Biochemical Pharmacology, Vol. 38, No. 18, pp. 2949–2955, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

PROPRANOLOL-INDUCED ALTERATIONS IN RAT ERYTHROCYTE MEMBRANE FLUIDITY AND APPARENT PHASE-TRANSITION TEMPERATURES

A DEPTH-DEPENDENT PROCESS

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(*Received* 4 *April* 1988; *accepted* 13 *December* 1988)

Abstract—Propranolol-induced alterations of membrane structure were studied in rat erythrocytes using electron spin resonance techniques. Propranolol produced a concentration-dependent change in membrane fluidity in hydrophobic membrane regions, while producing virtually no change in hydrophilic membrane regions. The changes were associated with depth-dependent alterations in "apparent" phase-transition profiles and transition temperatures. The effects of propranolol on these membrane characteristics were similar to those produced by cholesterol. Propranolol fluidized erythrocyte membranes in a depth-specific fashion, by virtue of its association with the rigid phospholipid acyl chains and cholesterol sterol rings in the hydrophilic regions of the membrane, which produced distant perturbations within the hydrophilic regions of the membrane.

The therapeutic efficacy of the non-selective beta-adrenergic antagonist propranolol has been well established for a variety of disease states including hypertension, angina pectoris and cardiac arrhythmias [1]. While the mechanisms of action of propranolol in these disease states are predominantly through stereo-specific blockade of the beta-receptor, the significance of its nonstereo-specific membrane-stabilizing properties remains unclear. The nonstereo-specific membrane actions of propranolol are thought to be partially responsible for its antipsychotic, anticonvulsant and antiarrhythmic activity [2–4]. The membrane effects of propranolol are probably produced indirectly, by the inhibition of various membrane associated functions; enzymatic activity (acetylcholinesterase and *p*-nitrophenyl phosphatase [5]); carrier mediated transport mechanisms (glucose transport [6]); and electrophysiologic changes (prolongation of ventricular effective refractory period [7, 8], and increased background outward current (i_{K_1}) in Purkinje fibers [9]).

Model membrane systems and isolated erythrocyte membranes have been used extensively to examine the interaction of propranolol with membrane components. Surewicz and Leyko [10] showed that propranolol preferentially associates with negatively charged acidic phospholipids (phosphatidylserine and phosphatidic acid). This resulted in a reduction of negative electrostatic interactions within the membrane matrix, since propranolol is positively charged at physiologic pH. This reduction in electrostatic forces was thought to account for the condensation effects of propranolol on acidic phospholipids (as expressed by an increase in order parameter), while its hydrophilic interaction with the phospholipids could be demonstrated by monolayer expansion. Work by Godin *et al.* [5] and Surewicz [11] suggested that propranolol caused perturbation of protein-lipid interactions at the membrane surface. In addition, Singer [12] and Akiyama and Igisu [13] demonstrated that propranolol increases mobility of phospholipid-associated fatty acyl chains. These observations led Surewicz *et al.* [14] to hypothesize a biphasic interaction of propranolol with erythrocyte membranes: at low concentrations interacting with membrane proteins and increasing "stability" and at high concentrations interacting with membrane fatty acid chains and decreasing "stability".

In a recent electron spin resonance (ESR) study, Janoff *et al.* [15] failed to show a significant alteration of erythrocyte membrane lipid fluidity after propranolol incorporation using 5-doxyl stearic acid and 5-doxyl stearic methyl ester spin-labels. Since their study only evaluated membrane fluidity characteristics at the phospholipid polar head-group region of the erythrocyte membrane, we attempted to examine a depth-dependent effect of propranolol on erythrocyte membrane fluidity. This was accomplished by using 5-doxyl, 12-doxyl and 16-doxyl stearic acids as the spin-labeled fatty acids, which sample membrane fluidity at the polar head-, middle- and inner-hydrophobic regions of the outer leaflet of the erythrocyte membrane. In addition, the effect of propranolol on temperature-dependent changes in membrane fluidity characteristics was evaluated for possible

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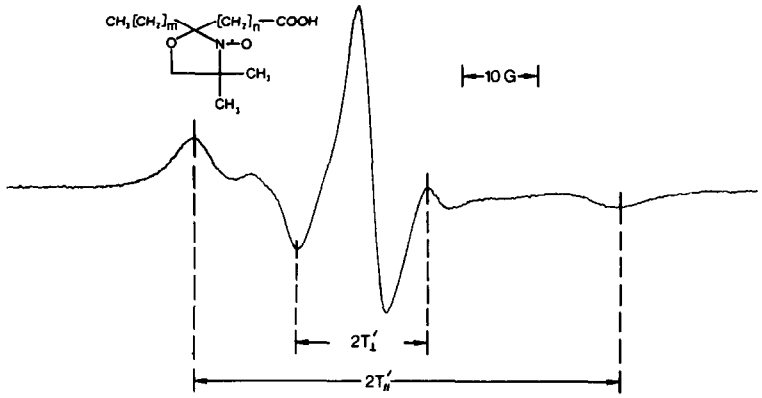

Fig. 1. Measurement of spectral parameters ($2T'_{\parallel}$ and $2T'_{\perp}$) obtained from 5-doxyl stearic acid incorporated into RBC membranes, for calculation of order parameter S value. Structural representation of the three doxyl stearic acids, demonstrating the location (m,n) on the acyl chain of the reporter group (nitroxyl radical): for 5-DS (12,3), 12-DS (5,10), and 16-DS (1,14).

changes in apparent phase-transition temperatures. The observed changes in phase-transition temperature profiles produced by propranolol were similar to those produced by cholesterol in erythrocyte (RBC) model phospholipid liposomes.

METHODS

Animals and drugs. Virgin female Sprague–Dawley rats were obtained from Harlan Sprague–Dawley, Inc. (Walkersville, MD). Rats were maintained on 12 hr light/12 hr dark photoperiod cycle and were supplied with food and water *ad lib.* DL-Propranolol hydrochloride and cholesterol were obtained from the Sigma Chemical Co. (St Louis, MO). Spin-labels [5-, 12- and 16-doxyl stearic acids (DS)] were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Figure 1 shows the structure of the spin-labels, with (m,n) values as follows: 5-DS (12,3); 12-DS (5,10); 16-DS (1,14). RBC phospholipids (No. 27-6202) were obtained from Pharmacia/P-L Biochemicals, Inc. (Piscataway, NJ).

RBC/Liposome preparation. Rats were anesthetized with 30 mg/kg, i.p., of pentobarbital sodium. Blood was obtained by cardiac puncture, using heparinized syringes, and was centrifuged immediately at 2000 rpm for 20 min. The RBCs was washed three times with a 1:1 (v/v) phosphate-buffered (0.1 M, pH 7.40) saline (PBS) and then incubated with the spin-label under study at 30 μM in the PBS (1:5, v/v) at 23° for 0.5 hr. Following incorporation of the spin-label, the RBCs were washed three times with PBS (1:10, v/v). The resulting pellet was then incubated with propranolol at concentrations ranging from 0.5 to 10 mM in PBS buffer (1:5, v/v) at 23° for 0.5 hr, washed three times with PBS (1:10, v/v), and the resulting pellet stored at 4° until analysis (less than 3 hr). Similarly, liposomes were formed from commercially available RBC phospholipids, after proteins and cholesterol were removed, whose phospholipid profile has been described previously [16]. Reconstitution of the phospholipids was conducted in the presence of the appropriate spin-label, and liposomal formation was induced by sonication [Branson Sonicator, power setting 6 (50 W), ~10 min at 4°], according to the technique of van Rooijen and Nieuwmegen [17]. Using these procedures, the resultant concentration of spin-label to lipid ratio was <1:120 (mg/mg), not considering cholesterol as a phospholipid component of the RBC membrane.

ESR measurements. All spectra were obtained on a Bruker model 200 D-SRC ESR spectrometer (IBM Instruments, Orchard Park, CT), using a central field strength of 3350 G, sweep width of 100 G, modulation frequency of 100 kHz, modulation amplitude of 2 Gpp, microwave power of 4.7 mW, time constant of 1.0 sec, and a scan time of 1 ksec. Order parameter (S value) calculations were employed for the estimation of membrane fluidity. The spectral parameters $2T'_{\parallel}$ and $2T'_{\perp}$ (Fig. 1) were employed to calculate S values, using the techniques of Hubbell and McConnell [18] and Gaffney [19]. At temperatures below 280°K it was difficult to clearly identify $2T_{\perp}$ without employing computer-assisted signal averaging [IBM-PC with software (EPRDS -1.00 B/2.89) supplied by Adaptable Laboratory Software, Rochester, NY], curve smoothing and amplification. As a result, the S values obtained below the temperature of 280°K should be viewed critically. However, the same trends suggested by S values were confirmed by calculating the order parameter $S_{\parallel}$, which is only dependent on $T'_{\parallel}$ and was clearly identified at all temperatures employed. $S_{\parallel}$ values were calculated in accordance with the technique of Sauerheber *et al.* [20] and Gordon *et al.* [21]. The order parameter calculations from the spectral characteristics are:

$$S = \frac{(T'_{\parallel} - T'_{\perp})(a_N)}{(T_{zz} - T_{xx})(a'_N)};$$

$$S_{\parallel} = \frac{1}{2}\left[\frac{3(T'_{\parallel} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right]$$

T_{xx} and T_{zz} are determined from host crystal studies, while a_N and a'_N are isotropic hyperfine coupling constants for the spin-label in membrane and crystal

Table 1. Propranolol-induced alteration of RBC membrane fluidity at different temperatures and different membrane depths

Propranolol treatment*	Order parameter (S)		
	283°K	296°K	310°K
5-DS			
0 mM P†	0.7306 ± 0.0043	0.6703 ± 0.0054	0.6110 ± 0.0034
5 mM P	0.7342 ± 0.0031	0.6725 ± 0.0062	0.6063 ± 0.0032
10 mM P	0.7313 ± 0.0056	0.6694 ± 0.0048	0.6120 ± 0.0007
12-DS			
0 mM P	0.6802 ± 0.0018	0.5824 ± 0.0088	0.4552 ± 0.0039
5 mM P	0.6963 ± 0.0066‡	0.5533 ± 0.0041‡	0.4461 ± 0.0034‡
10 mM P	0.6894 ± 0.0037‡	0.5632 ± 0.0051‡	0.4473 ± 0.0046
16-DS			
0 mM P	0.2914 ± 0.0058	0.2669 ± 0.0054	0.2211 ± 0.0022
5 mM P	0.2876 ± 0.0053	0.2440 ± 0.0022‡	0.1866 ± 0.0065‡
10 mM P	0.2959 ± 0.0053	0.2386 ± 0.0160‡	0.1607 ± 0.0091‡

Values are means ± SE.

* 5-DS (5-doxyl stearic acid), 12-DS (12-doxyl stearic acid), 16-DS (16-doxyl stearic acid) incubated at 296°K for 0.5 hr at 30 μM.

† P (propranolol HCl), where N = 3 for each group.

‡ $P < 0.05$, Student's t-test [23].

hosts respectively $[a'_N = 1/3(T'_{\parallel} - 2T'_{\perp}),\ a_N = 1/3(T_{zz} + 2T_{xx})]$. T_{zz} and T_{xx} splitting elements for 5- and 12-DS were taken from Seelig [22]. Since T_{xx} and T_{zz} were not available for 16-DS, we utilized the splitting elements for 5-DS.

Temperature and depth profiles. Once the intact RBCs or the RBC phospholipids (with various percent cholesterol content) had been prepared with the appropriate spin-label (5-, 12-, or 16-DS), full spectra were obtained at either 283°, 296° or 310°K. Using separate samples, temperature profiles of the $2T'_{\parallel}$ and $2T'_{\perp}$ spectral parameters were generated over a range from 274°K to 316°K, in 3°K increments. Sample-cavity temperature was maintained with a liquid nitrogen unit (Bruker, VT-100) within ±0.5°K. To ensure thermal equilibrium of the sample, it was maintained at each desired temperature for 5 min before each spectrum was obtained. In all cases, the samples were processed from low to high temperature and low to high magnetic field strengths. The rate of RBC spin-label reduction, although measurable, was not of sufficient magnitude to alter signal/noise relationships.

Statistics. Comparison of individual group means was accomplished by Student's t-test, at a significant level of $P \leq 0.05$ (Steele and Torrie [23]), while linear regressions were accomplished with the least-squares technique (Steel and Torrie [24]).

RESULTS

The results in Table 1 demonstrate that propranolol at a concentration as high as 10 mM did not alter intact rat RBC membrane fluidity characteristics sampled by the spin-label 5-DS at the three temperatures examined. Conversely, both 12-DS and 16-DS, which report on membrane fluidity characteristics deeper in the RBC membrane (towards the hydrophobic core), showed propranolol-induced concentration-dependent alterations in membrane fluidity, particularly at physiologic temperatures. The changes produced by propranolol in the membrane examined by 16-DS appear to be greater, both on a percent change basis and on an absolute value basis, than those reported by 12-DS.

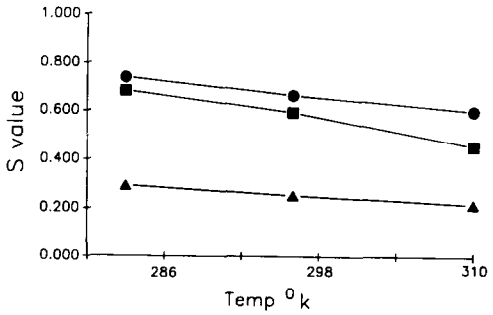

Fig. 2. Temperature-dependent changes in RBC membrane fluidity (S value) for 5- (●), 12- (■), and 16- (▲) doxyl stearic acids (SE values less than the size of symbols).

All three spin-labels demonstrated a temperature-dependent increase in membrane fluidity with increasing temperature (Fig. 2). Clearly, 12-DS shows a much larger change in membrane fluidity (33%) from 283° to 310°K than either 5-DS (16%) or 16-DS (26%). In addition, the results demonstrate that, at low temperatures, 5-DS and 12-DS reported almost the same molecular mobility, whereas 16-DS reported on a much more mobile environment. At physiologic temperatures, the depth-dependent fluidity profile became much more consistent with the depth-dependent increase in membrane fluidity demonstrated in model membranes (Seelig *et al.* [25]). It was this rapid temperature-dependent change in the environment reported by 12-DS that prompted us to examine closely RBC membranes for temperature-dependent phase-transitions at various membrane depths.

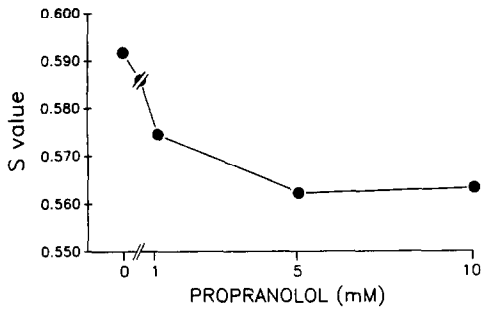

Fig. 3. Concentration-dependent alteration in RBC membrane fluidity (order parameter) produced by propranolol (<3%, w/w) as reported by 12-doxyl stearic acid. ($P < 0.05$, relative to control at 296°K, for all values of propranolol.)

Figure 3 shows the concentration-dependent change in RBC membrane fluidity produced by propranolol, as reported by 12-DS. The maximum dose-dependent increase in membrane fluidity occurred around 5 mM, with no further increase in membrane fluidity seen at 10 mM propranolol. In fact, at concentrations above 5 mM the RBC membrane appeared slightly more rigid, and is associated with increased RBC hemolysis [14]. In contrast, 16-DS showed a continued increase in RBC membrane fluidity at 10 mM (Table 1). However, further increases in propranolol concentration resulted in excessive RBC hemolysis. Propranolol is highly soluble in both aqueous and lipid environments. Work by Surewicz and Leyko [10] reported partition coefficients of 290 to 20,000 between phospholipids and buffer systems. At an exposure concentration of 10 mM propranolol, the maximal RBC membrane content of propranolol was less than 3% (w/w), under the conditions employed in this study. Thus, it is unlikely that the plateau in the ΔS value for 12-DS at ~5 mM propranolol is due to solubility limitations. This conclusion was supported by the fact that no plateau was observed at propranolol concentrations >5 mM for 16-DS, and the report by Ondrias *et al.* [26] that membrane disordering continues at concentrations as high as 25 mM (16-DS).

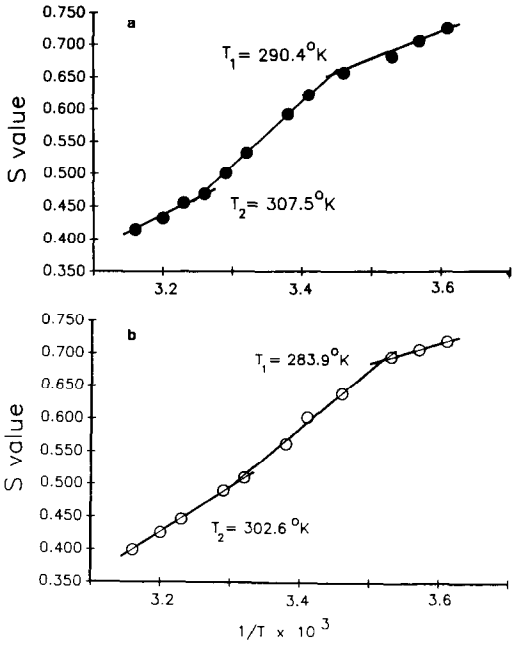

Fig. 4. (a) Phase-transition profile (1/temperature × 10^3 vs *S* value) in RBC membranes reported by 12-doxyl stearic acid (control—●), and (b) its alteration by exposure to 5 mM propranolol (○) following incubation for 0.5 hr at 296°K.

Figure 4 shows the effect of propranolol (5 mM) on the apparent thermotropic phase-transitions reported by 12-DS in rat RBC membranes. Propranolol shifted both transitions to lower temperatures, with a 6.5°K decrease at the low temperature transition and a 4.9°K decrease at the high temperature transtition. Table 2 lists apparent phase-transition temperatures calculated by multiple linear regression, for all three spin-labels. No phase-transitions were observed for 5-DS, contrary to the work of Janoff *et al.* [15], nor did propranolol alter membrane fluidity characteristics reported by 5-DS.

It was possible that lateral-phase separations occurred in the RBC membranes at lower temperatures, and produced the phase-transitions through spin-label concentration and resultant spin–

Table 2. Alteration of apparent phase-transition temperatures by propranolol as reported by 5-, 12-, and 16-doxyl stearic acids in rat RBCs

		Apparent phase-transition temperatures		
Spin-label*	T_n	Control	Propranolol†	ΔT‡
5-DS		None	None	
12-DS	T_1	290.4°K	283.9°K	−6.5°K
	T_2	307.5°K	302.6°K	−4.9°K
16-DS		None	None	

* 5-DS (5-doxyl stearic acid), 12-DS (12-doxyl stearic acid), 16-DS (16-doxyl stearic acid) incubated at 296°K for 0.5 hr at 30 μM.

† Propranolol incubation concentration of 5 mM at 296°K for 0.5 hr.

‡ Calculated from linear regressions of 1/temperature × 10^3 vs *S* value [24].

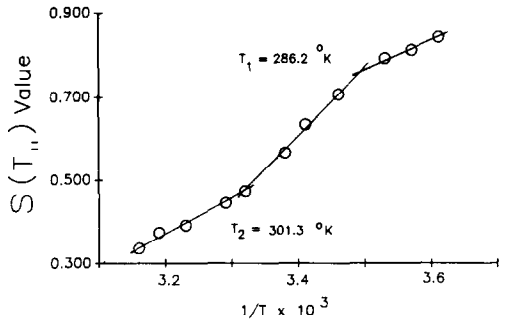

Fig. 5. Phase-transition profile (l/temperature × 10^3 vs $S_{\parallel}$ value) in RBC membranes reported by 12-doxyl stearic acid, after exposure to 5 mM propranolol (0.5 hr at 296°K), demonstrating the occurrence of phase-transitions independent of lateral-phase separation.

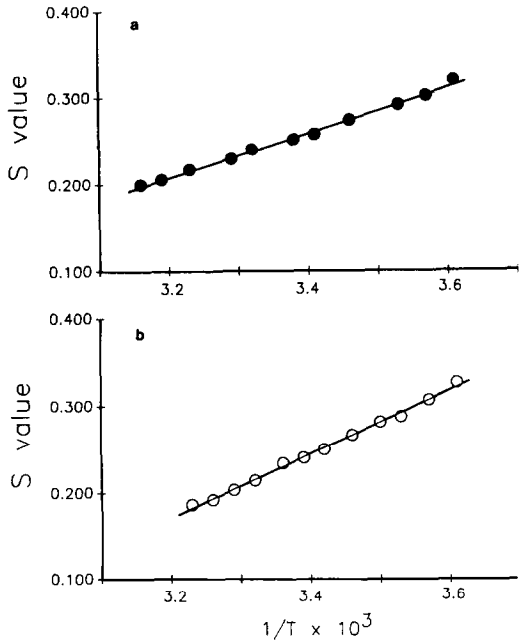

Fig. 6. (a) Phase-transition profile (l/temperature × 10^3 vs S value) in RBC membranes reported by 16-doxyl stearic acid (●); and (b) its alteration by exposure to 5 mM propranolol (○) following incubation for 0.5 hr at 296°K.

spin interactions. As a result, our data from Fig. 4 were plotted as l/temperature × 10^3 versus $S_{\parallel}$, which has been reported by Gordon *et al.* [21] to be insensitive to concentration-dependent spin–spin interactions. These data, in Fig. 5, demonstrate both apparent phase-transition temperatures as sharply as those shown in Fig. 4. Thus, it is doubtful that the apparent phase-transitions observed for 12-DS are due to lateral-phase separations using the criteria of Gordon *et al.* [21]. This was confirmed further by the absence of transition temperatures reported by 16-DS (Fig. 6).

Propranolol has been described as localizing in biological membranes at a membrane depth shared with cholesterol [27]. As a result, the effect of cholesterol on apparent phase-transition temperatures in liposomes formed from RBC phospholipids was evaluated in a depth-dependent manner. The results in Table 3 suggest that the appearance of the apparent phase-transitions in RBC phospholipids reported by 12-DS can be induced by the presence of cholesterol in a concentration-dependent fashion.

Table 3. Effect of cholesterol content on apparent phase-transition temperatures in erythrocyte liposomes

Cholesterol content* (%)	Apparent phase-transition temperatures†	
	5-DS	12-DS
0	None	None
10	None	288.4°K, 298.3°K
30	None	286.8°K, 303.2°K
50	None	292.9°K, 302.4°K

* 5-DS (5-doxyl stearic acid) and 12-DS (12-doxyl stearic acid) were incubated at 296°K for 0.5 hr at 30 μM in liposomes formed from erythrocyte phospholipids (no protein or cholesterol) reconstituted with various concentrations of cholesterol.

† Calculated from linear regressions of l/temperature (277–307°K) × 10^3 vs $S_{\parallel}$ values [24].

DISCUSSION

This investigation examined the RBC membrane for possible depth-dependent changes in fluidity characteristics induced by exposure to propranolol. Spin-labeled fatty acids (5-, 12- and 16-DS) were utilized to sample fluidity at various depths in the RBC membrane. As expected, RBC membrane fluidity increased (order parameter decreased) with both increasing membrane depth (5 > 12 > 16-DS) and increasing membrane temperature (Table 1). Propranolol produced a concentration-dependent increase in membrane fluidity, using 12-DS and 16-DS as the spin-labeled probes, while no change was observed with 5-DS (Table 1). The later observation agrees with the work of Janoff *et al.* [15], who was unable to observe significant propranolol-induced changes in RBC membrane fluidity with 5-DS.

It is difficult to rationalize the propranolol-induced alteration in membrane mobility deep within the RBC, when other studies have suggested that propranolol is predominately associated with the polar-head region of the phospholipids [10, 11]. The intercalation of propranolol within the outer aspect of the lipid membrane has been shown to increase significantly the intermolecular distances of the phospholipids as a result of its bulk lipid incorporation. However, the membrane fluidity as reported by 5-DS, up to a propranolol concentration of 10 mM, remained unchanged in both our study and that by Janoff *et al.* [15]. Since propranolol only inserts part-way into the membrane, there would be an increase in intermolecular distances further down the acyl fatty chains. Thus, the inner or hydrophobic portions of the acyl chains would experience a decrease in molecular packing density, and should demonstrate differences in acyl mobility (12- and 16-

DS) following propranolol exposure. This is supported by the observation that 12-DS showed no concentration-dependent increase in membrane fluidity beyond 5 mM (Fig. 3), whereas 16-DS continued to show a linear increase in membrane fluidity at propranolol concentrations as high as 10 mM (Table 1). These data are analogous to data obtained by Chin and Goldstein [28] for ethanol, and by Ondrias *et al.* [26] for a series of β-antagonists.

Our results are not consistent with the membrane models that have been offered previously to explain phase-transition changes. The model most frequently offered to account for the existence of phase-transitions in biological membranes is the cluster model described by Tsong *et al.* [29] as a two-dimensional Ising lattice, where lipid exists in either a solid (S) of fluid (F) state. This model was not designed to consider variations in acyl chain length, configuration or degree of unsaturation. Similar models have been suggested to explain phase-transition changes by Gent and Ho [30] using a F-19 NMR technique, by Verma and Wallach [31] using Laser–Ramen spectroscopy, by Lee [32] using a TEMPO partitioning technique, by Ziemann and Zimmer [33] using both ANS fluorescence and 5-DS spin-label, and by Klausner *et al.* [34] using ANS/DPH fluorescence. Although the phase-separation models are certainly supported by the various techniques, and are undoubtedly an accurate representation of events occurring near the phospholipid head group region, these models do not account for the phospholipid unsaturated acyl chain component of membrane fluidity.

A likely mechanism for our observation is the presence of unsaturated fatty acids in the RBC membrane, as alluded to in the review by Lands and Davis [35]. Most of the long chain unsaturated fatty acids have their double bonds located near the middle- and terminal-end (hydrophobic end) of the fatty acid. As a result, there is a considerable amount of conformational disorder introduced into biological membranes towards the hydrophobic core of the bilaminar leaflets. This disorder appears amplified by the partial penetration of both cholesterol and superficial protein molecules, which occupy space in the upper regions of the acyl chains of the phospholipids, resulting in a significant decrease in acyl chain density at the hydrophobic core. Biphasic apparent phase-transitions in biological membranes are not reported frequently because (1) it requires appropriate selection of the reporter molecule so that the correct depth of the membrane is sampled; (2) it requires the presence of long-chain unsaturated fatty acids, something not always utilized in model membrane systems; and (3) it requires the presence of cholesterol to decrease fatty acid chain packing density at the level of the reporter molecule. Thus, the biphasic apparent phase-transitions reported by 12-DS may occur as a result of a complex indirect interaction of cholesterol and proteins with unsaturated long-chain fatty acids in the RBC membrane. This interaction is altered by the incorporation of propranolol, which produced a concentration-dependent increase in membrane fluidity at both 12-DS and 16-DS reporter levels, but not at the 5-DS level. In addition, it decreased both apparent phase-transition temperatures reported by 12-DS. This proposed mechanism is in agreement with the model described previously by Ondrias *et al.* [26, 36, 37].

In conclusion, supra-pharmacologic doses of propranolol produced depth-dependent alterations in membrane fluidity relationships in rat RBCs. The data demonstrate that apparent phase-transitions in biological membranes are probably dependent on multiple mechanisms which have been elucidated only partially with current model membrane studies. Our data offer some explanation for the divergence of the results and conclusions drawn from a number of previous studies using a variety of techniques to explore the effects of propranolol on RBC membrane structure/function relationships.

Acknowledgements—This work was supported by HL-32134, ES 03493 and WHA84-FA-04. We thank Ms Janet Wolfe for manuscript preparation.

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