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Involvement of Erythrocyte Skeletal Proteins in the Modulation of Membrane Fluidity by Phenothiazines†

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Received December 24, 1986; Revised Manuscript Received June 16, 1987

ABSTRACT: The effects of phenothiazines (chlorpromazine, chlorpromazine sulfoxide, and trifluoperazine) and antimitotic drugs (colchicine and vinblastine) on the erythrocyte membrane have been investigated. Chlorpromazine and trifluoperazine induced a dose-dependent increase in the freedom of motion of stearic acid spin-labels bound to both intact erythrocytes and ghosts, but did not affect the freedom of motion of stearic acids bound to vesicles depleted of spectrin and actin or of ghosts resealed with anti-spectrin antibodies. Further, chlorpromazine and trifluoperazine were able to eliminate a protein 4.1 dependent membrane thermal transition detected by stearic acid spin-labels at 8.5 ± 1.5 °C. Antimitotic drugs and chlorpromazine sulfoxide did not change either the freedom of motion of stearic acid spin-labels or the 8.5 °C membrane thermal transition. Results indicate the involvement of skeletal proteins as possible membrane target sites of biologically active phenothiazines and suggest that the control of stearic acid spin-label freedom of motion is mediated by the spectrin–actin network and the proteins that link the skeletal network to the membrane.

The interaction of phenothiazines with the erythrocyte membrane has received particular attention in view of its role in phenothiazine transportation to and release from the brain by means of erythrocytes. At micromolar concentrations phenothiazines stabilized the cell against hypotonic hemolysis (Seeman & Weinstein, 1966), whereas above 0.3 mM they produced holes in the membrane (Lieber et al., 1984) and promoted cell–cell fusion (Lang et al., 1984). Results obtained with ESR[1] spectroscopy of erythrocytes treated with sublytic concentrations of phenothiazines showed an increase in the motional freedom of stearic acid spin-labels inserted into the membrane, which was interpreted as an increase in membrane fluidity (Suda et al., 1981).

Phenothiazines at sublytic concentrations imposed inward curvature (invagination or cupping) of red cell membranes (Deuticke, 1968), and Sheetz and Singer (1974) suggested that this effect may be due to preferential partitioning of the drug (and hence expansion) into the negatively charged cytoplasmic leaflet of the bilayer. Results obtained by Franks and Lieb (1981) suggested that a specific target site, rather than a general perturbation of membrane proteins (Seeman, 1972), was responsible for membrane expansion induced by anesthetic molecules. Consistent with the idea of a specific protein target, Nelson et al. (1983) found that molecules with cup-forming and antihemolytic activities were also calmodulin inhibitors.

† This research was partially supported by NATO Research Grant 236/84 and by a grant of the Italian National Research Council, Special Project "Oncology", Contract No. 86.00699.44.
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[1] Abbreviations: ESR, electron spin resonance; 12-NS, 2-(3-carboxypropyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyl-1-oxy; CPZ, chlorpromazine hydrochloride; TFP, trifluoperazine dihydrochloride; CPZ-SO, chlorpromazine sulfoxide hydrochloride; CC, colchicine; VB, vinblastine; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; Ig, immunoglobulin.

0006-2960/87/0426-8133$01.50/0

The anti-calmodulin activity of phenothiazines was shared with several drugs including vinblastine, a mitotic inhibitor and microtubule disrupting agent. Also, other studies reported that phenothiazines, in the micromolar concentration range, may have biological effects similar to those of antimitotic drugs (Poffenbarger & Fuller, 1977; Ryan et al., 1974; De Petris, 1974; Raizada & Fellows, 1979). In principle these similar biological effects may be due to ubiquitous protein target sites present in erythroid as well as nonerythroid cells.

These observations suggested a comparative study on the effects of chlorpromazine (CPZ), chlorpromazine sulfoxide (CPZ-SO), trifluoperazine (TFP), vinblastine (VB), and colchicine (CC) on membrane fluidity and membrane thermal transitions of the human erythrocyte. We found that biologically active phenothiazines (CPZ and TFP) produced rather specific membrane modifications in comparison to both chlorpromazine sulfoxide and the antimitotic drugs. Our findings suggest the involvement of skeletal proteins as possible membrane target sites of biologically active phenothiazines.

Materials and Methods

Materials. Stearic acid spin-labels were obtained from Syva (Palo Alto, CA). DFP and CC were obtained from Fluka (Buchs, Switzerland) and PMSF, CPZ, and TFP from Sigma (St. Louis, MO). VB was obtained from Eli Lilly & Co. (Indianapolis, IN), and CPZ-SO was generously given by Smith Kline & French Laboratories (Philadelphia, PA).

Preparation of Membranes, Extraction of Skeletal Proteins, and Drug Treatment. Ghosts were prepared from fresh human erythrocytes with 5 mM sodium phosphate, pH 8, at 0 °C. Inside-out vesicles were prepared by extraction of ghosts at 37 °C for 30 min with 0.2 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM PMSF, and 0.2 mM DFP, pH 9.4 (Morrow & Marchesi, 1981). Samples of 0.25 mL of packed cells or membranes were incubated for 1 h at 37 °C with 10 mL of 150 mM NaCl and 5 mM sodium phosphate, pH 8, buffer containing the indicated amount of drug. To avoid proteolytic degradation, 1 mM DFP, 0.1 mM PMSF, and 1 μg/mL leupeptin were added to ghosts and inside-out vesicles.

Reaction with Antibodies. Rabbit polyclonal affinity-purified anti-spectrin antibodies were obtained and purified by standard techniques. Antibodies were dissolved at 0.5 mg/mL in 5 mM sodium phosphate, pH 8, buffer and added to unsealed ghosts at 0 °C. Ghosts were resealed at 37 °C with a buffer containing 150 mM KCl, 5 mM sodium phosphate, 0.25 mM $MgCl_2$, 1 mM DFP, and 0.1 mM PMSF, pH 7.2. Membranes were checked for permeability to macromolecules after the resealing with fluorescent dextrans as previously described (Minetti & Ceccarini, 1982). The effects of these anti-spectrin antibodies on the thermal transitions of the red blood cell membrane have been described elsewhere (Minetti et al., 1986).

Spin-Labeling and Spectra Analysis. Before spin-labeling, intact erythrocytes, resealed ghosts, and inside-out vesicles were concentrated by centrifugation to 50% (v/v). Stearic acid spin-labels were dissolved in ethanol. To 3 μg of probe, evaporated under a nitrogen stream, was added an amount of membranes corresponding to 0.3 mg of lipids. At room temperature the time needed for the spin-label incorporation into membranes was very short and, after 5–10 min, the spin-label was completely associated with the membranes. Samples were centrifuged, and the packed cells were sucked into a capillary (50-μL capilette, Boehringer Mannheim GmbH, Mannheim, West Germany) and then sealed (Aszalos et al., 1985). Spectra were recorded on a Varian E-4 spectrometer (Varian Associates, Inc., Palo Alto, CA) operated at 9.5 GHz, with TE_{102} cavity resonator, 3260 G field set, 100-kHz field modulation, 1.25 G peak to peak modulation amplitude, and 18-mW microwave power. Amplification of the low- and high-field lines was obtained either by increasing the gain or by setting the modulation amplitude at 5 G without apparent changes in the $2T_{\parallel}'$ values. The instrumentation was equipped with a variable-temperature accessory, and temperature was monitored by a digital thermometer set above the cavity. After an equilibration time of 2 min, the temperature gradient measured from the top to the end of the sample did not exceed ±0.5 °C. The evaluated parameters for ESR spectrometric measurements, $2T_{\parallel}'$ and $2T_{\perp}'$, are components of the motionally averaged nitrogen hyperfine tensor from which the order parameter, S, can be calculated by the equation of Hubbell and McConnell (1971)

$$S = \frac{(T_{\parallel}' - T_{\perp}')a_{NxL}}{(T_{\parallel} - T_{\perp})_{xL}a_N'}$$

where xL refers to single-crystal parameters obtained from the date of Hubbell and McConnell (1971) and $a_N' = 1/3(T_{\parallel}' + 2T_{\perp}')$. The definitions of $2T_{\parallel}'$ and $2T_{\perp}'$ are shown in Figure 1. A correction factor of 1.6 G was used in the estimation of $T_{\perp}'$ values (Hubbell & McConnell, 1971). Due to the anisotropy of the erythrocyte membrane, the calculated order parameters are not true order parameters (Schreier et al., 1978), but the apparent order parameter as well as $2T_{\parallel}'$ measurements may be used to obtain information on the dynamic behavior of the membrane. ESR spectra of 12-NS-labeled erythrocytes showed contributions mostly from the membrane-bound spin-label with restricted motion and a small contribution from free-moving spin-label (Figure 1). Recently Gordon et al. (1985) showed that a measurable clustering of 5-NS may occur in ghosts at a spin-label/membrane lipid ratio much lower than has been previously reported (Butterfield et al., 1976; Gordon & Mobley, 1984). However, in this study the decrease in the apparent order parameter induced by CPZ and TFP on both intact cells and ghosts was mainly due to a decrease in $2T_{\parallel}'$ (see Table I and Figure 4). This spectral parameter was not affected by spin-probe concentration and clustering (Gordon & Mobley, 1984; Gordon et al., 1985). At each temperature four spectra were recorded, and standard deviations of the measurements of $2T_{\parallel}'$ and $2T_{\perp}'$ were ±0.05–0.2 G.

Results

Effects of Drugs on Stearic Acid Spin-Labeled Membranes. Stearic acid spin-label analysis of red blood cells treated with different drugs provided an evaluation of the effects of these drugs on the motional freedom of the inserted spin probe. Information on the dynamic behavior of the spin-labels was obtained by the outer hyperfine splitting, $2T_{\parallel}'$ (Figure 1), and by the percent difference of the apparent order parameter, % S (see Materials and Methods).

The ESR spectra of intact erythrocytes treated with 200 μM CPZ revealed an increase in the spin-label freedom of motion as indicated by the decrease of both $2T_{\parallel}'$ and S for all spin-labels utilized, 5-, 7-, 10-, and 12-NS (Table I). The order parameter of 200 μM CPZ-treated cells in comparison to that of untreated cells was reduced by 1.0 ± 0.2% for the 5-NS and by 7.0 ± 0.5% for the 12-NS. Figure 2 shows that % S in CPZ-treated cell increased linearly with the position of the nitroxide ring in the fatty acid chain, thus suggesting that the effects of CPZ were more evident with the increase of the flexibility gradient toward the center of the bilayer (Gaffney, 1974). With the 5-NS, changes of % S were de-

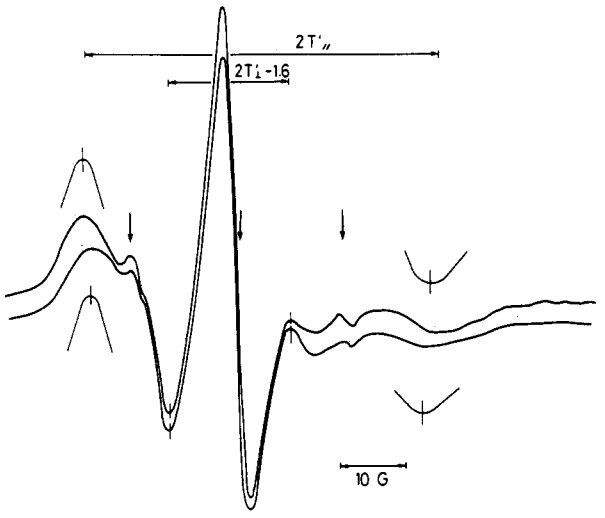

FIGURE 1: Typical ESR spectra of 12-NS-labeled intact erythrocytes at 20 °C: (upper) control erythrocytes; (lower) 100 μM CPZ treated erythrocytes. The arrows indicate the position of free-moving spin-label.

Table I: ESR Parameters of Spin-Labeled Intact Erythrocytes at 20 °C

spin-label	treatment[a]	$2T_{\parallel}'$ (G)	$2T_{\perp}' - 1.6$ (G)	S^b
5-NS	no treatment	60.6	17.0	0.731 ± 0.002
5-NS	+200 μM CPZ	60.0	17.0	0.724 ± 0.001
7-NS	no treatment	60.5	17.0	0.729 ± 0.001
7-NS	+200 μM CPZ	59.0	17.1	0.713 ± 0.003
10-NS	no treatment	59.6	18.1	0.686 ± 0.003
10-NS	+200 μM CPZ	56.4	18.1	0.652 ± 0.003
12-NS	no treatment	54.8	18.9	0.610 ± 0.004
12-NS	+200 μM CPZ	51.5	19.0	0.567 ± 0.004
12-NS	+100 μM CPZ	52.3	19.0	0.577 ± 0.004
12-NS	+100 μM CPZ + 2.5 mM EGTA	52.5	18.9	0.579 ± 0.005
12-NS	+100 μM CPZ + N_2, dark	52.3	19.0	0.577 ± 0.003
12-NS	+100 μM CPZ + washed CPZ-free	54.0	19.0	0.597 ± 0.005

[a] Erythrocyte treatments were performed in 5 mM sodium phosphate and 0.15 M NaCl, pH 7.6. [b] Values were calculated by the equation of Hubbell and McConnell (see Materials and Methods).

tectable at concentrations of CPZ near that reported to induce hemolysis (Lieber et al., 1984). For this reason we used concentrations of CPZ not exceeding 100 μM and the 12-NS in all the following experiments.

The increase of % S induced by both CPZ and TFP was dose dependent (Figure 3), whereas antimitotic drugs in the same concentration range were unable to change either $2T_{\parallel}'$ or % S. Especially noteworthy was that the sulfoxide derivative of chlorpromazine, CPZ-SO, did not affect significantly $2T_{\parallel}'$ and % S even at a concentration of 100 μM (Figure 3). CPZ-SO, in the micromolar concentration range, has been shown to be inactive also as a cup-former, anti-calmodulin, and antihemolytic agent (Nelson et al., 1983). The increase of 12-NS freedom of motion induced by CPZ was not affected by the presence of 2.5 mM EGTA or by incubation of cells in the dark in a N_2 atmosphere to avoid photoxidative damage (Table I). On the contrary, the drug effect was reverted when CPZ-treated erythrocytes were washed in a CPZ-free buffer (Table I). The membrane effects of CPZ were also evident with resealed ghosts, but the % S was slightly reduced in comparison to that of intact erythrocytes (Figure 3). The histograms in Figure 3 show that the CPZ-induced increase in the % S was observed also in ghosts prepared in the presence

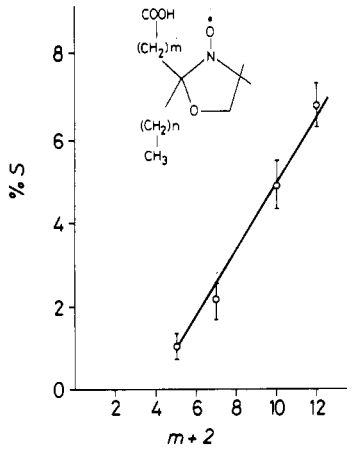

FIGURE 2: Effects of 200 μM CPZ on the freedom of motion of different stearic acid spin-labels bound to intact erythrocytes. $m + 2$ indicates the position of the nitroxide ring from the carboxylic end. Apparent order parameters of spin-labeled erythrocytes were calculated by the equation of Hubbell and McConnell (see Materials and Methods). The percent difference of the order parameter, % S, was calculated by % $S = [(S_c - S_d)/S_c] \times 100$, where S_c is the order parameter of control erythrocytes and S_d the order parameter of drug-treated cells. Spectra were recorded at 20 °C.

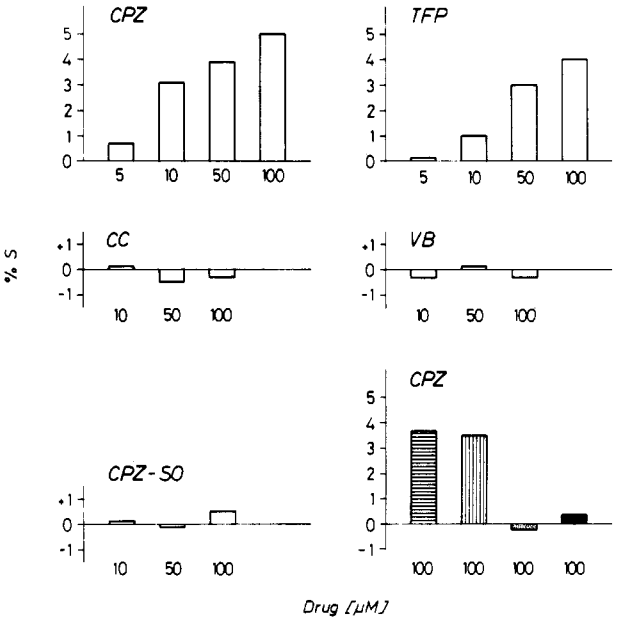

FIGURE 3: Histograms showing the effects of phenothiazines and antimitotic drugs on the freedom of motion of 12-NS-labeled membranes. % S was calculated as reported in Figure 2. Positive % S values indicate increased spin-label freedom of motion in comparison to that of the control sample, whereas negative values indicate decreased spin-label motion. The standard deviations observed were ±0.3–0.6 % S. Changes equal to or lower than 0.6 % S were considered meaningless. CPZ, chlorpromazine; TFP, trifluoperazine; CC, colchicine; VB, vinblastine; CPZ-SO, chlorpromazine sulfoxide. TFP produced 4% and 7% hemolysis, respectively, at 50 and 100 μM concentrations. Open bars, intact erythrocytes; shaded horizontal bar, resealed ghosts; shaded vertical bar, ghosts prepared with 0.1 mM EGTA and then resealed; dotted bar, ghosts resealed with anti-spectrin antibodies; black bar, inside-out vesicles. Spectra were recorded at 20 °C.

of 0.1 mM EGTA, a condition known to remove endogenous calmodulin from the red blood cell membranes (Quist & Roufogalis, 1975; Cohen & Foley, 1986). The differential effects of CPZ and CPZ-SO were not due to a different partitioning behavior into the erythrocyte membrane. CPZ, due to its high membrane solubility, strongly quenched the intrinsic fluorescence of ghost proteins (Yamaguki et al., 1985),

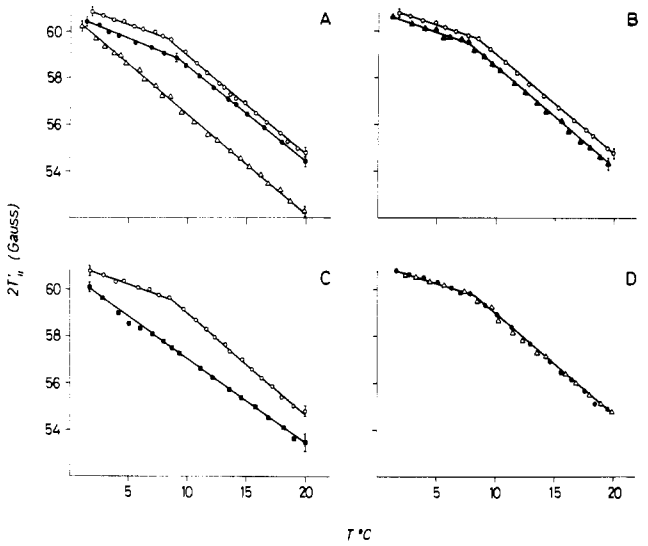

FIGURE 4: Effects of drugs on the thermotropic behavior of 12-NS-labeled intact erythrocytes. (A) (○) Control cells; (△) 100 μM CPZ treated cells; (●) 100 μM CPZ treated cells after washing in a CPZ-free buffer. (B) (○) Control cells; (▲) 100 μM CPZ-SO treated cells. (C) (○) Control cells; (■) 50 μM TFP treated cells. (D) (△) 100 μM CC treated cells; (●) 100 μM VB treated cells. Points are average values of three spectra; typical ±SEM are indicated. Break temperatures were determined by computer analysis as described elsewhere (Minetti et al., 1984).

and we observed a CPZ-SO quenching curve superimposable to that of CPZ (data not shown).

In order to test the role of skeletal proteins, ghosts were either extracted at low salt concentration or resealed with monospecific anti-spectrin antibodies. The low salt extraction is known to remove most of the spectrin and actin with the production of inside-out vesicles (Steck & Kant, 1974). As reported in Figure 3, both the removal of spectrin–actin and the anti-spectrin antibodies treatment were sufficient to virtually abolish the effects of CPZ on the order parameter of 12-NS-labeled ghosts. Control experiments with the IgG fraction of rabbit preimmune sera did not show changes in the phenothiazine-induced increase of % S, thus excluding nonspecific effects of IgG.

Erythrocyte Thermal Properties. In previous works (Minetti et al., 1984, 1986; Forte et al., 1985) we observed that in the 0–50 °C temperature range the erythrocyte membrane presented discontinuous changes in the freedom of motion of 16-NS spin-label that may be indicative of protein-dependent structural transitions.

In the 0–20 °C temperature range, the $2T_{\parallel}'$ for 12-NS-labeled intact erythrocytes detected a discontinuity in the spin-label motional freedom at 8.5 ± 1.5 °C (Figure 4). A discontinuity around this temperature was not specific for the 12-NS spin-label or the empirical parameter chosen for spectra evaluation. Similar thermal transitions were obtained with both 5-NS (Forte and Minetti, unpublished results) and 16-NS spin-labels. The spectra of 16-NS were analyzed by the amplitude ratio of h_0 and h_{-1} lines (Minetti et al., 1984).

When 100 μM CPZ or 50 μM TFP was added to intact red blood cells, the $2T_{\parallel}'$ value decreased and the thermal transition was abolished (Figure 4). In contrast, CC, VB, and CPZ-SO at 100 μM concentrations affected neither the $2T_{\parallel}'$ value nor the thermal transition (Figure 4). The 8.5 °C thermal transition was dependent upon the presence of peripheral proteins. Erythrocyte membrane vesicles stripped of peripheral proteins did not show this thermal transition on either 16-NS- (Forte et al., 1985) or 5-NS-labeled membranes (Minetti and Forte, unpublished results). Phenothiazine inhibition of the thermal transition was a reversible process. Erythrocytes treated with 100 μM CPZ and then washed with a CPZ-free buffer showed restoration of a thermal break at 9.0 °C (Figure 4).

Discussion

Biological and pharmacological properties of phenothiazines vary qualitatively as a function of their concentration (Seeman, 1972, 1977). This work has been focused on the effects of phenothiazines in the 10–100 μM concentration range where these drugs produced hemolysis protection or "pre-lytic" phenomena in the red blood cell (Seeman, 1972). At these concentrations CPZ- and TFP-treated erythrocytes showed a dose-dependent increase in the freedom of motion of a stearic acid spin-label, which was not observed with CPZ-SO and antimitotic drugs (as measured by the percent difference of the apparent order parameter, % S). Therefore, the increase in the freedom of motion of 5-NS labeled Chinese hamster ovary cells induced by antimitotic drugs (Aszalos et al., 1985), and possibly other biological effects in common with phenothiazines, likely involved different mechanisms or different cellular targets.

The effects of phenothiazines on 12-NS freedom of motion and membrane thermal properties were reversible phenomena, thus excluding mechanisms involving proteolysis (Jimbu et al., 1984; Lang et al., 1984), Ca^{2+}-activated irreversible processes (King & Morrison 1977), or photoxidation. Biologically active phenothiazines may perturb membrane lipid structure (changes in the apparent order parameter) through two possible mechanisms: (1) a direct interaction of the drug with membrane protein reflected in the lipid bilayer and (2) a perturbation of lipid structure that leads to perturbation of proteins via lipid–protein interactions. Experiments reported in this study do not provide evidence against either of the hypotheses but suggest that the membrane target site(s) may involve distinct proteins rather than simple drug solubilization in membrane lipids. Three lines of evidence suggest that the target sites involve erythrocyte skeletal proteins. First, we observed that spectrin–actin-depleted membranes did not show the CPZ-induced increase of % S. Second, the % S of ghosts treated with anti-spectrin antibodies was not changed by CPZ and, third, we observed that phenothiazines inhibited a skeletal protein-dependent thermal transition of the erythrocyte membrane. In a previous paper (Forte et al., 1985) we reported that this transition was abolished by specific anti-protein 4.1 antibodies and by the extraction of protein 4.1, whereas the rebinding of purified protein 4.1 to membranes was sufficient to restore the transition. Biochemical studies suggest a key role for this protein in the stabilization of a complex with spectrin and actin (Ohanian et al., 1984; Correas et al., 1986); more recently, the membrane interaction of protein 4.1 has been shown to involve glycophorin A and polyphosphoinositides as lipid cofactors (Anderson & Marchesi, 1985).

It should be noted that the extraction of spectrin and actin did not coextract the majority of protein 4.1 (Tyler et al., 1979). Moreover, we reported that both the extraction of spectrin–actin and the treatment with anti-spectrin antibodies were unable to eliminate the 8.5 °C transition (Forte et al., 1985; Minetti et al., 1986). In contrast, the same treatments were sufficient to abolish almost completely the effects of CPZ. These findings led to the assumption that (1) biologically active phenothiazines affect the submembranous network at the level of the interaction of protein 4.1 and its membrane binding sites and (2) the motion of membrane-bound stearic acid spin-labels is determined, at least in part, by the binding of the spectrin–actin network. Although the participation of other skeletal proteins cannot be ruled out, we suggest that the supramole-

cular domain formed by glycophorin, negatively charged lipids, and protein 4.1 is affected by phenothiazines. The hypothesis that membrane physicochemical properties are mediated by membrane–skeleton interactions is supported by results showing the control of skeletal proteins on the lateral mobility of intrinsic proteins and phospholipids (Golan & Veatch, 1980; Sheetz et al., 1982; Tsuji & Ohnishi, 1986; Gawrisch et al., 1986).

ACKNOWLEDGMENTS

We thank Prof. Vincent T. Marchesi and Drs. Thomas L. Leto and Nicholas J. F. Dodd for helpful discussions and critical reading of the manuscript. We are grateful to Drs. J. Schlosser and G. Scarpitta from Smith Kline & French Laboratories for providing chlorpromazine sulfoxide and to Dr. A. Di Nuccio from Centro Sperimentale della Croce Rossa Italiana for the generous gifts of blood samples.

Registry No. Chlorpromazine, 50-53-3; chlorpromazine sulfoxide, 969-99-3; trifluoperazine, 117-89-5.

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