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INTERACTION OF PROPRANOLOL WITH MODEL PHOSPHOLIPID MEMBRANES

MONOLAYER, SPIN LABEL AND FLUORESCENCE SPECTROSCOPY STUDIES

WITOLD K. SUREWICZ and WANDA LEYKO

Department of Biophysics, Institute of Biochemistry and Biophysics, University of Łódź, Banacha 12/16, Łódź (Poland)

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Summary

The interaction of propranolol with model phospholipid membranes was studied using various experimental techniques. The partition coefficient of propranolol in the negatively charged membranes of vesicles prepared from phosphatidylserine and phosphatidic acid was found to be more than 20-times higher than in neutral phosphatidylcholine membranes. Preferential interaction of propranolol with acidic phospholipid membranes was confirmed using the monolayer compression isotherm technique and the spin-labeling method. Phosphatidylserine monolayers were markedly expanded even at a relatively low drug concentration ($5 \cdot 10^{-6}$ M). In contrast, the effect of propranolol on phosphatidylcholine monolayers was much smaller, being detectable only at a higher concentration of the drug ($1 \cdot 10^{-4}$ M). Spin-labeling experiments show that propranolol exerts marked ordering effect on bilayers prepared from acidic phospholipids and does not change the order parameter of phosphatidylcholine membranes. The dependence of the propranolol fluorescence spectrum on the polarity of the solvent allowed us to identify the intercalation region of the drug in the membrane. The fluorophore moiety of propranolol was found to be localized in the lipid polar head groups region of the bilayer. The role of electrostatic and hydrophobic effects in propranolol-membrane interaction is discussed and the effect of propranolol on the ordering of phospholipid bilayers is compared with the effects of other anesthetic-like molecules.

Introduction

The role of propranolol as a beta-adrenergic receptor blocker is well established [1]. Detailed studies of propranolol-binding sites in various tissues

showed, however, that in most cases a substantial part of the drug is associated with binding sites which are not stereoselective and are unrelated to beta-adrenergic receptors [2,3]. This nonreceptor binding of propranolol may lead to more general membrane perturbations which could be connected, for example, with local anesthetic effects of the drug [4,5]. Moreover, it has been suggested that non-stereoselective membrane action of propranolol may contribute substantially to such complex pharmacological properties of the drug as its antipsychotic [6], anticonvulsant [7] and antiarrhythmic [8] effects.

Similarly, as in the case of other anesthetic-like molecules, membrane action of propranolol causes stabilization of biological membranes, as reflected in, for example, the antihemolytic effect of the drug on red blood cells [9]. Besides, propranolol has been shown to produce diverse functional perturbations in various membrane systems [10—15]. Attempts have been also made to study some aspects of propranolol-induced structural alterations in biomembranes. These studies have suggested that both lipids [10,16,17] and proteins [10] may be perturbed upon interaction with the drug. The molecular mechanism of propranolol-membrane interaction is, however, far from being clearly understood. Detailed knowledge of such a mechanism would be helpful for a better understanding of the relationship between functional perturbations of the membrane and structural alterations in its components. It could also contribute to the elucidation of the molecular basis of certain pharmacological properties of propranolol and other membrane-stabilizing drugs.

In the present study we explore the interaction of propranolol with model phospholipid membranes.

Materials and Methods

Chromatographically pure egg yolk lecithin was prepared by the method of Singelton et al. [18]. Phosphatidic acid (Grade I), bovine brain phosphatidylserine and DL-propranolol hydrochloride were obtained from Sigma and used without further purification. The spin label 5-doxylstearic acid was delivered by Syva, Palo Alto, CA.

Multilamellar liposomes for partition coefficient study were prepared by the method of Bangham et al. [19] in 20 mM Tris-HCl/0.1 mM EDTA buffer (pH 7.4), in the presence or absence of 100 mM NaCl. Phospholipid vesicles used in fluorescence spectroscopy studies were prepared by sonication of concentrated lipid suspension (15 mg/ml) for 30 min in an ice bath using an MSE Sonifier. In order to remove titanium particles and larger lipid aggregates the resulting mixture was centrifuged at $40\,000 \times g$ for 20 min, and the clear supernatant was used as a stock suspension of vesicles.

Phospholipid suspensions used in electron spin resonance (ESR) measurements were prepared as follows. Spin label was added to chloroform solution of phospholipids. After evaporation of the solvent under nitrogen Tris buffer (50 mM, pH 7.4) containing 100 mM NaCl and appropriate amount of propranolol was added and the mixture was shaken by means of Vortex rotamixer for 5 min. The resulting suspension was equilibrated before ESR measurements for at least 3 h at room temperature. Spin label/phospholipid molar ratio was 1 : 100. The final lipid concentration was kept at 15—20 mg/ml.

The partitioning of propranolol into liposomal membranes was determined essentially as described by Papahadjopoulos et al. [20]. Multilamellar liposomes were incubated in the presence of propranolol for 3 h at room temperature. Subsequently, lipids were separated from aqueous medium by centrifugation, using a Beckman L5 65 ultracentrifuge (200 000 × *g*, 23°C, 60 min). The separation was checked by means of phosphate assay [21]. Under experimental conditions usually less than 5% of total phosphate was found in the supernatant. The concentration of propranolol in the supernatant and in control buffer was measured spectrophotometrically at 290 nm, using a Carl Zeiss VSU2-P spectrophotometer. Partition coefficients were calculated according to the formula [22]:

$$P = \frac{C_T - C_S}{C_S m}(1 - m)$$

where C_T is the absorbance at 290 nm of control buffer containing a given amount of propranolol but no lipid, C_S is the absorbance of supernatant, corrected, if necessary, for the absorbance due to materials other than propranolol, and m represents the weight ratio of lipid to entire suspension.

Fluorescence spectra of propranolol were recorded in a Jobin Yvon JY3 spectrofluorimeter. In order to study the effect of phospholipid vesicles on propranolol fluorescence spectra, small amounts of concentrated vesicle suspension were sucessively added to the propranolol solution. After each addition the suspension was mixed and left to equilibrate for 10 min before recording of the spectrum.

ESR spectra were obtained at about 19°C with a SE/X-28 ESR spectrometer (Wroclaw Technical University) operating at 9.5 GHz.

Compression isotherms of phospholipid monolayers were obtained using a rectangular Teflon trough with a movable Teflon barrier and conventional Wilhelmy balance. Lipids were spread on the subphase as a solution in chloroform/ethanol (90 : 10, v/v) and the solvent was allowed to evaporate for 20 min. Surface pressure vs. surface area curves were recorded continuously. The aqueous subphase contained 20 mM Tris-HCl/100 mM NaCl buffer (pH 7.4) and propranolol in appropriate concentration. The cleanliness of the surface was checked before each experiment. Propranolol alone at the concentrations used did not produce any detectable surface pressure. Each experiment was performed in triplicate.

Results

Partition coefficient of propranolol in phospholipid membranes. The values of the partition coefficient of propranolol in model membranes prepared from acidic (phosphatidylserine, phosphatidic acid) and neutral (phosphatidylcholine) phospholipids are given in Table I. The unusually high values of the partition coefficient in liposomes prepared from acidic phospholipids were markedly reduced in the presence of monovalent salt (100 mM NaCl) but they still belong to the highest reported in literature for cationic anesthetics [23]. They were very similar to the value of about 5500 obtained using the same experimental procedure for partitioning of the potent local anesthetic dibu-

TABLE I

PARTITION COEFFICIENT OF PROPRANOLOL BETWEEN PHOSPHOLIPID MEMBRANES AND BUFFER

Propranolol concentration in all experiments was $1 \cdot 10^{-4}$ M. Each value is the mean ± S.D. of six to ten experiments.

Buffer	Lipid composition	Lipid concentration (mg/ml)	Partition coefficient	Bound propranolol per lipid (molar ratio)
20 mM Tris-HCl, pH 7.4	Phosphatidylserine	0.2	23 350 ± 5300	1/3.0
	Phosphatidic acid	0.2	19 800 ± 3240	1/3.5
	Phosphatidylcholine	1.0	292 ± 45	1/59
20 mM Tris-HCl/100 mM NaCl, pH 7.4	Phosphatidylserine	0.1	5860 ± 780	1/3.3
	Phosphatidylserine	0.2	6703 ± 1060	1/4.3
	Phosphatidic acid	0.2	5850 ± 950	1/5.2
	Phosphatidylcholine	1.0	276 ± 29	1/61

caine in phosphatidylserine membranes [20]. The value of the partition coefficient of propranolol in phosphatidylcholine liposomes is more than 20-times lower than in acidic lipid membranes. These data would suggest a very pronounced preference for occupation of negatively charged domains in biological membranes and the possibility of a high local concentration of the drug in these domains.

Fluorescence spectroscopy studies. Interaction of propranolol with phospholipid membranes was studied in more detail by means of fluorescence spectroscopy. The method is based on the dependence of the shape of propranolol emission spectrum on the environment of the fluorophore moiety. As can be seen in Fig. 1, three maxima may be distinguished in the propranolol fluorescence spectrum. The addition of phospholipid membranes to the solution of propranolol results in a marked change in the fluorescence spectrum of the drug. As a working parameter characterizing these spectral changes, the ratio I_1/I_3 of the emission peak intensities around 328 nm and 354 nm has been chosen. The intercalation of the drug into the bilayer structure of unilamellar vesicles could be thus conveniently followed by changes in the fluorescence peak intensity ratio I_1/I_3 upon the increase in phospholipid concentration. The titration curve of this type obtained for propranolol binding by phosphatidylserine vesicles is presented in Fig. 2. As can be seen at phosphatidylserine concentration above 0.5 mg/ml, virtually all drug present is associated with the membrane. Therefore, the fluorescence spectra of propranolol at concentrations of vesicles above 0.5 mg/ml could be used as a base for identification of the drug intercalation region in the membrane. For this purpose the fluorescence spectra of propranolol in water/ethanol mixtures of different polarities were recorded. The finding that there is a linear relation between the peak intensity ratio I_1/I_3 and the dielectric constant of the solvent, ϵ, (Fig. 3) gives a simple way for a rough estimation of the dielectric constant of the propranolol intercalation region in the membrane. The so estimated apparent dielec-

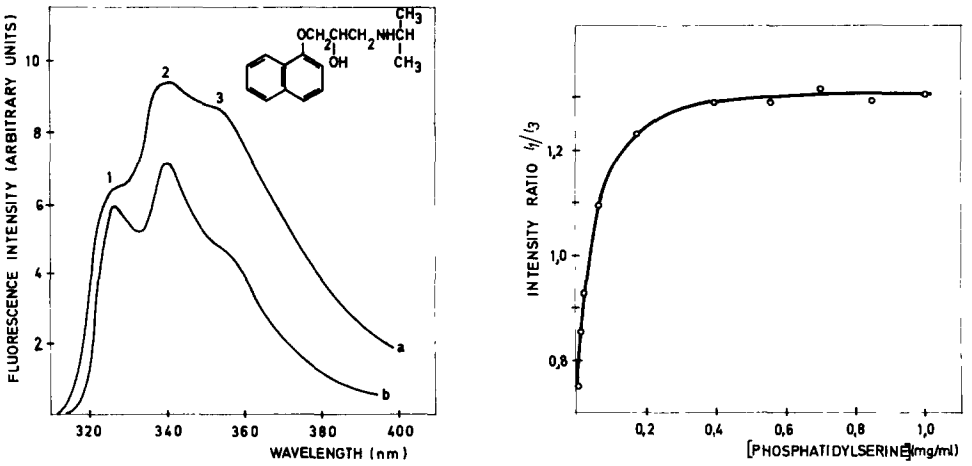


Fig. 1. Uncorrected fluorescence spectra of propranolol ($1 \cdot 10^{-5}$ M) in buffer (spectrum a) and in 0.5 mg/ml suspension of phosphatidylserine vesicles (spectrum b). Excitation wavelength: 290 nm.

Fig. 2. Fluorescence titration of propranolol binding to phosphatidylserine vesicles. Propranolol concentration $1 \cdot 10^{-5}$ M. Fluorescence intensities derived from spectra similar to the ones shown in Fig. 1.

tric constant for the environment of the fluorophore moiety in phosphatidylserine vesicles had a value of about 40. In the case of phosphatidic acid membranes (titration curve not shown) the respective value of dielectric constant amounted to about 43.

Comparison of these two values with the dielectric constants of water (about 80) and long chain hydrocarbons (about 2) [24] allows the conclusion that

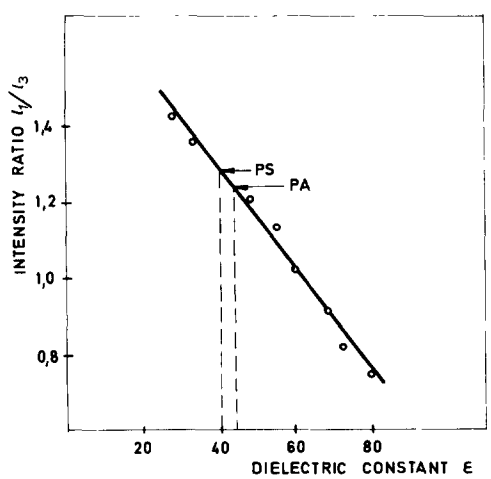


Fig. 3. The dependence of the ratio of fluorescence peak intensities I_1/I_3 of propranolol on the dielectric constant of the solvent (ethanol/water mixtures). Fluorescence intensities derived from spectra similar to the ones shown in Fig. 1. PS, phosphatidylserine; PA, phosphatidic acid.

the fluorophore moiety of propranolol is associated with polar head groups of lipids and does not penetrate deeper into hydrocarbon chains.

Titration curves such as presented in Fig. 2 are sometimes used also for calculation of the drug-membrane binding constants [25]. However, the rigorous analysis of titration curves of this type in terms of dissociation constants is limited to cases when the total number of binding sites greatly exceeds the concentration of the drug [26]. This requirement was not fulfilled in the present study, and, therefore, we limited our analysis of fluorescence spectra to identification of the propranolol intercalation region in the membrane.

Monolayer studies. The compression isotherm technique is a powerful method for studying interaction of ions, proteins and drugs with lipid films. Fig. 4 shows the surface pressure vs. area curves for monolayers of phosphatidylcholine (Fig. 4A) and phosphatidylserine (Fig. 4B), with and without addition of propranolol to the subphase. For both phospholipids propranolol shifts the pressure-area curves to the right. Such shifts indicate an increase in the area per one lipid molecule as a result of drug penetration into monolayer. The expansion of phosphatidylcholine monolayer was observed only at relatively high concentrations of propranolol ($1 \cdot 10^{-4}$ M) and was more marked at low surface pressure values. The effect of propranolol on phosphatidylserine film was much greater. The expansion of monolayer was found already at drug concentration of $5 \cdot 10^{-6}$ M, and at higher concentrations of propranolol this effect became very pronounced. The marked expansion of phosphatidylserine film persisted also at high values of surface pressure, indicating that propranolol is not squeezed out from the monolayer upon film compression. The persistance of phospholipid film expansion at high values of surface pressure is usually considered to be indicative of relatively strong interaction between lipids and the perturber added to the subphase [27]. In the case of weaker interaction the penetrant could be expelled from the area

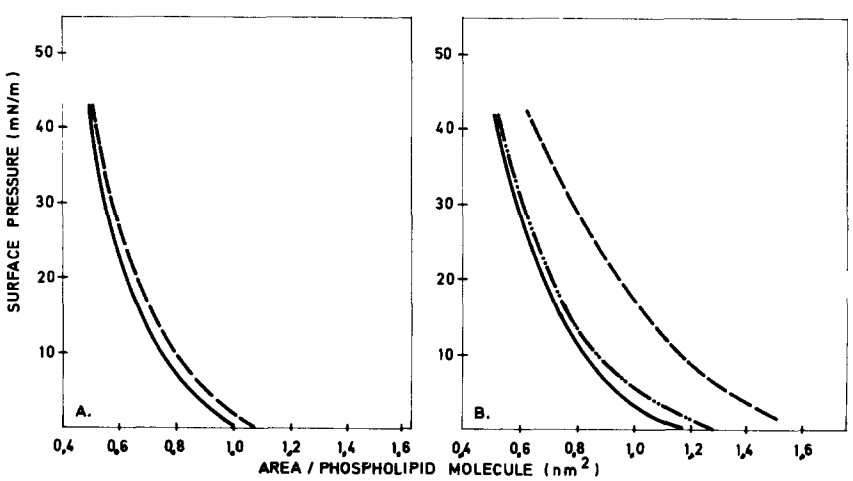
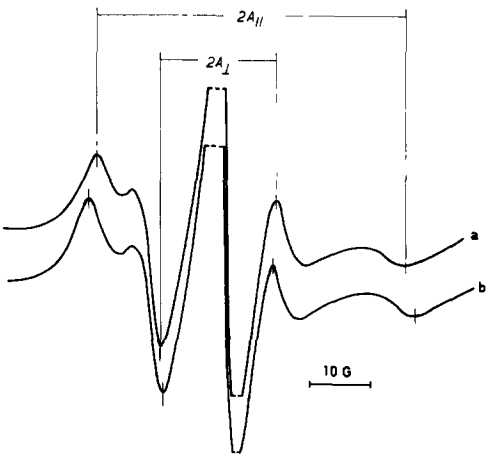


Fig. 4. The effect of propranolol on surface pressure-area curves of phospholipid films: (A) phosphatidylcholine monolayers; ———, no drug; - - - - -, $1 \cdot 10^{-4}$ M propranolol. (B) phosphatidylserine monolayers. ———, no drug; — · · —, $5 \cdot 10^{-6}$ M propranolol; - - - - -, $5 \cdot 10^{-5}$ M propranolol.



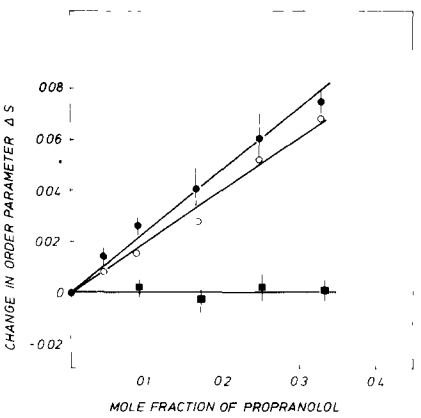


Fig. 5. ESR spectra of 5-doxylstearic acid spin probe incorporated into phosphatidic acid liposomes: (a) phosphatidic acid alone; (b) phosphatidic acid/propranolol (3 : 1 molar ratio).

Fig. 6. The effect of propranolol on the order parameter change of 5-doxylstearic acid spin-labeled liposomes prepared from phosphatidylcholine (■), phosphatidic acid (●) and phosphatidylserine (○). Propranolol mole fraction was calculated as propranolol/(phospholipid + propranolol). Each point represents the mean of four experiments. Vertical bars indicate standard deviations. The mean order parameters for control liposomes were 0.640 ± 0.006, 0.602 ± 0.005 and 0.612 ± 0.006 for phosphatidylcholine, phosphatidic acid and phosphatidylserine, respectively.

determining position at higher values of surface pressure.

ESR studies. ESR spectra of 5-doxylstearic acid spin probe incorporated into phosphatidic acid, phosphatidylserine and phosphatidylcholine liposomes are qualitatively very similar. They reflect the relatively high degree of order in the nitroxide reporter group intercalation regions of the bilayers. As a representative, the spectrum of spin-labeled phosphatidic acid multibilayers is presented in Fig. 5 (trace a). In the presence of propranolol in phosphatidic acid liposomes (trace b) the hyperfine splitting $2A_{\parallel}$ (being equal to the distance between outermost peaks) increases, whereas the hyperfine splitting $2A_{\perp}$ decreases. Qualitatively similar spectral changes were observed also upon addition of propranolol to phosphatidylserine bilayers, but not in the case of phosphatidylcholine liposomes. Spectral changes of this type may be characterized quantitatively using the order parameter, S, calculated according to the equation [28]:

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \frac{a}{a'}$$

where $(A_{xx}, A_{yy}, A_{zz}) = (5.9, 5.4, 32.9)$ G [29] are the components of hyperfine splitting tensor obtained by incorporating nitroxide derivatives into host crystals, $a = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$ and $a' = \frac{1}{3}(A_{\parallel} + 2A_{\perp})$.

Fig. 6 shows the effect of propranolol on the order parameter of phospholipid membranes prepared from different phospholipids. The order parameter of spin-labeled acidic phospholipid liposomes increases with increasing concen-

tration of propranolol. Moreover, it is interesting to note that the order parameter change vs. propranolol concentration curves do not deviate from linearity even at membrane concentrations of propranolol of one drug molecules per two phospholipid molecules. It indicates that even at such a high concentration the bilayer region is not fully saturated with the drug. The increase in order parameter is relatively very marked. In order to compare the ordering potency of propranolol with other membrane perturbers, slopes of the order parameter change vs. propranolol mole fraction plots were calculated. They amounted to 0.23 and 0.19 for phosphatidic acid and phosphatidylserine liposomes, respectively. Comparison of these two values with the value of 0.27 obtained for cholesterol in mixed phosphatidylcholine/phosphatidic acid multibilayers [30] shows that on the mole basis the effect of propranolol is not very much smaller than the effect of such a potent membrane orderer as cholesterol.

Propranolol has no effect on the order of neutral phosphatidylcholine membranes. The unchanged order parameter could indicate that the partitioning of the drug into bilayer is insufficient to produce any detectable changes and/or that propranolol, even when present in phosphatidylcholine membrane, is able neither to order nor to disorder its structure.

Data shown in Fig. 6 were obtained at relatively high ionic strength (100 mM NaCl). It should be noted, however, that the ordering effect of propranolol on acidic phospholipid membranes was observed also at lower and higher NaCl concentrations.

In spin-labeling experiments, because of technical reasons, much higher concentrations of propranolol were used (up to 14 mM) than in partition coefficient, monolayer and fluorescence spectroscopy studies. Therefore, a question may arise whether also in this case propranolol is incorporated into lipid membranes in monomeric form or whether it penetrates the bilayer in micellar form. This last possibility should be, however, excluded since the concentrations of the drug used are still far below the critical micelle concentration which amounts to about 100 mM [31].

Discussion

Results obtained in this study clearly demonstrate preferential interaction of propranolol with acidic phospholipids. Similar preference has been previously reported for other cationic drugs possesing local anesthetic activity [20,32]. Measurements of partition coefficients show that the concentration of the drug in acidic lipid membranes may reach very high values. For example, exposure of phosphatidylserine membranes (0.1 mg lipid/ml) to an aqueous concentration of $1 \cdot 10^{-4}$ M propranolol leads to membrane concentration of the drug of almost 0.4 molal which corresponds to approximately one propranolol molecule per three phosphatidylserine molecules.

The structure of biological membranes is much more complex than that of simple systems used in our study and, therefore, it is difficult to predict precisely the situation in biological membranes relying on the results of model experiments. Nevertheless, our experimental data suggest that the possibility of even those very high local concentrations of propranolol in domains rich

in acidic lipids has to be considered when interpreting the mechanisms of drug-induced functional perturbations in biological membranes. In model membranes acidic phospholipid domains have been shown to be associated with divalent cations and basic groups of proteins [33—38]. Perturbation of such domains in biological membranes could trigger a sequence of whole-membrane alterations, including changes in the structure and functioning of certain integral and peripheral proteins.

It is interesting to note that in the present study the propranolol-induced increase in the order parameter of acidic phospholipid bilayers was accompanied by monolayer expansion. Such behaviour might be explained assuming that the penetration of the drug leads to increased average separation between phospholipid molecules (monolayer expansion), but in regions where the lipid molecules are in direct contact with propranolol the degree of order of the bilayer increases as a result of strong interactions. Although propranolol interacts preferentially with polar head groups of acidic phospholipids and does not penetrate deeply into hydrocarbon chains it seems that both electrostatic and hydrophobic effects are involved in drug-membrane interaction. In the case of the purely electrostatic nature of the interaction, reduction of the negative charge of acidic phospholipids head groups would be the major effect (drug is positively charged at experimental pH). This would result in reduction of repulsive forces between phospholipids, and, consequently, in condensation of the monolayer.

Spin-labeling experiments demonstrated a very profound ordering effect of propranolol on acidic phospholipid membranes. Although here relatively very high and pharmacologically irrelevant concentrations of the drug (1—14 mM) were used we feel that these results may be also of some pharmacological importance. In spin-labeling studies, because of technical reasons, relatively concentrated lipid suspensions (15—20 mg phospholipid/ml suspension) were used. It is obvious that in this case pharmacologically relevant concentrations of propranolol would lead to much lower concentrations of the drug in bilayer region than may be reached when the drug is incubated with lipid suspensions of lower concentration (see Table I). Therefore, in model studies the membrane concentration of the drug appears to be more representative than its aqueous concentration and under certain conditions application of even very high total suspension concentrations of the drug may be justified.

The effect of other small lipophilic molecules of pharmacological importance on the degree of order and fluidity of phospholipid bilayers has been extensively studied in the last years. Spin-labeling experiments have shown that local anesthetics may increase or decrease the degree of molecular order in phospholipid membranes, depending upon lipid composition, anesthetic concentration and solution pH [30,39,40]. The presence of cholesterol in the membrane appears to be of special importance since it has been demonstrated that some drugs may exert disordering or ordering effects on phospholipid bilayers, depending on their cholesterol content [30]. The present study gives additional evidence that in the absence of cholesterol the addition of anesthetic-like molecules may result in ordering effects on phospholipid bilayers. It should be noted here, however, that when the effect of propranolol on the organization of erythrocyte membrane was studied, a small decrease in the

order parameter of membrane-incorporated spin probe was observed (Surewicz, W. and Karvaly, B., unpublished results).

Previous studies on the effect of local anesthetics on synthetic phosphatidylcholine membranes showed that propranolol is able to depress the phase-transition temperature from gel to liquid crystalline state [16,17]. This phenomenon was referred to as fluidizing or disordering effect of the drug. The results of the present study, performed at a temperature above the phase-transition of lipids used, demonstrate that a decrease in phase transition temperature does not imply further disordering of phospholipid membranes when they are already in liquid crystalline state. Below and above the transition temperature the effect of the drug on membrane order and fluidity may be even opposite, and, therefore, great caution should be exercised when extrapolating results of phase-transition experiments of synthetic phospholipids to events at biomembranes.

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