Biochimica et Biophysica Acta 820 (1985) 157–164
Elsevier

BBA 72752

ESR spectral changes induced by chlorpromazine in spin-labeled erythrocyte ghost membranes

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(Received June 3rd, 1985)

Key words: ESR; Spin label; Lipid-protein interaction; Chlorpromazine; (Erythrocyte membrane)

chlorpromazine interacted preferentially with membrane proteins rather than membrane lipids in the initial incorporation into human erythrocyte ghosts, as demonstrated by means of the fluorescence quenching and a maleimide spin label. In this state the membrane fluidity increased. At higher concentrations of chlorpromazine, the membrane fluidity decreased and a motionally restricted signal from fatty acid spin labels appeared predominantly. However, no such signal appeared in protein-free vesicles. The temperature and pH dependences of the outer hyperfine splitting of this restricted signal were very similar to those of bovine serum albumin. On the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chlorpromazine-treated and -untreated ghosts, it was found that there was no significant difference in membrane proteins between both samples except for the changes of a few bands which were not directly concerned with the occurrence of this restricted signal. These results suggest that the fatty acid spin labels bind preferably to membrane proteins as the lipid domain becomes packed with chlorpromazine.

Introduction

Tranquilizers and anesthetics are amphiphilic drugs and surface-active compounds. These drugs at a low concentration cause a stabilization of many types of biomembranes against hypotonic and mechanical hemolysis, whereas at a high concentration they cause hemolysis [1,2]. To examine such a biphasic effect, extensive studies have been carried out on the interaction of various drugs with biological membranes [3–5]. In particular, the interactions of chlorpromazine with erythrocyte membranes are very interesting because of the possibility of chlorpromazine transportation to the brain by means of erythrocytes. Seeman and Weinstein demonstrated the relation of hemolysis and K^+ release [1]. One possibility considered was that the antihemolytic effect by chlorpromazine was affected by the interaction of the drug with sialic acid moieties of glycoproteins [6]. Lieber et al. [7] reported that chlorpromazine induced holes with a diameter of approximately 14 Å in erythrocyte membranes. Salesse et al. [8] have demonstrated that the membrane fluidity of pigeon erythrocytes changes multiphasically, depending on the concentration of chlorpromazine and is largely correlated with adenylate cyclase activity. All these studies were performed at relatively low concentrations of chlorpromazine. In general, surface-active compounds form the ordered aggregates known as micelles. Ogiso et al. [9] have demonstrated that the critical micelle concentration of chlorpromazine is 0.3 mM in isotonic

Abbreviations: 5-nitroxide stearic acid, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl; 12-nitroxide stearic acid, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl; 16-nitroxide stearic acid, 2-(14-carboxyltetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl; TEMPO, 2,2,6,6-tetramethylpiperidinooxyl.

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solution at 25°C. Above the critical micelle concentration, it is expected that the size and shape of micelles change greatly. Therefore, the interaction of biomembranes with the drugs above the critical micelle concentration is very interesting from a physicochemical point of view, although such a high concentration of the drugs is not used physiologically.

Spin-label techniques have been widely used to study the changes in membrane lipids and proteins induced by drug-membrane interactions. In chlorpromazine-treated erythrocytes, the results of lipid spin label show the increase in membrane fluidity [10]. On the other hand, in membrane protein spin labeling, two components appear, i.e., the strongly immobilized component and the weakly immobilized one [11,12]. The ratio of the ESR signal amplitude in both components is significantly changed by the chlorpromazine treatment of spin-labeled ghosts. Holmes and Piette [11] regarded the changes as the conformational change of membrane proteins. Recently, Benga et al. [12] proposed that the weakly immobilized signal is specifically reduced. When the fatty acid spin labels are incorporated into biomembranes, their ESR spectra reveal the motionally restricted lipid component in addition to the fluid lipid one. In protein-free vesicles, only the fluid lipid component is observed. Therefore, it is thought that the motionally restricted lipid component usually reflects the lipid-protein interactions [13,14]. Such restricted lipid signals have been obviously observed in photobleached rod outer segment disc membranes [15,16] and H_2O_2-treated erythrocytes [17]. However, each signal comes from a quite different origin.

In this paper, the interaction of chlorpromazine with human erythrocyte ghosts was studied in the drug concentration range of 1–50 mM. We describe the properties of the motionally restricted signal observed using fatty acid spin labels and propose the mechanism of the appearance of such a signal.

Materials and Methods

Materials. Three kinds of fatty acid spin labels, 2-(3-carboxypropyl)-2-tridecyl-, 2-(10-carboxydecyl)-2-hexyl- and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as 5-nitroxide stearic acid, 12-nitroxide stearic acid and 16-nitroxide stearic acid, respectively), a cholestane spin label, 4′,4′-dimethylspiro-(5α-cholestane-3,2′-oxazolidin)-3′-yloxyl, a maleimide spin label, 4-maleimide-2,2,6,6-tetramethylpiperidinooxyl and 2,2,6,6-tetramethylpiperidinooxyl (TEMPO) were purchased from Syva Co. chlorpromazine hydrochloride, dibucaine hydrochloride, tetracaine hydrochloride and dipalmitoylphosphatidylcholine were obtained from Sigma Chemical Co. Other chemicals were of analytical grade.

Chlorpromazine treatment of erythrocyte ghosts and their lipid vesicles. Erythrocyte ghosts were prepared according to Dodge et al. [18]. The preparation of lipid vesicles was carried out as described previously [19]. chlorpromazine solutions were prepared using 0.15 M NaCl/10 mM sodium phosphate (phosphate-buffered saline, pH 7.3). Each 50 μl of ghosts or vesicles was added to 1 ml of chlorpromazine solution preincubated at 0 or 37°C. After the incubation, the suspension was immediately centrifuged for 10 min at 2000 rpm for ghosts or for 20 min at 3000 rpm for vesicles. The samples were washed twice with 1 ml of phosphate-buffered saline.

Spin labeling. To label the membranes with a fatty acid spin label, 50 μl of washed cells were suspended in 5 ml of phosphate-buffered saline and the suspension was mixed with 5 μl of label reagent (10^{-2} M) dissolved in ethanol. This suspension was incubated for 10 min at 37°C. After being washed once with the buffer, labeled ghosts were packed into a hematocrit capillary tube. Cholestane spin-labeled ghosts were prepared by the incubation with the spin-labeled vesicles in a shaking water-bath at 37°C up to 24 h, according to the method of Cooper et al. [20]. The labeling of membrane proteins with a maleimide spin label was carried out as previously described [21].

pH dependence. The effect of pH on membrane fluidity of chlorpromazine-treated ghost was examined in the following buffers: 0.15 M NaCl/10 mM sodium acetate (pH 3.5), 0.15 M NaCl/10 mM sodium phosphate (pH 7.3), 0.15 M NaCl/10 mM Tris-HCl (pH 9.1). In each buffer, the chlorpromazine-treated ghosts were incubated for 30 min at 0°C and then spin-labeled.

Electrophoresis. Sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis of membrane proteins and the nomenclature of the polypeptide bands were performed according to Fairbanks et al. [22].

ESR measurement. ESR spectra were recorded on a JEOL JES FE-1X spectrometer (9.2 GHz) equipped with a variable temperature accessory. The usual spectrometer settings were 100 kHz modulation amplitude, 1 gauss; microwave power, 20 mW; scan range, 200 G; scan speed, 8 min. Unless stated otherwise, ESR measurement was carried out at 23°C.

Fluorescence measurement. The fluorescence quenching of membrane proteins by chlorpromazine was measured with a Japan Spectroscopy Company FP-550A spectrometer with excitation at 285 nm (slit width 3 nm) and emission at 327 nm.

Results

Fig. 1 shows the mobility of TEMPO added into chlorpromazine solution (0.1–50 mM). At 37°C, chlorpromazine solution became largely turbid in the concentration range of 1–10 mM and gradually transparent at higher concentrations. The rotational correlation time (τ_c) [23] of TEMPO remained almost constant in the range of 0.1–10 mM chlorpromazine but at higher concentrations it increased drastically. On the other hand, no such a clear-cut change in the τ_c was observed at 3°C.

Fig. 2 reveals the ESR spectra of three kinds of fatty acid spin labels, 5-, 12- and 16-nitroxide stearic acids, and a cholestane spin label incorporated into chlorpromazine-treated ghosts. In fatty acid spin labels, the ESR spectra were changed drastically by chlorpromazine, i.e., in addition to the fluid lipid component, the motionally restricted lipid component appeared clearly with increasing chlorpromazine concentration. Such a restricted component was also observed using local anesthetics such as dibucaine (50 mM) and tetracaine (50 mM). Of the fatty acid spin labels used, the ESR spectra changed dramatically in 12-nitroxide stearic acid. Unless stated otherwise, 12-nitroxide stearic acid was used to examine the properties of the motionally restricted lipid component. On the other hand, the ESR spectra of cholestance spin-labeled ghosts did not show such a restricted signal as that induced by chlorpromazine. The observed spectrum was similar to that in erythrocyte lipid vesicles treated with 50 mM chlorpromazine.

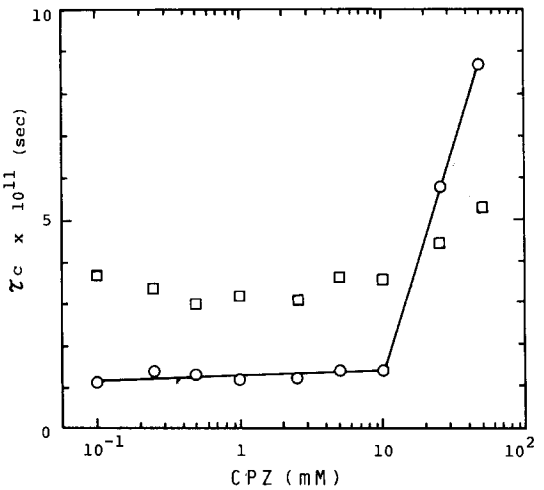

Fig. 1. Effect of chlorpromazine (CPZ) concentration on the mobility of TEMPO. chlorpromazine solutions were prepared using phosphate-buffered saline (pH 7.3). The ESR spectra of TEMPO in chlorpromazine solution were measured at 3°C (□) and 37°C (○). The rotational correlation time (τ_c) was obtained by the method of Stone et al. [23].

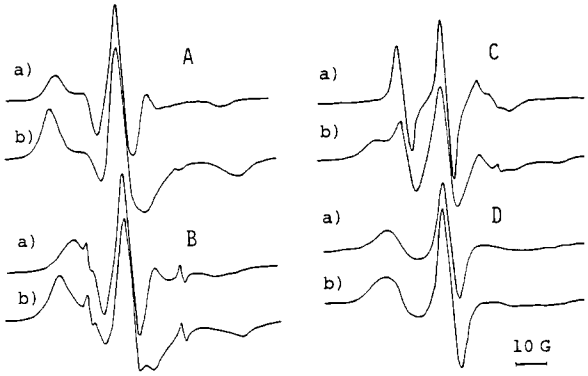

Fig. 2. ESR spectra of fatty acid spin labels and cholestane spin label incorporated into ghosts incubated for 30 min at 0°C without chlorpromazine (a) and with 50 mM chlorpromazine (b). For fatty acid spin labels, i.e., 5-nitroxide stearic acid (A), 12-nitroxide stearic acid (B) and 16-nitroxide stearic acid (C), ghosts were treated with chlorpromazine and then spin-labeled. In cholestane spin label (D), labeled ghosts were treated with chlorpromazine. ESR spectra were measured at 23°C.

The effect of chlorpromazine concentration on the ESR spectra of 12-nitroxide stearic acid in ghosts was studied in the range of 1–50 mM at incubation temperatures 0 and 37°C. At 0°C, with up to 4 mM chlorpromazine, the ESR spectra were almost the same as that of control ghosts. However, at higher concentrations of chlorpromazine the motionally restricted lipid component appeared gradually. On the other hand, at 37°C, the ESR spectra changed slightly with up to 10 mM chlorpromazine. At higher concentrations, the motionally restricted component appeared suddenly (Fig. 3).

The temperature and pH dependences of the outer hyperfine splitting ($T_{\parallel}$) of the motionally restricted lipid component in chlorpromazine-treated ghosts were studied in the ranges of 4–47°C and pH 3.5–9.1, respectively. In this temperature range, the values of $T_{\parallel}$ changed from 31.5 G to 30.1 G. On the other hand, over the entire pH range, the $T_{\parallel}$ values remained constant (31.1 G).

Fig. 4 shows the chlorpromazine concentration dependence of the fluid lipid component from 12-nitroxide stearic acid in ghosts treated with the drug at 0 or 37°C. At 0°C, the values of $T_{\parallel}$ decreased with increasing chlorpromazine concentration up to 4 mM ($T_{\parallel} = 22.7$ G) and increased to 23.9 G at 5 mM chlorpromazine. At higher concentrations, the $T_{\parallel}$ value could not be obtained because of the superposition of the motionally restricted lipid component. In the case of 37°C, the values of $T_{\parallel}$ decreased gradually with up to 10 mM chlorpromazine. At higher concentrations, only the motionally restricted lipid component appeared.

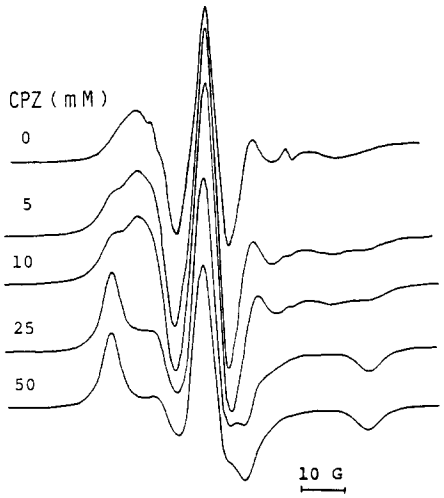

Fig. 3. Effect of chlorpromazine (CPZ) concentration on the appearance of the motionally restricted component from 12-nitroxide stearic acid. Ghosts were incubated in the chlorpromazine solution for 30 min at 37°C. Samples were washed with phosphate-buffered saline and spin-labeled.

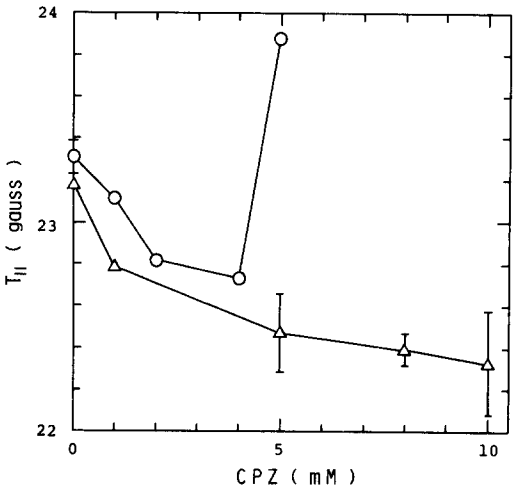

Fig. 4. Effect of chlorpromazine (CPZ) concentration on the outer hyperfine splitting ($T_{\parallel}$) of the fluid lipid component in ghosts. Ghosts treated with chlorpromazine for 30 min at 0°C (○) or 37°C (△) were spin-labeled with 12-nitroxide stearic acid. At higher concentrations of chlorpromazine, no $T_{\parallel}$ value was obtained because of superposition of the motionally restricted lipid component.

To examine the attribution of membrane proteins to the motionally restricted lipid component, erythrocyte lipid vesicles were treated with 1–50 mM chlorpromazine for 30 min at 0°C. However, no such restricted component appeared. The bilayer fluidity decreased linearly up to 10 mM and at higher concentrations remained almost constant ($T_{\parallel} = 23.7$ G).

Fig. 5 displays SDS-polyacrylamide gel electrophoretic pattern of ghosts incubated with chlorpromazine for 30 min at 37°C. The phoretic patterns were mainly characterized by a high molecular weight polymer on top of the gel and the diminution of band 6. Furthermore, the diffuse, nondiscrete bands mainly appeared between spectrin and the top of the gel in 10, 25 and 50 mM chlorpromazine-treated ghosts. However, there was no distinct difference among these three lanes. The

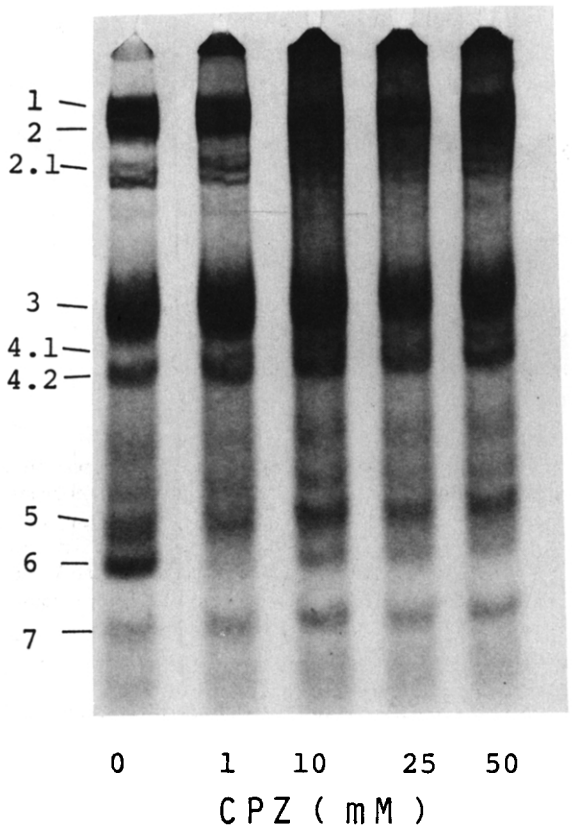

Fig. 5. SDS-polyacrylamide gel electrophoresis of chlorpromazine-treated ghosts. Ghosts were incubated for 30 min at 37°C in each chlorpromazine (CPZ) solution.

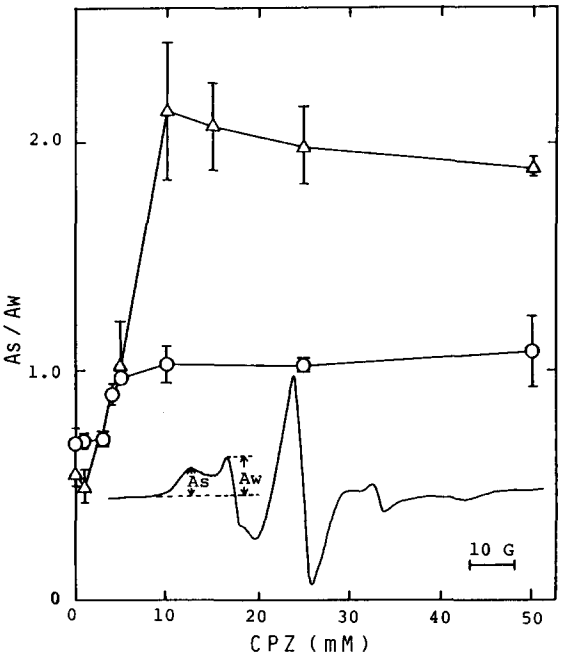

Fig. 6. Effect of chlorpromazine (CPZ) concentration on the A_s/A_w ratio of the ESR signal in maleimide spin-labeled ghosts. Maleimide spin-labeled ghosts were incubated in chlorpromazine solutions for 30 min at 0°C (○) or 37°C (△). Samples were washed with phosphate-buffered saline as for stearic acid spin label and packed into capillary tube. 'A_s' and 'A_w' were defined as indicated in the ESR spectrum. Each point represents the average of at least two experiments. Standard deviations are shown by vertical bars.

electrophoretic patterns of ghosts treated at 0°C were the same as those at 37°C (data not shown).

The effect of chlorpromazine concentration on the ESR spectra of membrane proteins labeled with a maleimide spin label was investigated at incubation temperatures of 0 and 37°C. The ESR spectrum of unperturbed ghosts reveals two components, i.e., the weakly immobilized component (w) and the strongly immobilized one (s). The signal intensity of the weakly immobilized component decreased drastically with increasing chlorpromazine concentration. The ratio, A_s/A_w, of the amplitude from the horizontal base line to each peak at the low field of both components was plotted against chlorpromazine concentration (Fig. 6). The values of A_s/A_w at 0 and 37°C increased linearly up to 4 and 10 mM, respectively, and remained almost constant at higher concentrations.

The reactivity of chlorpromazine with the sulfhydryl groups of membrane proteins was examined by labeling 50 mM chlorpromazine-treated ghosts with a maleimide spin label. The signal intensity and the A_s/A_w value of the ESR spectrum were almost the same as those obtained by the chlorpromazine treatment of spin-labeled ghosts.

In order to explain the spectral change by chlorpromazine of maleimide spin-labeled ghosts, the following experiments were performed. First, 10 mM chlorpromazine-treated ghosts, in which the weakly immobilized component diminished, were exposed to 10 mM $K_3Fe(CN)_6$ solution for 10 min at 37°C. No spectral change occurred. On the other hand, when chlorpromazine (10 mM)

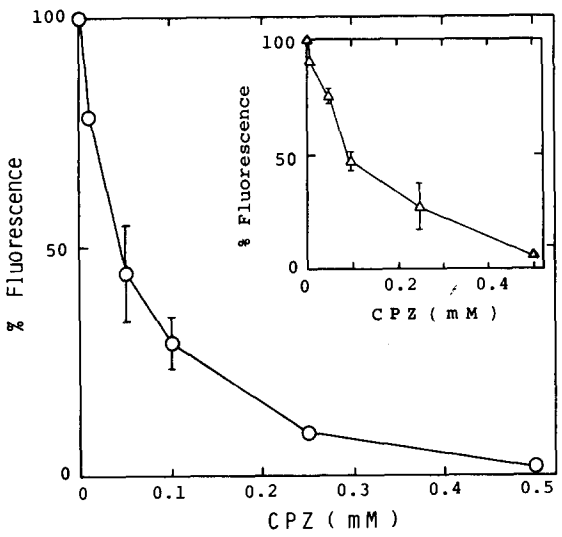

Fig. 7. Effect of chlorpromazine (CPZ) concentration on fluorescence quenching. Chlorpromazine was added to the ghost suspension. The suspension was incubated for 30 min at 37°C. Inset shows the fluorescence of aqueous tryptophan solution ($2.5 \cdot 10^{-5}$ M) treated with chlorpromazine for 30 min at 37°C. Each point represents the average of at least two experiments. Standard deviations are shown by vertical bars.

-treated ghosts were incubated in 5 mM ascorbate solution for 30 min at 37°C, their ESR signals disappeared completely. However, the ESR signals reappeared by incubating this sample in 10 mM $K_3Fe(CN)_6$ solution for 10 min at 37°C. Secondly, the signal intensity of TEMPO (0.25 mM) in the suspension containing 50 μl ghosts in 0.5 ml buffer was unchanged by the addition of chlorpromazine (final concentration 25 mM). Thirdly, the decreased signal intensity of the weakly immobilized component increased by washing this sample with the buffer.

Fig. 7 shows the interaction of chlorpromazine with membrane proteins on the basis of the measurement of the fluorescence quenching. The fluorescence from membrane proteins, perhaps tryptophan, was significantly quenched by chlorpromazine.

Discussion

Tranquilizers such as chlorpromazine are amphiphilic molecules and show surfactant-like behavior. The ESR signal of TEMPO in surfactant solution can provide information on the aggregation of the surfactant molecules. Considering that the critical micelle concentration of chlorpromazine is 0.3 mM in isotonic solution at 25°C [9], a remarked increase in τ_c values above 10 mM chlorpromazine may reflect the change in the size and/or shape of the micelles, due to the increase of the solubility.

Recently, Benga et al. [12] have claimed that the changes in ESR spectra of melimide spin-labeled ghosts by chlorpromazine are radical reductions, in contrast with the conformational changes of membrane proteins proposed by Holmes and Piette [11]. The spectral changes in our case are discussed as follows. (a) For the oxidation of reduced nitroxide radicals, $K_3Fe(CN)_6$ is widely utilized [24]. The ESR signal in chlorpromazine-treated ghosts disappeared with ascorbate but reappeared with $K_3Fe(CN)_6$, indicating that the activity of $K_3Fe(CN)_6$ exists even in the presence of chlorpromazine. The ESR spectra of the chlorpromazine-treated sample did not change with the addition of $K_3Fe(CN)_6$. (b) If the weakly immobilized component is specifically reduced by chlorpromazine, as pointed out by Benga et al. [12], the different ESR spectra should be obtained with the order of chlorpromazine treatment and spin labeling. However, the ESR spectrum was unaffected by the order. (c) It seems likely that chlorpromazine is localized at the interface between membrane lipids and membrane proteins [5]. The ESR spectrum of TEMPO in vesicle suspensions demonstrates two components [25]: one component is due to the radical in bulk and the other to that in vesicles. That is, TEMPO is soluble in both polar and apolar solvents. It is expected that TEMPO in suspensions of ghosts can approach the interface between the phospholipids and the proteins of membranes. So, if the radicals are reduced, a similar change for TEMPO in the ghost suspension should occur. The ESR signal intensity of TEMPO did not change with chlorpromazine. (d) Chlorpromazine loaded in ghosts was released from their membranes by washing with the buffer so that the ESR spectrum approached that of chlorpromazine-untreated sample. From these results, it is considered that the ESR spectral changes observed in the present work

are not radical reduction but the conformational change of membrane proteins.

Bilayer fluidity is mediated by the conformational change of membrane proteins as well as the chain length and the degree of unsaturation of lipids. When erythrocyte ghosts are incubated in the medium of low pH (pH 3.0–5.5), the folding of membrane proteins occurs and the membrane fluidity increases, as demonstrated by the spin label method [26]. The increased bilayer fluidity may be partially explained in terms of the increment of the internal membrane volume of lipids due to the protein folding, which decreases the volume occupied by membrane proteins [27]. The fluorescence of membrane proteins due to mainly tryptophan is not quenched by lowering of the pH [28], indicating that the fluorescence of membrane proteins is unaffected by the conformational change of membrane proteins caused by the variation in pH. On the other hand, the fluorescence of ghost membranes, as in tryptophan solution, is dramatically quenched by chlorpromazine, suggesting the interaction of chlorpromazine with membrane proteins. Thus, the spectral change in maleimide spin-labeled ghosts by chlorpromazine reveals the conformational change of membrane proteins due to interaction of chlorpromazine with membrane proteins. The A_s/A_w values increased with the increment of chlorpromazine up to 4 mM for 0°C and 10 mM for 37°C, and remained almost constant at higher concentrations. The different responses of the A_s/A_w values to incubation temperatures are attributed to the decrease of the solubility due to the precipitation of chlorpromazine. In chlorpromazine-treated ghosts, the bilayer fluidity increases in the chlorpromazine concentration range in which the A_s/A_w values increase, as demonstrated at lower pH [26], and then decreases to the $T_{\parallel}$ value observed in 50 mM chlorpromazine-treated vesicles at the chlorpromazine concentration at which the A_s/A_w values become constant. With further increment of chlorpromazine, the motionally restricted lipid component appears predominant. For chlorpromazine-treated lipid vesicles, no such a change in $T_{\parallel}$ values is observed. chlorpromazine induces the release of small but significant amounts of membrane proteins from erythrocyte membranes [5,9]. Therefore, the increase of membrane fluidity may be explained in terms of the folding and the release of membrane proteins by chlorpromazine. We propose that chlorpromazine interacts preferentially with membrane proteins rather than membrane lipids in the initial incorporation into ghost membranes and then enters into the lipid region as indicated by the decrease of the bilayer fluidity. Furthermore, the results of spin labels mentioned here indicate that the bilayer fluidity is largely mediated by membrane proteins [26].

The motionally restricted lipid component is often observed using fatty acid spin labels and phospholipid spin labels in biomembranes. No such a component appears in protein-free vesicles. It is expected that cholestane spin label as well as cholesterol is segregated from membrane proteins in erythrocyte ghost membranes [21,28]. In cholestane spin-labeled ghosts, only the fluid lipid component was observed by chlorpromazine. Therefore, such a restricted lipid component is attributed to the lipid-protein interactions [14]. In 1973, Jost et al. [13] proposed the existence of boundary lipid on the basis of the ESR spectra observed in membranous cytochrome oxidase with various phospholipid/protein ratios. The temperature dependence of the ESR signal due to boundary lipids indicates that the lipids in the first shell are exchangeable with other shell lipids [29]. Recently, Watts et al. [15,16] demonstrated that the motionally restricted lipid component observed by the extensive bleaching or delipidation of rod outer segment disc membranes is due to the lipid trapping within aggregated proteins. The ESR signal in oxidant-treated erythrocytes has quite a different origin compared with these two. On the basis of both the temperature dependence of the ESR signal and SDS-polyacrylamide gel electrophoresis of membrane proteins, we have proposed that the ESR signal in the oxidant-treated erythrocytes reflects the interaction of the lipids with the cross-linked products of membrane proteins [17]. In the present work, the properties of the motionally restricted lipid component which appeared in ghosts by using chlorpromazine have been examined. The electrophoretic patterns of ghosts treated with chlorpromazine were essentially different from those of H_2O_2-treated erythrocytes. The results of a maleimide spin label and the electrophoresis of ghosts treated with 10 mM

chlorpromazine were very similar to those with 50 mM chlorpromazine, whereas the ESR spectra of fatty acid spin label in both samples were significantly different. After the perturbation of membrane proteins by chlorpromazine, the motionally restricted lipid component is observed to be predominant. The temperature and pH dependences of this component are similar to those observed in bovine serum albumin [17].

From the results of a maleimide spin label and the fluorescence quenching, chlorpromazine interacts preferentially with membrane proteins at low concentrations of the drug. The folding of membrane proteins and their release from membranes occur so that the membrane fluidity increases. As the concentration of chlorpromazine increases, the lipid domain of membranes is packed with the drug. In such a membrane state, fatty acid spin labels bind preferably to membrane proteins than the rigid lipid domains. Thus, the motionally restricted lipid component appears. A similar mechanism may also explain the properties of the ESR signal which appeared with local anesthetics such as tetracaine and dibucaine.

Acknowledgement

This work was partly supported by a grant from the Central Research Institute of Fukuoka University.

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