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Distribution of 5-doxylstearic acid in the membranes of mammalian cells

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Concentration-dependent spin broadening of ESR spectra of the nitroxide 5-doxylstearic acid has been used to evaluate the distribution of 5-doxylstearic acid in the membranes of intact mouse thymus-bone marrow (TB) and Chinese hamster ovary (CHO) cells. TB cells, CHO cells, erythrocytes, and isolated plasma membranes from CHO cells were labelled with 5-doxylstearic acid and the peak to peak linewidths of the central line of the resulting ESR spectra were measured. The measured line widths were linearly dependent on the amount of 5-doxylstearic acid incorporated into the sample over the range of 0–0.18 mol nitroxide per mol lipid. In erythrocytes, the relationship between linewidths approximated a linear function at lower concentrations of 5-doxylstearic acid, up to 0.07 mol nitroxide per mol lipid. The amount of broadening of the central line for a given amount of 5-doxylstearic acid was far less for intact cells than for either erythrocytes or plasma membrane, indicating that the 5-doxylstearic acid samples a much larger lipid pool in the intact cells. With the broad assumption that the mobility of the 5-doxylstearic acid is similar in different membranes, the size of the lipid pool sampled by 5-doxylstearic acid is approximately equal to the total cellular lipid in intact cells. If a given concentration of 5-doxylstearic acid sampled only the plasma membrane of TB or CHO cells, we would expect to see a linewidth corresponding to a 12–20-fold greater local concentration of 5-doxylstearic acid than was observed, since the plasma membranes of CHO and TB cells represent only 5–8 percent of the total cellular lipid. Therefore, the 5-doxylstearic acid must distribute into most or all cellular membranes of intact cells and is not localized in the plasma membrane alone.

Introduction

Nitroxides derived from stearic acid are highly lipophilic and readily partition into liposomes and cell membranes. Although they are used extensively in biophysical studies of intact cells, their exact location in these cells has not been established firmly [1]. Many of the published studies assume that, in intact cells, the stearic acid nitroxides are located in the outer layer of the plasma membrane. The principal basis of this assumption is the observation that, at physiological temperatures, 5-doxylstearic acid is reduced by the cells to the ESR-inactive hydroxylamine and that the ESR signal could be regenerated by the membrane impermeable oxidizing agent $K_3Fe(CN)_6$ [2]. This oxidation of reduced nitroxides by ferricyanide has been demonstrated in a wide variety of cells (see, for example, Refs. 3–6). In addition, it often is assumed, on the basis of the observed reduction

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of the nitroxides in cells, that if slow migration of the nitroxides to the intracellular compartment did occur, the nitroxide would be reduced when it entered the cell interior and would no longer contribute to the ESR spectrum. Consequently, the ESR signals of the doxylstearic acid nitroxides and in particular 5-doxylstearic acid, have been widely assumed to arise from the plasma membrane in intact cells.

However, an alternative explanation of these experimental findings, recently outlined by Morse [1], is that these nitroxides are in equilibrium with all membranes of the cell and can redistribute between the membranes on a time scale that is at least as fast as the observed rate of oxidation of the hydroxylamines by ferricyanide. This would account for the observed rapid reduction at 37 °C of some doxylstearic acid nitroxides, since such reduction is thought to occur intracellularly.

We report here a series of experiments designed to resolve this problem for 5-doxylstearic acid using the phenomenon of spin-spin broadening [7–9] to examine the size of the lipid pools available to 5-doxylstearic acid. We compared spin-spin broadening of 5-doxylstearic acid as a function of 5-doxylstearic acid/lipid ratio in human erythrocytes, TB, and CHO cells. When 5-doxylstearic acid is added in increasing quantities to membranes, interactions between the nitroxides cause line broadening. We make use of this property to determine the average concentration of 5-doxylstearic acid in the cell membranes. If 5-doxylstearic acid partitions only into the plasma membrane of TB and CHO cells, then 5-doxylstearic acid would be expected to give equivalent linewidths in these cells and in erythrocytes when present at equivalent 5-doxylstearic acid to cell surface area ratios If, on the other hand, 5-doxylstearic acid partitions into all cell membranes of TB and CHO cells, any particular concentration of 5-doxylstearic acid would give a narrower linewidth in these cells than in erythrocytes, because of the much larger proportion of internal membrane associated with these cells.

Materials and Methods

All chemicals were analytical grade unless noted otherwise. 5-Doxylstearic acid (2,2'-dimethyl-5-undecyl-5' pentanoic acid oxazolidine-*N*-oxyl) was purchased from Molecular Probes (Junction City, OR) and used without further purification. Stock solutions of 5-doxylstearic acid were prepared in ethanol and stored at −20 °C. Medium, serum, and antibiotics were purchased from GIBCO Laboratories, Grand Island, NY.

All cell samples were labelled as follows: An appropriate aliquot of 5-doxylstearic acid in ethanol was pipetted into a 6 × 50 mm glass culture tube and dried to make a uniform film of 5-doxylstearic acid around the sides of the tube. The cell sample was then added ($1 \cdot 10^7$ cells in 100 µl), vortexed for 30 s in intervals of 5 s on and 5 s off, and then removed for ESR studies. The tube was then washed once with ethanol. The amount of nitroxide in the ethanol wash was determined by double integration of the ESR signal and comparison with a known standard of 2,2,6,6-tetramethylpiperdine-*N*-oxyl-4-ol, $6 \cdot 10^{23}$ spins/mol. This value was then subtracted from the known amount of nitroxide in the glass tube to determine the amount of nitroxide in the cell sample. The ratios of nitroxide to total lipid (Figs. 2–4) were calculated on the basis of the measured amount of nitroxide in each sample of cells.

All spectra were taken on a Varian E109 E spectrometer equipped with an IBM PC data acquisition system [10]. Modulation intensities were two gauss (p-p) to prevent over-modulation and subsequent artifactual broadening of the spectral lines. Samples were placed in gas permeable Teflon tubes (Zeuss Industries, Raritan, NJ) and air was blown across them to maintain adequate oxygen concentrations in the samples to prevent reduction of 5-doxylstearic acid. Measurements were made at room temperature using a Varian gas flow temperature controller. Under these conditions, no reduction of 5-doxylstearic acid took place over a period of an hour.

Various spectral parameters (central line peak height, slope of the central line where it crossed the baseline, half-height width of the central line above the baseline) were evaluated as a means of quantitating spin-spin broadening and, while all showed similar trends, the most sensitive was found to be the peak-to-peak linewidth of the central line of the ESR spectrum (W_o).

Chinese hamster ovary (CHO) cells were ob-

tained from Dr. L. Hopwood, Medical College of Wisconsin, Milwaukee. CHO stock cultures were maintained as monolayers and subcultured three times a week. 24 h before the experiment, cells were transferred into a spinner flask at a density of $1 \cdot 10^5$ cells/ml. McCoy's 5A medium [11,12] supplemented with 10% fetal bovine serum and penicillin/streptomycin was used for monolayer and suspension cultures. Cells routinely were counted in a hemocytometer. Mouse thymus-bone marrow (TB) cell suspensions were prepared as described previously [13]. TB cells were maintained as monolayers and were subcultured three times a week in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. They were harvested at confluence and resuspended in medium without fetal bovine serum. ESR measurements were carried out on TB and CHO cells that were washed and resuspended in Dulbecco's phosphate-buffered saline [14] (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 290 mosM) to 10^8/ml and then immediately labelled with 5-doxylstearic acid. Except as noted, all studies were carried out at room temperature with air flowing around gas permeable Teflon tubes, to retard biological reduction of the doxylstearic acid. Cell samples were kept in the spectrometer for 0.5 h or less. Viability of the cells, as determined by Trypan blue exclusion, was greater than 95% both before and after treatment for ESR spectroscopy.

Red cells were obtained by venipuncture of healthy adults. They were washed in 0.15 M NaCl, 5 mM phosphate buffer (pH 7.4) and used immediately. Plasma membranes were isolated by a modification of the method of Fleischer and Kervina [15]. All operations were carried out at 4°C. CHO and TB cells were washed and resuspended in 0.25 M sucrose, 0.01 M Hepes (pH 7.5) to obtain a final concentration of 10^8 cells/ml. The cells were then broken by homogenization in a Brinkmann homogenizer in 30 second bursts with microscopic examination between bursts. Once lysis was complete, the homogenate was centrifuged at $950 \times g$ for 15 min in a Beckman JA-20 centrifuge rotor. The pellets were resuspended to 10 ml in 0.25 M sucrose, 0.01 M Hepes with 1 mM $MgCl_2$ and membrane purification continued as described. The final pellet, containing the plasma membranes from the lysed cells, was resuspended to 1 ml in 0.25 M sucrose, 0.01 M Hepes, 1 mM EDTA (pH 7.5).

TABLE I

LIPID CONTENT OF WHOLE CELLS AND PLASMA MEMBRANES

Cell	Total lipid ($mg/10^8$ cells)	Plasma membrane lipid	
		($mg/10^8$ cells)	% of total
Red blood cells	0.046	0.041	89.0
CHO	3.27	0.23	7.0
TB	3.23	0.17	5.3

Plasma membranes from $1.32 \cdot 10^9$ TB or $4.15 \cdot 10^8$ CHO cells, as well as whole cells, $3.3 \cdot 10^8$ TB or $2.4 \cdot 10^8$ CHO cells, were subjected to chloroform/ methanol lipid extraction to quantitate the amount of lipid in each [16,17]. The extracts were transferred to tared conical tubes, dried under nitrogen, and weighed. The weight of extracted lipid was divided by 10^{-8} times the number of cells used. These values are reported in Table I. The molar ratio of nitroxide to lipid for each sample was then calculated from the values in Table I, the number of cells used, the number of nmoles of nitroxide added, and the arbitrary assumption of an average lipid molecular weight of 750.

Results

Fig. 1 shows the spectra of 5-doxylstearic acid in intact TB cells at molar ratios of 0.011 and 0.069 5-doxylstearic acid to phospholipid. Both spectra are characteristic of 5-doxylstearic acid in eukaryotic membranes and have only a very small rapidly mobile or water soluble component. The signal did not decrease in amplitude or change otherwise over a period of 0.5 h at room temperature with air as the perfusing gas.

Fig. 2 shows a plot of the width of the mid-field line vs. the ratio of 5-doxylstearic acid to lipid obtained for erythrocytes and plasma membranes of CHO cells. The slope of the line for erythrocytes decreases at molar ratios of 5-doxylstearic acid/lipid above 0.075, possibly due to binding of 5-doxylstearic acid to hemoglobin at high concentrations of 5-doxylstearic acid, while the line

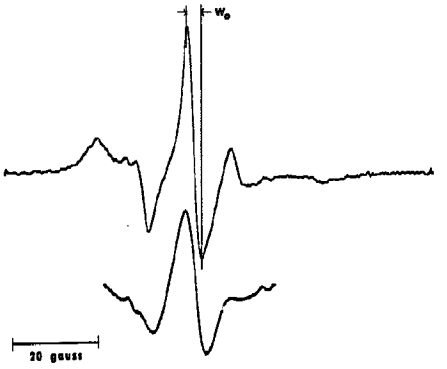

Fig. 1. Spectrum of 5-doxylstearic acid in TB cells. Spectrometer setting were 3247 G field center, 2.0 G modulation amplitude, and 5 mW power. Labelling conditions are described in the text. The cells were kept at room temperature and air was used as the perfusing gas. The upper spectrum arises from 5 nmoles of 5-doxylstearic acid in $1 \cdot 10^7$ TB cells (5-doxylstearic acid/lipid ratio = 0.011). Total sample volume was 100 μl. The lower spectrum arises from 37 nmoles 5-doxylstearic acid in 10^7 TB cells (5-doxylstearic acid : lipid ratio = 0.069). Sample volume was 100 μl.

for the plasma membranes is straight to ratios of 0.18, the highest ratio studied. In the region between 0 and 0.075, the slopes for both membranes are the same within experimental error (21.11 and average of 19.53, respectively). This shows that at low 5-doxylstearic acid concentrations, erythrocytes and plasma membrane have similar environments with regard to the frequency of collisions of 5-doxylstearic acid.

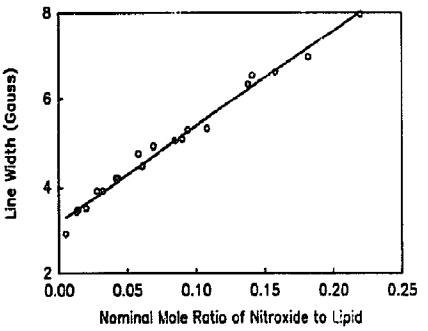

Fig. 3. Plot of mid-field line width of 5-doxylstearic acid as a function of 5-doxylstearic acid to lipid ratio in intact TB cells. Labelling conditions are described in the text. The data points are combined from three different series of experiments performed on different batches of cells.

We next looked at labelling of whole TB cells (Fig. 3) and CHO cells (Fig. 4). The data for the CHO cells are more scattered than for TB cells because the TB cells were obtained from confluent plates and therefore were mostly plateau phase cells, while the CHO cells were taken from spinner flasks after different times of incubation and, therefore, the amount of lipid per cell may have differed due to differences in cell phase distribution [18]. Nitroxide to lipid molar ratios are calculated on the basis of lipid measurements performed on a large pool of cells. Any variation in lipid content from one batch of cells to another would, therefore, produce a proportional error in the nitroxide to lipid molar ratio. However, each

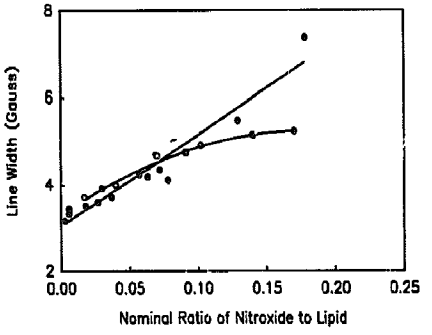

Fig. 2. Plot of mid-field line width of 5-doxylstearic acid as a function of 5-doxylstearic acid/lipid ratio in erythrocytes (○) and plasma membranes of CHO cells (●). Labelling conditions are described in the text. The lines are drawn from best fit linear or quadratic regression calculations.

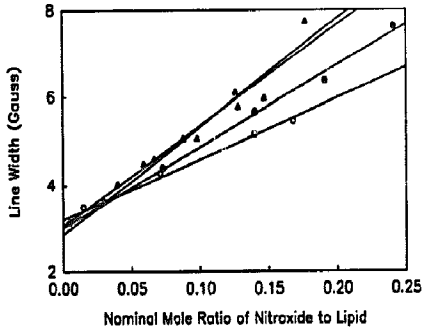

Fig. 4. Plot of mid-field line width of 5-doxylstearic acid as a function of 5-doxylstearic acid to lipid ratio in intact CHO cells. Labelling conditions are described in the text. The different symbols (○, ●, △, ▲) represent separate experiments performed on different batches of cells.

experimental series on CHO cells taken at a given time is very consistent. The slope of the curves for TB and CHO cells are similar to each other (TB 21.94 ± 2.62; CHO 18.57 ± 5.19) and to the slopes of the curves for erythrocyte and plasma membranes (see above). Again, this indicates that there are no significant differences between plasma membrane and total membranes sampled by 5-doxylstearic acid which could be reflected in differences in collision frequency of 5-doxylstearic acid.

Labelling CHO cells at 4°C and recording a spectrum at 4°C gave identical results as labelling and incubation of CHO cells at 37°C, then recording at 4°C (data not shown). This indicates that labelling of CHO cells by 5-doxylstearic acid is a passive process.

Discussion

If 5-doxylstearic acid partitioned only into the plasma membrane of TB and CHO cells, the slope of the curves in Figs. 3 and 4 should be more than 10-times greater than observed since the plasma membrane is only approx. 5–7.5% of the total lipid in these cells (Table I). Since this is not the case, we conclude that 5-doxylstearic acid is distributed into all of the membranes in these cells.

Line broadening due to spin exchange is dependent on both the nitroxide to lipid molar ratio and the rate of lateral diffusion (D_{diff}) of the nitroxide [7]. Thus, similar linewidths would be observed in two different systems if one system had double the viscosity but only one-half the amount of lipid of the other. Consequently, comparisons of effective molar ratios are only valid assuming a consistent rate of lateral diffusion. The slopes (see above) and intercepts (CHO plasma membrane = 3.05 g, erythrocytes = 3.31 g, TB cells = 3.19 g, CHO cells = 3.20 g) of the curves in Figs. 2–4 are the same, within experimental error, which shows that lateral diffusion of 5-doxylstearic acid is similar in TB cells, CHO cells, the plasma membrane of CHO cells, and erythrocytes. The values of D_{diff} calculated in TB and CHO cells $(2\text{–}4) \cdot 10^8$ $cm^2 \cdot s^{-1}$) were consistent with published values for isolated biological membranes using spin-spin broadening [8,19–21] and ELDOR [22,23].

Potential sources of error include the quantitation of the lipid in intact cells and isolated plasma membranes and the quantitation of the amount of 5-doxylstearic acid incorporated into the sample. The lipid content of erythrocytes was taken from the literature and typically shows a 20% variation, 90% of the lipid being in the plasma membrane (Documenta Geigy). We measured the lipid content of intact TB and CHO cells and of their isolated plasma membranes with an estimated accuracy of 10% and 25%, respectively. In TB and CHO cells the plasma membrane lipid is < 10% of the total lipid. Consequently, one would expect at least a 10-fold difference in the nitroxide/total lipid ratio, depending on whether the nitroxide was located in all areas of lipid or only in the plasma membrane. 5-Doxylstearic acid was added to the samples in the form of a film obtained by evaporation of an ethanolic solution. Errors in measuring the quantity (2–60 μl) are estimated to be less than 5% and any error in the concentration of the original solution would only produce a constant systematic error. Uptake of the nitroxide was measured for each sample and was typically 85–95% of available 5-doxylstearic acid (see Fig. 5). Uptake tended to be less at very small and very large amounts of nitroxide, perhaps reflecting nitroxide binding sites on the glass tube and saturation of the cells, respectively. At high nitroxide to lipid ratios, the ESR spectra of the cells occasionally showed the presence of nitroxide in the aqueous medium, but this represented < 1% of the total nitroxide. Also, small differences in W_o

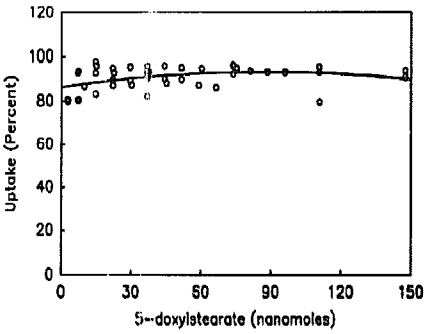

Fig. 5. Uptake of 5-doxylstearic acid as a function of nmoles of 5-doxylstearic acid in sample tube. Data are taken from labelling calculations for Figs. 3 and 4.

were not detectable at low nitroxide concentrations due to decreased signal to noise.

Finally, the possibility of local clustering of the nitroxide must be considered [24,25]. If clustering was observed in the TB cells, CHO cells, or erythrocytes, we would expect to see relatively steeper slopes of the lines in Figs. 2, 3 and/or 4, which would lead us to underestimate the size of the available lipid pools. Since the extracted lipid pools of whole cells and plasma membranes bear the same relationship as the lipid pools available to the 5-doxylstearic acid (similar slopes in Figs. 2–4), we conclude that, if such clustering occurs, it is similar in magnitude in all these cell types. Furthermore, our calculation of nitroxide concentrations are based on uniform distribution of 5-doxylstearic acid, which is a conservative and unfavorable assumption, if we interpret our results as showing that 5-doxylstearic acid partitions into all cellular membranes.

The results clearly demonstrate that 5-doxylstearic acid is not restricted to the plasma membranes of TB or CHO cells, but must also distribute into the internal cell membranes. Neither local clustering nor viscosity differences would be able to reconcile the data with a model in which only the plasma membrane of TB or CHO cells was labelled. It, therefore, seems reasonable to interpret experiments in intact TB and CHO cells with the assumption that the contribution in the total signal of 5-doxylstearic acid in the plasma membrane is less than 10%, the major contribution coming from other membrane systems. If cells under study have lipid distributions similar to hepatocytes, the major sites of nitroxide would be the endoplasmic reticulum and the mitochondria [15].

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