively charged compounds such as dicetyl phosphate, cardiolipin, or phosphatidic acid suggests that electrostatic interactions are important in the observed binding of the drug. The presence of cholesterol in the model lipid vesicles causes a decrease in both drug binding affinity and stoichiometry. Furthermore, differences observed in drug binding to lipid vesicles containing phosphatidylserine and cholesterol at molar fractions resembling those found in plasma membranes from drug-sensitive and -resistant cells are similar to those observed with entire plasma membranes. On the basis of these observations, we conclude that the lipid components of plasma membranes from the drug-sensitive and -resistant P-388 cells are important in determining the extent of daunomycin binding to the membranes and in establishing the observed resistance-related differences in drug binding.

Anthracycline antibiotics such as daunomycin (DNM)[1] are potent cytotoxic drugs currently used as antitumor agents. The classical model to explain anthracycline cytotoxicity (Arcamone, 1981; Waring, 1981) has been based upon interference with nucleic acid function. However, because of the cytotoxicity demonstrated for nonpenetrating, polymer-immobilized drug (Tritton & Yee, 1982), effects at the level of the cell membrane have also been proposed.

Clinical use of anthracyclines is partly limited by the appearance of drug-resistant tumor cells during treatment. Furthermore, cell lines that develop resistance to these drugs become cross-resistant to many other chemotherapy agents (Myers et al., 1987). This acquired cross-resistance, referred to as "multidrug-resistance" (MDR), constitutes a major obstacle to the success of chemotherapy programs, and, thus, the elucidation of its molecular basis is of interest.

MDR cell lines have often been shown to exhibit a reduced net accumulation of drug relative to the parental drug-sensitive line (Myers et al., 1987; Bradley et al., 1988). Most authors

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* Author to whom correspondence should be addressed.

[1] Abbreviations: DNM, daunomycin (daunorubicin); MDR, multiple drug resistance; P388/S, wild (drug-sensitive) P388 murine leukemia cells; P388/R, multidrug-resistant P388 murine leukemia cells selected for resistance to daunomycin; P-170, high molecular weight (~170K) glycoprotein; PC, phosphatidylcholine; PS, phosphatidylserine; FRET, fluorescence resonance energy transfer; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

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have explained these observations in terms of differences in the transport of the drugs in and out of the cells. For this, an energy-requiring drug efflux and/or an impaired influx mechanisms have been proposed (Riordan & Ling, 1985; Siegfried et al., 1985). In fact, the degree of drug resistance in several drug-resistant cell lines has been correlated with the overexpression of a plasma membrane, high molecular weight glycoprotein known as P-170 (Kartner et al., 1983, 1985). Such a protein has a structure predictably similar to that of bacterial hemolysin export protein, has been identified as an acceptor for analogues of vinblastine in photoaffinity labeling studies, and is believed to act as an ATP-dependent protein pump to actively eliminate drugs from cells [for a review, see Bradley et al. (1988) and Gottesman and Pastan (1988)].

The previous paragraphs suggest that both mechanisms of drug cytotoxicity and MDR could have in common an important role for the plasma membrane of the tumor cell, as either being a cytotoxic site itself and/or regulating the transport of the drugs in and out of the cell. Both of these possibilities should imply a previous interaction of the drugs with the membrane, and indeed, for the anthracycline family or antineoplastics, drug binding to artificial liposomes (Burke & Tritton, 1985; Nicolay et al., 1984; 1988; Henry et al., 1985) and to isolated heart mytochondrial (Griffin et al., 1986) and *Torpedo* postsynaptic membranes (Ferrer-Montiel et al., 1988) have been demonstrated.

We report here equilibrium binding studies on the interaction between the anthracycline DNM and plasma membranes isolated from drug-sensitive and drug-resistant tumor cells. Use of isolated plasma membranes in binding studies eliminates some of the difficulties encountered when using whole cell systems, such as simultaneous drug internalization, metabolism, or binding to intracellular components. Our purpose is (i) to identify plasma membrane components of the tumor cells which could be responsible for drug binding and (ii) to evaluate the possible relevance of drug binding to the plasma membrane in drug resistance.

Materials and Methods

DNM hydrochloride was from Sigma. DNM concentration in dilute solutions (below 10 μM) was determined by the absorbance at 480 nm, using a molar extinction coefficient of 11 500 M^{-1} cm^{-1} (Barcelo et al., 1986). Egg phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) were from Avanti Polar Lipids, and free cholesterol was from Sigma. Trypsin and soybean trypsin inhibitor were from Worthington. Tissue culture reagents were from Gibco. 4-Heptadecyl-7-hydroxycoumarin was from Molecular Probes.

Production of Cells and Isolation of Plasma Membranes. DNM-sensitive P388 murine leukemia cells (P388/S) and a 50-fold-resistant stable subline (P388/R) (LD_{50}'s for DNM in "in vitro" proliferation assays were 0.008 and 0.4 μM, respectively) were either continuously propagated by serial passage into the abdominal cavity of DBA-2J mice (Yanovich & Taub, 1982) or cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 μM mercaptoethanol, 2 mM glutamine, 50 units/mL penicillin base, and 50 μg/mL streptomycin base (Ramu et al., 1983). Plasma membrane fractions were prepared through a procedure similar to those described by Riordan and Ling (1979) and Ramu et al. (1983). Approximately 10^9–10^{10} cells of each line, washed in Dulbecco's phosphate-buffered saline, were used in every membrane preparation. Cells suspended in 50 mL of 10 mM Tris buffer, pH 7.4, containing 200 mM NaCl, 5 mM EDTA, 5 mM iodoacetamide, and 0.5 mM phenylmethanesulfonyl fluoride (both iodoacetamide and phenylmethanesulfonyl fluoride were added to the homogenization buffer immediately prior to use) were homogenized by using a Parr nitrogen pump (800 psi for 30 min at ~4 °C), followed by a Polytron homogenizer (3 × 90 s periods at high speed). The homogenate was centrifuged, first at 450*g* for 5 min and then at 25000*g* for 20 min. Both pellets were discarded, and the supernatant was submitted to ultracentrifugation at 200000*g* for 1 h. The resulting pellet was resuspended in 5 mM Tris buffer, pH 7.0, layered on top of a discontinuous sucrose density gradient [two layers of 4 mL each of 35% and 45% (w/v) sucrose in 5 mM Tris, pH 7.0], and centrifuged in a Beckman SW 41 rotor at 37 000 rpm for 2 h. Membranes migrating at the 35% sucrose interface were identified as a plasma membrane vesicle fraction by (i) electron microscopy, (ii) specific radioactivity exhibited by this membrane fraction when intact P388 cells were radioiodinated by a glucose oxidase–lactoperoxidase procedure (Hubbard & Cohn, 1972), and (iii) binding of anti-HLA antibodies for P388 cells. Plasma membrane fractions were collected, dialyzed, and stored in liquid N_2. Yields of 5–10 mg of protein (as plasma membrane protein) were obtained, depending upon the initial number of cells. Protein and lipid phosphorus concentrations were determined by the methods of Lowry et al. (1951) and Kyaw et al. (1985), respectively, except that the latter included an acid hydrolysis step (35% perchloric acid at 190 °C, for 90 min) to release inorganic phosphate from phospholipids.

Lipid Analysis and Formation of Liposomes. The procedures used for the extraction, fractionation, and characterization of membrane lipids have been described in detail (Gonzalez-Ros et al., 1982). To prepare lipid vesicles from PC, PS, and cholesterol, thin dried films of the appropriate lipid mixtures were formed on the inner surface of glass vials by evaporation under argon from a chloroform solution. Aliquots of 10 mM Hepes buffer, pH 7.4, containing 100 mM NaCl were then added to give a final concentration of 2–4 mM in lipid phosphorus. Lipids were suspended by sonication in a Soniprep 150 apparatus, until the samples were completely transparent. The sonicated samples were submitted to two successive rapid freezing–slow thawing cycles (Pick, 1981).

Equilibrium Binding of DNM. Binding of DNM to liposomes and plasma membrane fractions from P-388 cells was determined by using ultracentrifugation and fluorescence anisotropy procedures. The latter is based on the change in steady-state fluorescence anisotropy that occurs when the drug becomes associated with membranes or lipid vesicles (Burke & Tritton, 1985a). Steady-state anisotropy was determined as described by Lakowicz (1983) for "L-format" (single-channel detection) instruments, using a Perkin-Elmer LS-5 spectrofluorometer equipped with automatic polarization and total emission accessories, a thermostated cell holder, and 0.2 × 1.0 cm quartz cells. A battery of samples containing a fixed concentration of DNM (usually 2.5 μM) and increasing concentrations of membranes or lipid vesicles (ranging 20–500 μM and 20 μM–2 mM for membranes and lipid vesicles, respectively, in lipid phosphorus) in 10 mM Hepes buffer, pH 7.4, and 100 mM NaCl was incubated at 25 °C for ~1 h, prior to fluorescence measurements to assure equilibrium conditions. The excitation wavelength was 472 nm, and the fluorescence emission of the drug was observed by using a Corning sharp-cut 3.68 filter (CGF Corning Bulletin, Corning, NY). The observed anisotropy results from contributions by free and membrane-bound fluorophore as well as from a small contribution by scattered light. The latter was eliminated by using the correction procedure suggested by Shinitzky et al. (1971). Under these conditions, including the occurrence of

changes in the quantum yield of the fluorophore upon binding, the observed corrected anisotropy (a) is related to the fraction of membrane-bound fluorophore (f_b) by the expression (Rawitch & Weber, 1972; Lakowicz, 1983):

$$f_b = \frac{a - a_f}{(a_b - a)R + a - a_f}$$

where a_f and a_b correspond to the all-free and all-bound anisotropies of the fluorescence drug, respectively, and R respresents the ratio of the fluorescence intensity of the membrane or lipid vesicle bound (I_b) and free (I_f) forms of DNM. R values were equal to 0.4 and 1.4, respectively, for the native plasma membrane and the lipid vesicle samples. I_f is measured directly, in the absence of membranes, and I_b is estimated from the y intercept of a $1/I$ vs $1/[\text{phospholipid}]$ plot obtained from measurements in the presence of increasing concentration of membranes. The anisotropy of the free drug (a_f) is determined in the absence of membranes while that of the bound drug (a_b) is estimated from the y intercept of a $1/a$ vs $1/[\text{phospholipid}]$ plot (Rawitch & Weber, 1972). Knowledge of f_b and the total DNM concentration provides directly the concentration of free and membrane-bound drug at each membrane concentration used in the assays. No chemical degradation of DNM was observed during the course of the experiments.

The ultracentrifugation assay requires a larger amount of sample than the anisotropy procedure and, therefore, was used only with lipid vesicles. DNM at concentrations ranging from 1 to 8 μM was added to a fixed amount of liposomes (ranging 0.1–0.3 mM final concentration in lipid phosphorus) in 10 mM Hepes, pH 7.4, and 100 mM NaCl. Samples (1-mL total volume) were kept in the dark and incubated overnight, at 4 °C. Aliquots (0.4 mL) were removed to determine the concentration of total drug in each sample, and the remaining incubation mixtures were centrifuged at 23 000 rpm for 120 min at 4 °C, using a type 25 Beckman rotor. Aliquots (0.4 mL) from the supernatants were used to determine free, unbound DNM. The concentration of bound drug was calculated as the difference between total and unbound DNM. The concentration of DNM in either the incubation mixtures or the supernatants was determined by fluorescence intensity measurements, in the presence of 1% sodium dodecyl sulfate.

Other Fluorescence Methods. pH titration of 4-heptadecyl-7-hydroxycoumarin incorporated into plasma membrane fractions from P388/S and P388/R cells was performed as described previously (Gonzalez-Ros et al., 1982). Experimental conditions used here for fluorescence resonance energy transfer (FRET) in membranes have been previously described (Ferrer-Montiel et al., 1988), as well as the process of anthracycline excitation by transfer of tryptophan fluorescence emission (Griffin et al., 1986).

Results

Figure 1 shows direct equilibrium binding data for the interaction of DNM with plasma membrane fractions from P388/S and P388/R cells, obtained by using the fluorescence anisotropy procedure described under Materials and Methods. The steady-state fluorescence anisotropy of the free drug was 0.065, while that corresponding to the all-bound drug ranged from 0.18 to 0.20 for membrane samples, and from 0.26 to 0.30 for lipid vesicles, depending upon lipid composition. This emphasizes the advantages of monitoring drug binding by fluorescence anisotropy (3–5-fold increase from the all-free to the all-bound drug), rather than by using the smaller changes in fluorescence intensity (Lakowicz, 1983; Burke & Tritton, 1985a). The total DNM concentration used in these experiments did not exceed 8 μM, and, therefore, the binding data should represent relatively uncomplicated equilibria where both bound and free forms of DNM are in the monomeric state (Chaires et al., 1982).

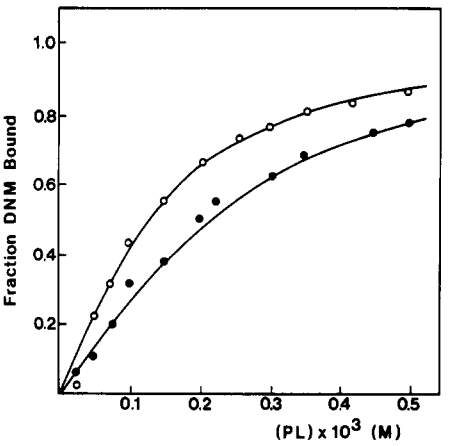

FIGURE 1: Representative equilibrium binding data for the interaction between DNM and plasma membrane fractions from DNM-sensitive (○) and -resistant (●) P388 cells. Total DNM concentration was maintained constant (2.5 μM) in all samples, and the fraction of bound drug was determined, at 25 °C, by using a steady-state fluorescence anisotropy procedure (see Materials and Methods).

Table I: Equilibrium Binding Parameters for the Interaction of DNM with Plasma Membrane Fractions from P388/S and P388/R Cells[a]

	$K_S \times 10^{-4}$ (M^{-1})	n
P388/S membranes	1.50	0.016
P388/R membranes	0.98	0.012

[a] Equilibrium binding data were obtained at 25 °C, using a steady-state fluorescence anisotropy assay, as described under Materials and Methods. K_S and n are, respectively, the overall binding constant and the stoichiometry (moles of DNM bound per mole of phospholipid) and were estimated from C_f/r vs C_f plots assuming a single class of DNM binding sites or multiple classes of noninteracting sites with identical binding constants. Experimental error was ±10% (n = 3).

Binding data, such as that shown in Figure 1, were analyzed by using C_f/r vs C_f plots (Klotz & Hunston, 1971), where C_f is the concentration of free drug and r represents the concentration of bound drug (C_b) divided by the phospholipid concentration. It should be noted that all the drug binding experiments were conducted at low r values (low bound drug/phospholipid ratios) and, thus, the fluorescence monitoring of the binding process should not be masked by aggregation (self-association) of the drug at the membrane surface (Tritton & Burke, 1984). Also, since the phospholipid to protein ratios in both membrane types are identical (~0.79 μmol of phospholipid/mg of protein), our data were easily referred to either phospholipid or protein concentration. Plots of the drug binding data always resulted in straight lines which, according to the Klotz and Hunston model, suggested the existence of either a single class of drug binding sites or multiple classes of noninteracting sites with identical binding constants. For these cases, the slope of the C_f/r vs C_f plot is equal to $1/n$, where n is the apparent maximum number of binding sites per phospholipid molecule, while the y-axis intercept equals $1/K_S$, where K_S is the overall binding constant. Values of n and K_S obtained from four independent experiments using three different sets of plasma membrane preparations from each cell line (two preparations from cells propagated in mice and one from both cell lines in tissue culture) are shown in Table I. Identical results were also obtained by Scatchard analysis of the binding data. Drug binding constants for the plasma membranes from P388/S are

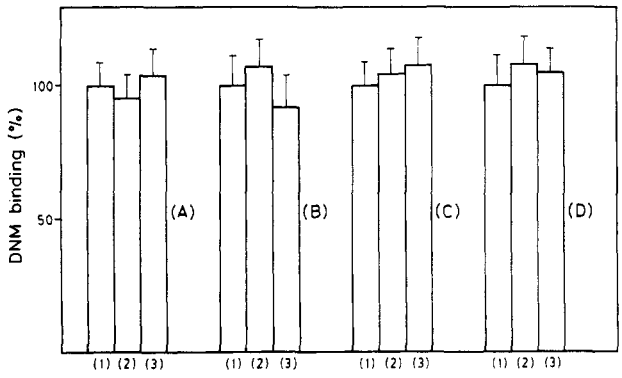

FIGURE 2: DNM binding to heat-denatured (bars numbered "2") or trypsin-treated (bars numbered "3") plasma membrane fractions from P388/S (A and B) and P388/R (C and D) cells. Values for the DNM binding constant (A and C) and stoichiometry (B and D) are given as the percentage with respect to those binding parameters exhibited by untreated membrane samples (bars numbered "1"). Heat-denatured samples were prepared by heating membranes at 80 °C for 15 min, prior to the binding assay. For trypsin treatment, membrane samples were preincubated with 20 ng of trypsin/mg of membrane protein, at 37 °C for 30 min, followed by the addition of an equimolar amount of soybean trypsin inhibitor. DNM binding to heat or trypsin-treated samples was determined, at 25 °C, by using the fluorescence anisotropy procedure described under Materials and Methods.

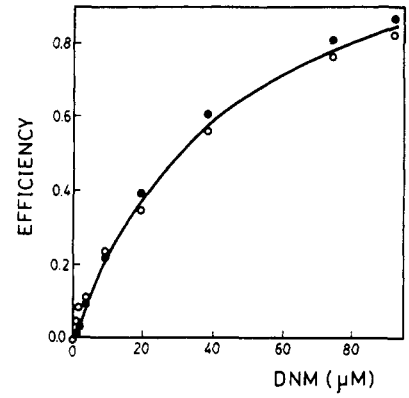

FIGURE 3: Changes in the efficiency of fluorescence resonance energy transfer between protein tryptophan residues (energy donors) present in plasma membranes from P388/S (○) and P388/R (●) cells, and DNM (energy acceptor), as a function of the acceptor concentration. Membrane concentration was held constant at 20 μM in terms of lipid phosphorus. Samples were excited at 280 nm, and fluorescence emission was recorded at 340 nm. The efficiency of energy transfer (E) was determined from $E = 1 - (I/I_0)$, where I and I_0 are the fluorescence intensities of the energy donor in the presence and in the absence of the energy acceptor, respectively. Temperature was 25 °C.

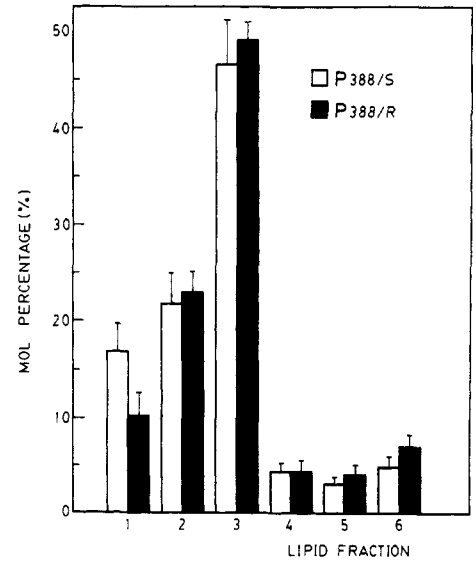

FIGURE 4: Phospholipid composition of plasma membrane fractions from P388/S (open bars) and P388/R (dark bars) cells. Results are given as the percentage of phospholipid classes with respect to total lipid phosphorus. Numbers in the abscissa indicate (1) PS, (2) PE, (3) PC, (4) phosphatidic acid, (5) cardiolipin, and (6) lysophosphatidylcholine plus sphingomyelin.

approximately 1.5–2-fold higher than those corresponding to the drug-resistant samples. At saturation, our stoichiometry values predict an average of 62 and 83 phospholipid molecules of P388/S and P388/R plasma membranes, respectively, to be involved in the binding of a single DNM molecule.

To determine whether the observed differences in drug binding parameters are mediated by protein components of the plasma membranes, we performed equilibrium binding experiments using plasma membranes samples which were previously (i) treated with trypsin at different protease/membrane protein ratios and incubation times or (ii) heated at temperatures which cause thermal denaturation of membrane proteins (Sanchez-Ruiz & Mateo, 1987). The effects of trypsin treatment or heat denaturation on the integrity of plasma membrane proteins were monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Trypsin-treated samples exhibited electrophoretic patterns in which most of the observed proteins bands were present at apparent molecular weight values lower than those observed in untreated samples. On the other hand, heat-denatured samples were characterized by the presence of considerable amounts of protein aggregates, unable to enter the separating gel (data not shown). Nevertheless, as indicated in Figure 2, neither heat denaturation nor trypsin treatment produces significant effects in the drug binding parameters. Furthermore, the differences in drug binding observed between native (untreated) plasma membranes from drug-sensitive and -resistant cells are maintained unperturbed upon either heat or trypsin treatment, thus suggesting the lack of a major involvement of protein components of the membranes in the observed drug binding.

Additional evidence to disregard a major role for membrane proteins in determining the observed differences in drug binding between the two plasma membranes was provided by FRET between tryptophan residues in proteins, acting as Förster's energy donors and DNM as the acceptor (Griffin et al., 1986). The efficiency of the transfer process depends upon the concentration of the energy acceptor (Figure 3). Nevertheless, because of the limited spectral overlap between tryptophan emission and daunomycin excitation (Griffin et al., 1986), fluorescence energy transfer to DNM does not occur very efficiently, and, thus, relatively high concentrations of DNM have to be used to achieve high transfer efficiencies. Within the DNM concentration range assayed, however, no significant differences in the transfer efficiency were found when plasma membranes from drug-sensitive or -resistant cells were used in the experiments.

To determine whether there are membrane components other than proteins which might be involved in defining the differences observed in drug binding, the lipid composition of plasma membranes from P388/S and P388/R cells was determined. Differences in the phospholipid components of the membranes are shown in Figure 4, and refer mainly to the relative abundance of the acidic PS, whose levels in plasma membranes from P388/S cells are almost 2-fold higher than those found in P388/R. We also found small differences in the cholesterol to phospholipid molar ratios, which were 0.40 and 0.45, respectively, for plasma membranes from P388/S

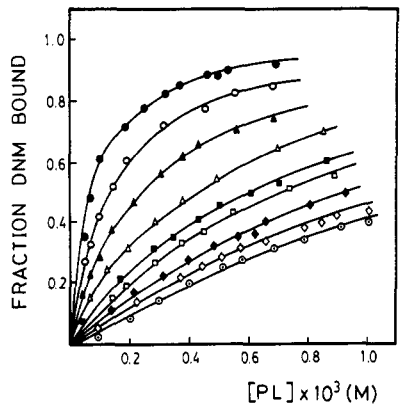

FIGURE 5: Equilibrium binding of DNM to artificial lipid vesicles made from (i) egg yolk PC/PS mixtures at PS molar percentages of 0 (■), 5 (△), 10 (▲), 20 (○), and 40 (●) or (ii) egg yolk PC/cholesterol mixtures at cholesterol molar fractions of 9 (□), 16 (♦), 28 (◇), and 33 (⊙). The total concentration of DNM was 2.5 μM, and the fraction of bound drug was determined, at 25 °C, by using the fluorescence anisotropy procedure described under Materials and Methods.

Table II: Effects of Lipid Composition on Equilibrium Binding of DNM to Artificial Lipid Vesicles[a]

vesicle composition (molar ratios)	$K_S \times 10^{-4}$ (M^{-1})		n	
	A (25 °C)	B (4 °C)	A (25 °C)	B (4 °C)
pure egg PC	0.19	0.28	0.015	0.022
PC/cholesterol				
91/9	0.16	0.24	0.013	0.029
87/13		0.21		0.017
84/16	0.11	0.19	0.011	0.015
77/23		0.15		0.012
72/28	0.088		0.0098	
67/33	0.077		0.0092	
PC/PS				
95/5	0.29	0.47	0.023	0.040
90/10	0.52	0.78	0.030	0.060
85/15		1.35		0.090
80/20	1.23	1.70	0.057	0.150
60/40	1.72	2.10	0.070	0.200

[a] Equilibrium binding data were obtained either at 25 °C, using the steady-state fluorescence anisotropy assay (columns "A"), or at 4–5 °C, using the ultracentrifugation procedure (columns "B"). K_S and n were estimated from C_f/r vs C_f plots, as in Table I. Values shown are the average of at least two different experiments.

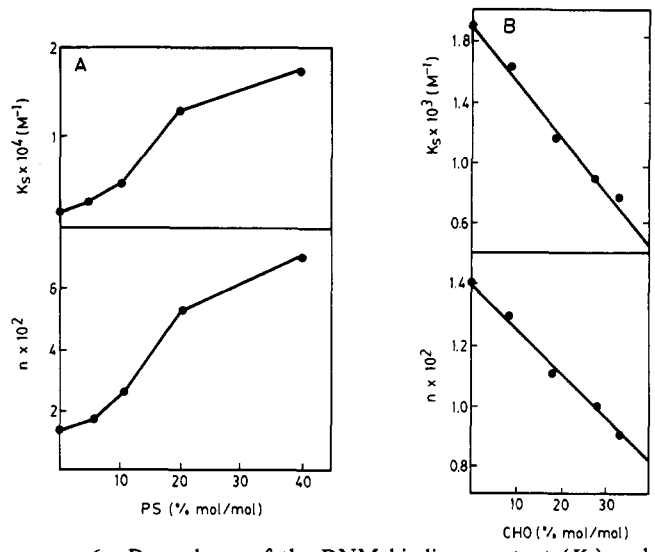

FIGURE 6: Dependence of the DNM binding constant (K_S) and stoichiometry (n) on the molar percentage of PS (panel A) or cholesterol (panel B) contained in the lipid vesicles from Figure 5. Drug binding parameters (K_S and n) were obtained from Klotz and Hunston plots of the data presented in Figure 5.

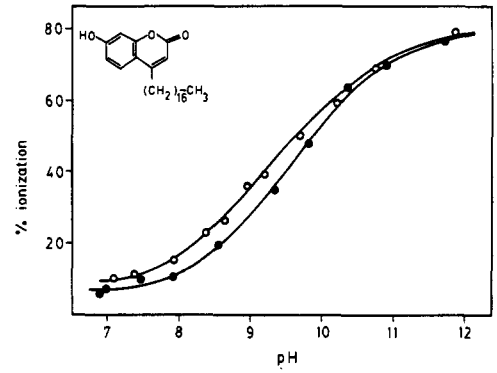

FIGURE 7: Titration of 4-heptadecyl-7-hydroxycoumarin incorporated into plasma membrane fractions from anthracycline-sensitive (○) and -resistant (●) P-388 cells. Fluorescence excitation spectra were taken at the indicated pHs, using a 450-nm fixed emission wavelength. The degree of ionization of the coumarin probe was calculated from the ratio between the fluorescence intensities at the excitation maxima for the ionized (383 nm) to that of the nonionized (343 nm) fluorophore at the indicated pHs (Gonzalez-Ros et al., 1982).

and P388/R cells. Therefore, plasma membranes from P388/S cells have more PS and slightly less cholesterol than those derived from the drug-resistant cell line.

The possible role of both cholesterol and PS in modulating drug binding to the membranes was tested by using liposomes made from egg PC and different molar fractions of cholesterol and/or PS, as a model. Representative results from equilibrium binding experiments using such liposomes and DNM are shown in Figure 5. Binding parameters, as calculated from Klotz and Hunston plots, are given in Table II. Increasing the molar percentage of PS in the liposomes greatly increases the binding constant and the stoichiometry. Furthermore, such an increase in drug binding with increasing PS exhibits sigmoidal behavior (Figure 6A), in which the more pronounced differences in drug binding are produced within the 10–20 molar percentage range, that is, at PS relative abundances similar to those present in the native plasma membranes. Acidic phospholipids such as PS, phosphatidic acid, or cardiolipin were previously proposed as potential sites for binding of the cationic anthracycline to artificial liposomes (Goormaghtigh et al., 1980; Nicolay et al., 1984, 1988; Henry et al., 1985). Moreover, the importance of having groups with net negative charges at the surface of the liposomes in determining DNM binding has been emphasized recently (Ferragut et al., 1988).

Since the observed differences in the relative abundance of acidic phospholipids were expected to result in a different ionic environment at the membrane surface, we used the pH dependence of the excitation spectrum of the fluorophore 4-heptadecyl-7-hydroxycoumarin to probe the surface of plasma membranes from P388/S and P388/R cells. The hydrophilic nature of the hydroxycoumarin head group dictates the position of the probe near the membrane surface, aligned with the phospholipid polar head groups, with the heptadecyl moiety anchored into hydrophobic domains of the bilayer (Petri et al., 1981). Figure 7 shows the results obtained from pH titrations of the coumarin probe incorporated into plasma membranes. The apparent pK value of the probe into the environment provided by the P388/R membranes was approximately 0.3 pH unit higher than that corresponding to membranes from P388/S cells. Such differences in pK values appear to be consistent with the observed differences in relative levels of PS between plasma membranes from the drug-sensitive and -resistant cells.

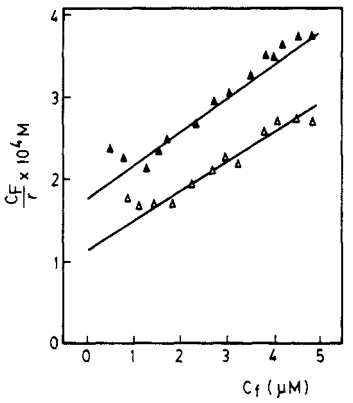

FIGURE 8: Equilibrium binding of DNM to artificial lipid vesicles containing PC, PS, and cholesterol at molar fractions resembling those found in plasma membranes from P388/S and P388/R cells. Binding data were obtained, at 4–5 °C, by using an ultracentrifugation procedure (see Materials and Methods) and are shown in the form of the Klotz and Hunston plot. Molar ratios of PC, PS, and cholesterol in the vesicles were 83/17/40 (△) and 90/10/45 (▲), respectively.

Increasing the presence of cholesterol in egg PC liposomes (Figure 5 and Table II) causes an effect opposite to that observed with PS; i.e., the binding constant and stoichiometry decrease as the cholesterol molar fraction increases. Figure 6B illustrates that, within the molar fraction range used in the studies, the amount of daunomycin bound to cholesterol-containing liposomes, as a function of the cholesterol molar fraction, decreases linearly. It should be noticed, however, that such a decrease in DNM binding induced by the presence of cholesterol is not quantitatively as dramatic as the increase in DNM binding induced by the presence of PS at similar molar percentages.

Additional evidence to support the hypothesis that lipid components have a major role in determining drug binding to membranes is provided by equilibrium binding experiments using PC/PS/cholesterol liposomes, which contained PS and cholesterol at molar fractions resembling those found in the plasma membranes from drug-sensitive and -resistant cells (Figure 8). Our results indicate that the differences in DNM binding parameters between those liposomes are similar to the observed differences in drug binding exhibited by native plasma membranes from P388/S and P388/R cells.

Discussion

A main objective of this work has been to establish whether cellular drug resistance in a model tumor cell line is accompanied by differences in the extent of drug binding to the plasma membrane, a cellular structure considered critical for drug cytotoxicity (Tritton & Yee, 1982). Our conclusion from equilibrium binding experiments using DNM and isolated plasma membrane fractions is that plasma membranes from drug-sensitive cells bind more DNM than those derived from drug-resistant cells, which seems consistent with the decreased drug uptake characteristic of the MDR phenotype (Myers et al., 1987; Bradley et al., 1988).

The observed binding of DNM to plasma membranes does not appear to be due to protein components of the membranes. This conclusion arises from equilibrium binding experiments in which the plasma membrane fractions from both cell lines are previously (i) heated at temperatures at which membrane proteins are denatured or (ii) treated with trypsin under conditions at which extensive proteolysis of membrane protein occurs. Heat-denatured or trypsin-treated membranes used in the drug binding assays exhibit binding parameters which are very similar to those from untreated samples, thus indicating that the protein components of the membranes do not play a major role in establishing the observed resistance-related differences in drug binding. In support of this, FRET between membrane protein tryptophan residues and DNM indicates that, regardless of whether plasma membranes from P388/S or P388/R cells are used, the transfer process occurs with a similar efficiency. This suggests that although membrane proteins may be in close proximity to the incorporated DNM, there are no resistance-related proteins markedly involved in the interaction with the drug. This conclusion is in contrast with reports by Pastan and co-workers (Cornwell et al., 1986) in which binding of vinblastine to plasma membranes from drug-sensitive and -resistant KB cells is reported to be EXCLUSIVELY mediated by a resistance-related, high molecular weight membrane protein, likely to be P-170. We do not have a satisfactory explanation for this apparent discrepancy, although it is possible that cellular drug resistance induced by different drugs could be differently manifested in different cell lines (Riordan & Ling, 1985; McGrath & Center, 1987). In any case, it should be noticed that Pastan's group measures drug binding by using a filtration assay in which they reported that dilution, filtration, and wash steps take approximately 15 s. Under those conditions, having an association constant in the micromolar range, and assuming a moderate rate for the dissociation of drug–acceptor complexes (Bennet, 1978), it is likely that a good amount of the membrane-bound vinblastine would have dissociated in less than 15 s, and, therefore, the published data could represent residual drug binding remaining upon partial dissociation from sites of higher affinity (i.e., the high molecular weight protein), rather than the total drug binding to the plasma membrane. On the other hand, anthracyclines have been shown to bind to artificial lipid vesicles (Burke & Tritton, 1985a; Nicolay et al., 1984, 1988; Henry et al., 1985) and to native heart mitochondrial (Griffin et al., 1986) and *Torpedo* postsynaptic membranes (Ferrer-Montiel et al., 1988), where binding of the drug is preferentially, if not exclusively, mediated by membrane lipids.

A comparison between the lipid composition of plasma membranes from P388/S and P388/R cells indicates differences in the relative abundances of certain lipid components, namely, PS, and to a much lesser extent cholesterol. Differences in the contents of the acidic phospholipid are apparently translated into providing a different ionic environment at the surface of the two plasma membrane samples, as indicated by probing such an environment with a pH-sensitive coumarin probe. To test whether these lipid components could partly be responsible for the observed differences in drug binding, we have used artificial egg PC liposomes containing PS and/or cholesterol at different molar ratios. It should be noticed that the liposome binding studies were carried out by using two unrelated techniques which, nonetheless, provide very similar results. Our data indicate that both the drug binding constant and the stoichiometry are decreased and increased, respectively, when the molar proportion of cholesterol and PS within the liposomes increases. The similarity between the effects caused by increasing PS and those observed in the presence of dicetyl phosphate (Ferragut et al., 1988), cardiolipin (Nicolay et al., 1984, 1988), or phosphatidic acid (Nicolay et al., 1984; Henry et al., 1985) in "negatively-charged" liposomes indicates that, in addition to hydrophobic forces (Burke & Tritton, 1985a), electrostatic interactions between the drug and the liposome surface are also important in determining total drug binding to membrane sites. Also, the observed effects of cholesterol could be interpreted as a competition between cholesterol and the drug for the occupation of internal sites within the membrane. This interpre-

tation seems consistent with the observation that daunomycin partitioning into membranes occurs at both membrane surface and lipid-intercalated domains of the bilayer (Henry et al., 1985; Burke & Tritton 1985b; Griffin et al., 1986; Ferrer-Montiel et al., 1988; Dupou-Cezanne et al., 1989). Considering all of the above, the observed differences in drug binding to plasma membranes from drug-sensitive and -resistant P388 cells seem compatible with the observation that plasma membranes from drug-resistant cells contain less PS and more cholesterol than those from drug-sensitive cells. Moreover, when liposomes are made from PC/PS/cholesterol mixtures at molar ratios resembling those found in P388/S and P388/R plasma membranes, the differences in DNM binding between these liposomes are very similar to those exhibited by the corresponding native plasma membranes. Our conclusion is, therefore, that mainly PS and also cholesterol have an important role in modulating the binding of the drug to the plasma membrane and in establishing the observed resistance-related differences. In fact, membrane lipids were also proposed to be responsible for anthracycline binding to natural heart mitochondrial (Griffin et al., 1986) and *Torpedo* postsynaptic membranes (Ferrer-Montiel et al., 1988).

At the present time, we cannot evaluate the relative importance of our observations, as compared to the proposed drug efflux pumping activity of the P-170 glycoprotein, in determining the extent of drug accumulation by whole drug-resistant cells. It seems obvious that overexpression of the P-170 glycoprotein constitutes the basis for drug resistance in many drug-resistant cells (Bradley et al., 1988; Gottesman & Pastan, 1988). Nevertheless, it is reasonable to assume that decreased drug binding to the plasma membrane in the drug-resistant cells could provide an additional mechanism for these cells to survive the presence of the antineoplastic. This is an attractive possibility since there are drug-resistant cell lines and tumors in which the presence of P-170 could not be demonstrated (Bell et al., 1985; McGrath & Center, 1987; Fojo et al., 1987; Cole et al., 1989; Norris et al., 1989).

Our observations on DNM binding to plasma membranes may also offer some insight into the possible mechanism(s) of transport of anthracyclines across plasma membranes. Three alternatives have been entertained to explain drug accumulation in intact cells: passive diffusion through the plasma membrane, with or without the selective removal of the drug via a metabolic energy-dependent efflux pump, or facilitated diffusion involving a protein carrier (Skovsgaard & Nissen, 1982; Siegfried et al., 1985). The preferential binding of the drug to membrane lipids and its ability to interact with both surface and core domains of the bilayer suggest that anthracyclines may diffuse through the membrane passively and that such diffusion would be controlled by properties of the bilayer.

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References

Arcamone, F. (1981) in *Doxorubicin: Anticancer Antibiotics* (Sartorelli, A. C., Ed.) pp 1–369, Academic Press, New York.

Barcelo, F., Barcelo, I., Gavilanes, F., Ferragut, J. A., Yanovich, S., & Gonzalez-Ros, J. M. (1986) *Biochim. Biophys. Acta 884*, 172–181.

Bell, D. R., Gerlach, J. H., Kartner, N., Buick, R. N., & Ling, V. (1985) *J. Clin. Oncol. 3*, 311–315.

Bennet, J. P., Jr. (1978) in *Methods in Binding Studies. Neurotransmitter Receptor Binding* (Yamamura, H. I., Enna, S. J., & Kuhar, M. J., Eds.) pp 58–89, Raven Press, New York.

Bradley, G., Juranka, P. F., & Ling, V. (1988) *Biochim. Biophys. Acta 948*, 87–128.

Burke, T. G., & Tritton, T. R. (1984) *Anal. Biochem. 143*, 135–140.

Burke, T. G., & Tritton, T. R. (1985a) *Biochemistry 24*, 1768–1776.

Burke, T. G., & Tritton, T. R. (1985b) *Biochemistry 24*, 5972–5980.

Chaires, J. B., Dattagupta, N., & Crothers, D. M. (1982) *Biochemistry 21*, 3927–3932.

Cole, S. P. C., Downes, H. F., & Slovak, M. L. (1989) *Br. J. Cancer 59*, 42–46.

Cornwell, M. M., Gottesman, M. M., & Pastan, I. (1986) *J. Biol. Chem. 261*, 7921–7928.

Dupou-Cezanne, L., Sautereau, A. M., & Tocanne, J. F. (1989) *Eur. J. Biochem. 181*, 695–702.

Ferragut, J. A., Gonzalez-Ros, J. M., Ferrer-Montiel, A. V., & Escriba, P. V. (1988) *Ann. N.Y. Acad. Sci. 551*, 443–445.

Ferrer-Montiel, A. V., Gonzalez-Ros, J. M., & Ferragut, J. A. (1988) *Biochim. Biophys. Acta 937*, 379–386.

Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M., & Pastan, I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 265–269.

Gonzalez-Ros, J. M., Llanillo, M., Paraschos, A., & Martinez-Carrion, M. (1982) *Biochemistry 21*, 3467–3474.

Goormaghtigh, E., Chatelain, P., Caspers, J., & Ruysschaert, J. M. (1980) *Biochim. Biophys. Acta 597*, 1–14.

Gottesman, M. M., & Pastan, I. (1988) *J. Biol. Chem. 263*, 12163–12166.

Griffin, E. A., Vanderkooi, J. M., Maniara, G., & Erecinska, M. (1986) *Biochemistry 25*, 7875–7880.

Henry, N., Fantine, E. O., Bolard, J., & Garnier-Suillerot, A. (1985) *Biochemistry 24*, 7085–7092.

Hubbard, A. L., & Cohn, Z. A. (1972) *J. Cell Biol. 55*, 390–405.

Kartner, N., Riordan, J. R., & Ling, V. (1983) *Science 221*, 1285–1288.

Kartner, N., Evernden-Porelle, D., Bradley, G., & Ling, V. (1985) *Nature 316*, 820–823.

Klotz, I. M., & Hunston, D. L. (1971) *Biochemistry 10*, 3065–3069.

Kyaw, A., Maung-U, K., & Toe, T. (1985) *Anal. Biochem. 145*, 230–234.

Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, pp 111–150, Plenum Press, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McGrath, T., & Center, M. (1987) *Biochem. Biophys. Res. Commun. 145*, 1171–1176.

Myers, C., Cowan, K., Sinha, B., & Chabner, B. (1987) in *Important Advances in Oncology* (DeVita, V. T., Hellman, S., & Rosenberg, S. A., Eds.) pp 27–38, J. B. Lippincott, Co., Philadelphia, PA.

Nicolay, K., Timmers, R. J. M., Spoelstra, E., Van der Neut, R., Fok, J. J., Huigen, Y. M., Verkleij, A. J., & De Kruijff, B. (1984) *Biochim. Biophys. Acta 778*, 359–371.

Nicolay, K., Sautereau, A. M., Tocanne, J. F., Brasseur, R., Huart, P., Ruysschaert, J. M., & De Kruijff, B. (1988) *Biochim. Biophys. Acta 940*, 197–208.

Norris, M. D., Haber, M., King, M., & Davey, R. A. (1989) *Biochem. Biophys. Res. Commun. 165*, 1435–1441.

Petri, W. A., Pal, R., Barenholz, Y., & Wagner, R. R. (1981) *Biochemistry 20*, 2796.
Pick, V. (1981) *Arch. Biochem. Biophys. 212*, 186-194.
Ramu, A., Glaubiger, D., Magrath, I. T., & Joshi, A. (1983) *Cancer Res. 43*, 5533-5537.
Rawitch, A. B., & Weber, G. (1972) *J. Biol. Chem. 247*, 680-685.
Riordan, J. R., & Ling, V. (1979) *J. Biol. Chem. 254*, 12701-12705.
Riordan, J. R., & Ling, V. (1985) *Pharmacol. Ther. 28*, 51-75.
Sanchez-Ruiz, J. M., & Mateo, P. L. (1987) *Cell Biol. Rev. 11*, 15-45.
Shinitzky, M., Dianoux, A. C., Gitler, C., & Weber, G. (1971) *Biochemistry 10*, 2106-2113.
Siegfried, J. M., Burke, T. G., & Tritton, T. R. (1985) *Biochem. Pharmacol. 34*, 593-598.
Skovsgaard, T., & Nissen, N. I. (1982) *Pharmacol. Ther. 18*, 293-314.
Tritton, T. R., & Yee, G. (1982) *Science 217*, 248-250.
Waring, M. J. (1981) *Annu. Rev. Biochem. 50*, 159.
Yanovich, S., & Taub, R. (1982) *Cancer Res. 42*, 3583-3586.

Regulation of Chloride Transport in Parotid Secretory Granules by Membrane Fluidity†

Kenneth W. Gasser,‡ Adam Goldsmith, and Ulrich Hopfer*
Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106
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ABSTRACT: Zymogen granule membranes contain Cl^- conductance and Cl/anion exchange activities that become important for primary fluid production after fusion with the apical plasma membrane of the acinar cell. We have used steady-state fluorescence anisotropy of diphenylhexatriene derivatives and measurements of Cl^- transport in isolated secretory granules to determine the contribution of membrane fluidity to the regulation of transport across the granule membrane. Secretory granules from several unstimulated glands (rat pancreas and parotid, rabbit gastric glands) were shown to have low membrane fluidity compared to plasma membranes. In addition, Cl^- transport activity in different granule preparations showed a strong correlation to the membrane fluidity when measured with 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH), but not with 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl)-phenyl]propionic acid (PA-DPH). These data suggest that TMA-DPH preferentially partitions into a specific lipid environment associated with, or which exerts an influence on, the Cl^- transport proteins and that increases in the fluidity of this environment are associated with higher transport rates. Data from other types of plasma membranes indicate that TMA-DPH partitions much more than PA-DPH into the cytoplasmic leaflet, suggesting that this part of the granule membrane is involved in the observed fluidity changes. Furthermore, increasing the bulk membrane fluidity with the local anesthetics benzyl alcohol and *n*-alkanols increased the Cl^- transport rates up to 10-fold. This increase was apparently through specific transporters as anion selectivity was maintained in spite of the higher absolute rates. Temperature also influenced membrane fluidity and transport in a highly correlative manner. These observations are consistent with membrane fluidity acting as a major modulating factor for Cl^- transport activity in secretory granules and with exocrine acinar cells selectively regulating the fluidity of this environment.

Apart from serving as a simple boundary, biological membranes are involved in many complex physiological functions, including interactions with the integral proteins which reside within the lipid bilayer. These membrane-associated proteins typically contain large hydrophobic sequences which span or anchor in the lipid bilayer. The lipids serve as a solvent for the hydrophobic segment of the proteins and as such can have a profound effect on their properties. Studies on receptors, enzymes, and transport proteins (Abney & Owicki, 1985; Carruthers & Melchior, 1988; Farias, 1987) have shown optimal activity to require specific lipids, which serve as cofactors or stabilize the proteins through hydrophobic or electrostatic interactions and hydrogen bonding. In addition to these specific biochemical requirements, such bulk phase bilayer properties as fluidity, thickness, and surface potential may also influence the physiological activity of many integral proteins (Shinitzky, 1984; Deuticke & Haest, 1987; Watts & DePont, 1985).

Since the presentation by Singer and Nicolson (1972) of the fluid mosaic model of biological membranes, considerable attention has been directed toward the regulation and importance of membrane fluidity. Many organisms (e.g., bacteria, fish, hibernating mammals) have the ability to change the lipid composition and fluidity of membranes in response to such gross environmental conditions as temperature and thereby maintain a requisite fluidity for cellular functions (Cossins & Sinensky, 1984). However, evidence is lacking for specifically controlled fluidity changes serving as part of a regulatory system for membrane-bound enzymes or transport proteins in mammalian systems. In vitro alterations in membrane lipid composition and fluidity, or imposed in vivo changes brought about by diet, have had varied effects on the functioning of integral proteins (Shinitzky, 1984; Deuticke & Haest, 1987; Dudeja et al., 1987). Although membrane

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* To whom correspondence should be addressed.
‡ Present address: Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115-2861.

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