LATERAL DIFFUSION OF LIPID PROBES IN THE SURFACE MEMBRANE OF HUMAN PLATELETS

An Electron-Electron Double Resonance (ELDOR) Study

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ABSTRACT Electron-electron double resonance (ELDOR) techniques employing [^{14}N], [^{15}N] 16-Doxylstearate spin-label pairs have been used to measure the lateral diffusion constant, D, of lipids in the surface membrane of intact human blood platelets. For freshly prepared platelets, D is 1.0×10^{-8} cm^2/s at 37°C and for platelets stored for 3 d at room temperature under accepted routine blood bank conditions, D is 2.6×10^{-8} cm^2/s at 37°C. This is the first time that D in the surface membrane of platelets is reported. The marked increase in D for stored platelets may be attributed at least partly to loss of cholesterol during storage, suggesting a correlation between lipid lateral diffusion and cholesterol levels in cell membranes.

INTRODUCTION

Lipid lateral diffusion in cell membranes has been shown to play a role in many important cellular functions including differentiation (1), enzyme-acceptor interaction (2), electron transfer in mitochondrial membranes (3), and egg fertilization (4). There are a number of biophysical techniques for determining lipid lateral diffusion constant, D, including fluorescence recovery after photobleaching method, eximer formation, pulsed gradient nuclear magnetic resonance and electron spin resonance techniques employing line-width analysis, and recently, electron-electron double resonance (ELDOR)[1] methods. It is generally accepted that D in intact cells ranges from 10^{-7} to 10^{-8} cm^2/s (see reference 5 for a review). However, so far there is no report on D in the surface membrane of platelets.

During the past several years, ELDOR methods have been employed to measure D in synthetic phospholipid membranes (6, 7). The ELDOR technique is based on the effects of the Heisenberg exchange on saturation transfer between hyperfine lines of the spin labels. The rate of Heisenberg exchange is directly proportional to the rate of lateral motion of the spin labels in membranes. Additional factors affecting ELDOR signal are the electron spin relaxation time of the spin label (T_{1e}) and the nuclear spin relaxation time of the nitrogen nucleus of the spin label (T_{1n}). Recently, Feix et al. (7) have described an ELDOR method employing [^{14}N], [^{15}N] spin-label pairs to measure D in DMPC membranes. This technique eliminates the problem of intramolecular contributions to the ELDOR effect from nitrogen nuclear relaxation. Moreover, very recently Hyde and his colleagues (8) have developed a loop-gap resonator ELDOR method employing [^{14}N], [^{15}N] spin-label pairs and demonstrated its feasibility for the measurement of D in the erythrocyte membrane. This new ELDOR technique was shown to improve the sensitivity of ELDOR method by a factor of 20 (8).

In this communication, we report for the first time the use of both conventional bimodal cavity and loop-gap resonator ELDOR methods using [^{14}N], [^{15}N] 16-Doxylstearate spin-label pairs to determine D in intact human blood platelets. A significant difference in D values was found between freshly prepared and stored platelets.

MATERIALS AND METHODS

Platelet Preparations

Platelets were isolated as described by Kunicki et al. (9). Briefly, six volumes of whole blood were drawn into one volume acid-citrate-dextrose

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[1] *Abbreviations used in this paper:* ELDOR, electron-electron double resonance; D, lipid lateral diffusion constant; HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Doxyl, 4,4-dimethyl-3-oxazolinyloxy; ESR, electron spin resonance; H_{ex}, Heisenberg spin exchange.

(ACD, NIH formula A). Platelet-rich plasma (PRP) was separated by centrifugation at 150 *g* for 15 min. All procedures were carried out at ambient temperature. To 9 ml of PRP were added 1 ml of ACD NIH formula A (to adjust the pH of the suspension to 6.5), 2.5 units of apyrase (Grade III; Sigma Chemical Co., St. Louis, MO), and prostaglandin E-1 (PGE-1) to a final concentration of 35 nM. Platelets were then pelleted at 1200 *g* for 15 min and washed three times in wash buffer (36 mM citric acid, 5 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 103 mM NaCl, made to pH 6.5 with concentrated NaOH) to which had been added, just before use, bovine serum albumin to a final concentration of 0.35% (wt/vol), 2 units/ml apyrase and 20 nM PGE-1. Contaminating red and white blood cells were removed by intermittent slow-speed centrifugation (150 *g*; 10 min). The final platelet pellet was resuspended in resuspension buffer (5 mM HEPES, 0.3 Mm NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM KCl, 137 mM NaCl, pH 7.4) without BSA. For stored platelet studies, platelet concentrates, prepared by routine blood bank procedures in Fenwal PL-732 containers, were stored in an end-to-end rotating incubator at 22° ± 1°C for 72 h. Stored platelets were isolated and processed as described above for fresh platelets.

Spin Labeling of Platelets

Aliquots of [^{14}N] 16-Doxylstearate (Aldrich Chemical Co. Inc., Milwaukee, WI) and [^{15}N] 16-Doxylstearate (a gift from Dr. Jane Park) in chloroform at a two-to-one molar ratio were mixed in 2-ml polystyrene vials. After the solvent was evaporated with a stream of N_2 gas, about 100 μl of platelets (1×10^{10} cells) in resuspension buffer without BSA were added to the vials coated with the spin probes, and the mixtures were incubated at ambient temperature for 15 min with gentle agitation. The amount of [^{14}N]16-Doxylstearate in the sample was about 0.16 μmol. The spin-labeled platelets were then transferred into a TPX capillary (10), which in turn was placed in the ESR cavity and purged with N_2 gas for 30 min before measurement. No glass material was used throughout the manipulations to avoid platelet adhesion and loss.

ELDOR Measurement

Both conventional bimodal cavity ELDOR and loop-gap resonator ELDOR techniques were employed in this study for historical reasons. The loop-gap methods became available to us in the midst of the work. Bimodal cavity ELDOR instrumentation was as described by Popp and Hyde (6). An ELDOR bridge (model E800; Varian Associates Inc., Palo Alto, CA) with a microwave bridge (model E101; Varian Associates Inc.) was used to obtain ELDOR spectra. The cavity supports orthogonal rectangular TE_{103} and TE_{102} modes with the TE_{103} mode used as a pump mode and the TE_{102} used as the observing mode. $^{14}N/^{15}N$ dual-label ELDOR methodology was as described by Feix et al. in the previous study on lateral diffusion of stearic acid spin labeles in model membranes (7). W_e, electron spin relaxation rate, of the ^{14}N observing species in platelets measured independently by using saturation-recovery ESR methods (6) was found to be 0.24 MHz at 37°C. No differences in W_e values were observed for [^{14}N] 16-Doxylstearate spin label in freshly prepared and stored platelets.

Loop-gap resonator ELDOR instrumentation and methodology were as described by Hyde et al. (8). Briefly, pump and observing powers were fed to a normal loop-gap resonator. Pump power reflected from the resonator was separated from observing power by use of a tunable pump-trap cavity, thereby absorping the pump carrier. All ELDOR measurements were carried out at 37° ± 1°C. The results obtained from conventional bimodal cavity ELDOR and a loop-gap resonator ELDOR measurements were substantially identical. The data presented in Figs. 1 and 2 were obtained using the loop-gap resonator ELDOR method.

The ELDOR reduction factor R is defined as [(signal with pump off) − (signal with pump on)]/(signal with pump off) or $R = 1 - (I_p/I_o)$, where I_p and I_o are the peak-to-peak amplitudes of the observed transition with the pumping field turned on and off, respectively. Reductions were determined for each sample at several pump field intensities from 120 to 400 mW when using the bimodal cavity and from 12.5 to 50 mW when using the loop-gap resonator. It is noted that the measured field for a given incident power is much higher in the loop-gap resonator. The reduction at infinite power (R_∞) was obtained from a linear regression least-squares extrapolation of R^{-1} vs. P^{-1} (see Fig. 1), where P is the measured incident pump power.

The conventional ESR measurements were performed on a Century Line Varian Spectrometer as described previously (11). The microwave power was 5 mW. The field sweep was 100 G and the modulation amplitude 1.0 G. The effective rotational correlation times for isotropic rotation of the spin probes were calculated using the linear and quadratic terms of the ESR motional narrowing formalism.

$$\tau_2 = 6.51 \cdot 10^{-10}\,\Delta H(0)[(h_0/h_{-1})^{1/2} - (h_0/h_{+1})^{1/2}] \times s \text{ (linear term)}$$

$$\tau_2 = 6.51 \cdot 10^{-10}\,\Delta H(0)[(h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2] \times s \text{ (quadratic term)},$$

where $\Delta H(0)$ is the peak-to-peak linewidth of the central field line and h_0, h_{-1} and h_{+1} are the peak-to-peak amplitudes of the first derivative resonances of the central, high- and low-field peaks, respectively.

RESULTS AND DISCUSSION

The ELDOR effect on [^{14}N], [^{15}N] 16-Doxylstearate spin-label pairs in platelets at 37°C is demonstrated in Fig. 1. The dotted line is an ordinary superimposed [^{14}N], [^{15}N] ESR spectrum with the pump klystron off. The solid line is an ELDOR spectrum with the pump klystron on. The frequency difference between the pump field (↑, $M_I = -1/2$ of ^{15}N) and the observing field (↓, $M_I = 0$ of ^{14}N) was 26 MHz. The ELDOR effect, a reduction in signal amplitude of the $M_I = 0$ transition, can be seen in the solid line. A slight reduction in signal amplitude of the $M_I = -1/2$ transition probably due to nitroxide reduction in platelet membranes was noted (Fig.1).

The reduction in intensity is related to R, the ELDOR reduction factor (see the Methods section). A plot of R^{-1} vs. P^{-1} yields the reduction at infinite power, R_∞ as shown in Fig. 2. The R_∞ values for freshly prepared and stored

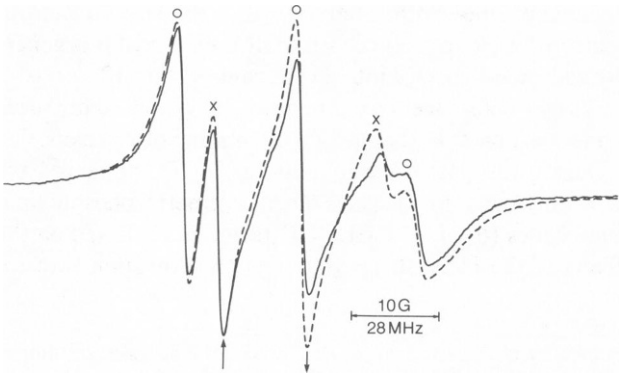

FIGURE 1 The ELDOR effects for the spectra of [^{14}N], [^{15}N] 16-doxylstearate spin labels (2:1 molar ratio) in freshly prepared platelets at pH 7.4. Superimposed spectra (O;^{14}N and ×;^{15}N) were recorded with pump off (broken line) and at 50-mW incident power (solid line). Arrows indicate the positions of pump field (↑) and observing field (↓). Field calibrations in Gauss and megahertz are given.

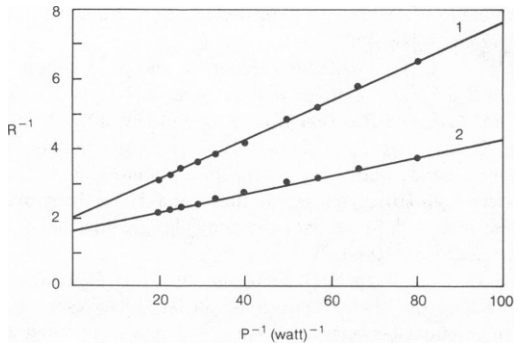

FIGURE 2 Determination of R_∞^{-1}. Plots of $1/R$ vs. the inverse of the measured pump power for [^{14}N], [^{15}N] 16-doxylstearate spin labels in (*a*) freshly prepared platelets and (*b*) stored platelets.

platelets are clearly different (Fig. 2 and Table 1). These values were used to calculate the bimolecular collision rates based on the following equations (12).

$$b'' = (R_\infty^{-1} - 1)^{-1} \tag{1}$$

and

$$W_{Hex} = 2\, b''\, W_e, \tag{2}$$

where W_{Hex} and W_e are Heisenberg exchange rate and electron spin relaxation rate, respectively. The lateral diffusion constant, D, was then calculated from W_{Hex} by using the Träuble and Sackmann equation

$$W_{Hex} = 2\, d_c \frac{c}{1 + c} \frac{1}{F} \frac{D}{\lambda} \frac{p}{\theta}, \tag{3}$$

where F, d_c and λ are the area per lipid molecule (60 Å^2 for $T > T_m$), the critical interaction distance (approximately 20 Å) and the length of one diffusional jump in the lipid lattice (8 Å), respectively. p and θ are the probability of spin exchange upon collision and a geometrical factor related to the lattice structure of the bilayer. C, the molar ratio of the pumped species to the total lipids in the plasma membrane of platelet cells, was estimated based upon the assumption that total incorporation of added spin labels into platelets had occurred (as evidenced from the lack of free-spin components), and taking into consideration the published values for phospholipid, cholesterol, and free fatty acid content of the platelet plasma membrane (13). A value of 0.02 for the molar ratio, C, was obtained based on these calculations. The D's for freshly prepared and stored platelets were found to be 1.0×10^{-8} and 2.6×10^{-8} cm^2/s, respectively (Table I). The order of magnitude of these data is in good agreement with that of literature values obtained for fluorescent lipid probes in the plasma membrane of lymphocytes and fibroblasts using fluorescence recovery after photobleaching techniques (14, 15). D in the plasma membrane of platelets reported here is about twofold to fourfold smaller than D in phospholipid membranes above main phase transitions (6, 7), a finding consistent with the observations made by Jacobson et al. in some other cell systems using fluorescence recovery after photobleaching techniques (16).

The 2.6-fold difference in D between freshly prepared and stored platelets indicates that the latter have a more fluid membrane than the former. To determine whether the rotational diffusion of lipid spin probes in these two platelet preparations is affected in a similar fashion, we incorporated [^{14}N] 16-Doxylstearate spin label alone into the platelets and measured the rotational correlation times using the line narrowing formalism. The results are shown in Table II. The difference in D by 260% between freshly prepared and stored platelets is far greater than the difference in τ_c (only ~20%, Table II). In DMPC membranes using the same techniques, the ratio of D measured at 47°C and at 37°C, and the ratio of τ_c measured at these two temperatures were shown to be about the same, i.e., 1.4 (Feix et al., unpublished observations), indicating that in pure phospholipid membranes, lateral diffusion and rotational diffusion exhibit similar temperature-dependent changes. The difference in the changes between D and τ_c for freshly prepared and stored platelets implies that membrane components other than phospholipids affect molecular motion. Hamid et al. (17) showed previously that platelets stored under similar experimental conditions lose ~15% of total cholesterol, 7–11% of total phospholipid, and 2–4% of total platelet protein content. Moreover, Jacobson et al. (16) demonstrated using fluorescence

TABLE I
CALCULATION OF LIPID LATERAL DIFFUSION CONSTANTS IN PLATELETS

Platelets*	R_∞^{-1} ± SD	b''§	W_{Hex}	D
			MHz‖	cm^2/s¶
Fresh	2.49 ± 0.37(7)‡	0.70	0.33	1.0×10^{-8}
Stored	1.54 ± 0.05(6)	1.85	0.88	2.6×10^{-8}

*Fresh and stored platelets were prepared as described in the Methods section.
‡Data are presented as mean ± standard deviation from least-squares linear regression analysis (number of independent measurements). Two of these measurements were obtained using a loop-gap resonator ELDOR method and the rest were obtained using conventional ELDOR method.
§,‖ and ¶ are from Eqs. 1, 2, and 3, respectively.

TABLE II
ROTATIONAL DIFFUSION CONSTANTS OF [^{14}N] 16-DOXYLSTEARATE SPIN LABEL IN FRESH AND STORED HUMAN PLATELETS

Platelets*	$\tau_c \times 10^9$ s‡	
	Linear	Quadratic
Fresh	1.37 ± 0.03	1.83 ± 0.03
Stored	1.17 ± 0.02	1.57 ± 0.01

*Fresh and stored platelets were prepared as described in the Methods section.
‡Rotational correlation times are calculated based on the line narrowing formalism as described in the Methods section.

recovery after photobleaching technique that the presence of membrane proteins or cholesterol reduces D in cell membranes. Since the change in protein content upon storage is small, it is plausible that the observed increase in D for stored platelets is at least partly due to a decrease in cholesterol content. Alternatively, changes in cytoskeleton-membrane association mediated by protein–protein interaction that are labile under storage conditions may result in significant alterations in membrane lipid mobility. In this regard, human platelet glycoprotein Ib, which appears to be selectively modified during the course of blood bank storage (18), has recently been shown to be associated with actin-binding protein of the cytoskeleton, an interaction that is readily dissociated when either or both actin-binding protein and Ib are cleaved by proteases (19).

In summary, we demonstrate here that the ELDOR method can be used to measure D in intact platelets. D for platelets stored for 3 d at room temperature after current blood bank regulations is 2.6 times greater than D for freshly prepared platelets. Even though numerous metabolic and structural changes have been observed for stored platelets, no specific abormalities have yet been directly linked to the loss of hemostatic effectiveness associated with normal storage (20, 21). It is tempting to speculate that changes in D on storage may result from alterations in membrane cholesterol content or from altered cytoskeleton-membrane interaction, thereby causing a loss of hemostatic effectiveness. The relationship between lipid lateral diffusion and hemostatic effectiveness of platelets merits further investigation, and the ELDOR method should prove a useful tool for this purpose.

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