Modulation of Adenylate Cyclase Activity by the Physical State of Pigeon Erythrocyte Membrane. 1. Parallel Drug-Induced Changes in the Bilayer Fluidity and Adenylate Cyclase Activity†

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ABSTRACT: The fluorescence anisotropy probe perylene and the spin-labels 5-doxylstearate and 16-doxylstearate were used to estimate the order and internal microviscosity of the pigeon erythrocyte membrane upon perturbation by cationic or neutral amphipathic drugs (chlorpromazine, methochlorpromazine, tetracaine, and octanol) and an anionic drug, octanoic acid. Both methods gave identical results. The fluidity changes were found to strictly correlate with those of adenylate cyclase activity in the presence of GTP when perturbed by the drugs [Salesse, R., & Garnier, J. (1979) *Biochim. Biophys. Acta 554*, 102–113]. The cationic or neutral drugs, in an intermediate range of concentration, decreased the degree of organization and the internal microviscosity of the lipids together with the activity of the adenylate cyclase. At a higher concentration they reincreased them up to or higher than their initial level before the final destruction of the membrane structure and functions. This concentration effect was time dependent with tetracaine. The quaternary amine methochlorpromazine acted as chlorpromazine only on open ghosts. On intact cells, it inhibited catecholamine receptors at higher concentration and monotonously decreased the order and microviscosity, as the anionic amphipath octanoic acid did. This is taken as evidence that the inner leaflet of the bilayer is the seat for the observed multiphasic changes of viscosity and the control of adenylate cyclase and catecholamine receptors. This could stem from either a preferential intercalation or a surface effect of the amphipaths in the inner leaflet of the membrane. Since the basal activity of adenylate cyclase was not affected in the presence of drugs, it may be inferred that the enzyme holds its activity but that its stimulation is modulated by the membrane physical state.

The physicochemical state of the lipid bilayer is generally thought to be largely responsible for the maintenance and control of most membrane enzyme activities (Sandermann, 1978). However, enhancement of the adenylate cyclase activity has been found either upon membrane ordering by cholesterol in chinese hamster ovary cells (Sinensky et al., 1979) or upon fluidization by benzyl alcohol in rat hepatocytes (Gordon et al., 1980).

Since in a previous work we showed that the adenylate cyclase in pigeon erythrocyte displayed successively inhibition and then reactivation following incubation with increasing concentrations of amphipathic cationic or neutral drugs (first class of drugs) but a simple inhibition with amphipathic anionic ones (second class) (Garnier & Singer, 1977; Salesse & Garnier, 1979), it was interesting to measure in this system the evolution of microviscosity and order evoked by these chemical compounds in the membrane.

The amphipathic drugs chlorpromazine (CPZ;[1] cationic), octanol (neutral), and octanoic acid (anionic) were chosen as representatives of the two classes, and complementary experiments were performed with the cationic tetracaine and methochlorpromazine (MCPZ).[1]

Fluorescence anisotropy and electron spin resonance (ESR)[1] measurements showed that the two classes of drugs have a different effect on the membrane physical state. In every case, the changes in the lipid fluidity paralleled those of the activity of the enzyme in the presence of GTP; fluidization led to inhibition while reordering involved recovery of the enzyme activity. The exposure of the inner leaflet of the membrane to the drugs appeared as an important step of their effect.

Experimental Procedures

Materials. Chlorpromazine hydrochloride (CPZ) and its quaternary ammonium derivative methochlorpromazine iodide (MCPZ) were a generous gift of Rhône-Poulenc. The origin of all other chemicals has been described previously, as well as the preparation of pigeon erythrocytes and nucleated ghosts (Salesse & Garnier, 1979) and plasma membranes (Salesse, 1980). Intact erythrocytes were kept at 4 °C in isotonic buffer, pH 7.4 (146 mM NaCl–20 mM Tris-HCl–20 mM D-glucose), and ghosts and membrane preparation in hypotonic buffer (10 mM Tris-HCl–4 mM $MgCl_2$, pH 7.4).

Fluorescence Experiments. Perylene (from Aldrich) was chosen as the fluorescence probe specific for membrane phospholipids (Shinitzky & Barenholz, 1978) to avoid the absorption bands of the drugs used (excitation wavelength = 415 nm; emission wavelength = 445 nm). It was stored at room temperature in the dark as a 0.1 mM solution in acetone. At each temperature and drug concentration, a 0.5-mL aliquot of the frozen membrane preparation was thawed and labeled with 2 μL of the perylene stock solution. After 15 min at 25 °C, 1.5 mL of drug in hypotonic buffer was added and the incubation carried on for 15 min at 25 °C. The resulting 2 mL was then poured in a 1 × 1 cm quartz cuvette and allowed to equilibrate at the temperature of experiment.

Fluorescence anisotropy measurements were performed on a Jobin-Yvon JY 3C spectrofluorometer equipped with ex-

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[1] Abbreviations: CPZ, chlorpromazine hydrochloride; MCPZ, methochlorpromazine iodide; ESR, electron spin resonance.

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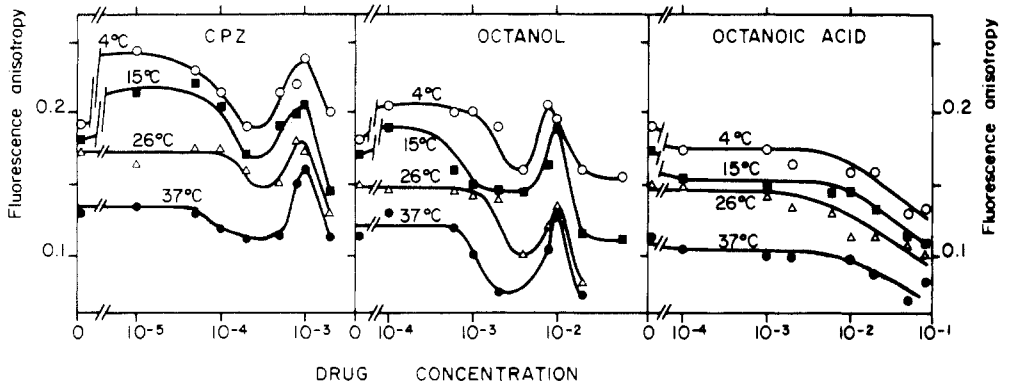

FIGURE 1: Effect of CPZ, octanol, and octanoic acid at different temperatures on the fluorescence anisotropy of perylene in a purified membrane preparation.

citation and emission polarizers. Spectra were corrected for light scattering, and the anisotropy of fluorescence was calculated as

$$r = \frac{I_{vv} - I_{vh}(I_{hh}/I_{hv})}{I_{vv} + 2I_{vh}(I_{hh}/I_{hv})}$$

(Pesce et al., 1971) where the emission intensities (I) were recorded in the vertical (v) and horizontal (h) positions of the polarizers. Measurements were usually duplicated. Relative errors on r were in the 5–10% range. Less than 1% of the perylene added to the membrane preparations could be extracted with ether from the supernatant.

It is now established that the value of the fluorescence anisotropy (r) may be expressed as the sum of a dynamic and of a structural contribution and that the structural contribution can reach 90% of the r value near 0 °C and 50–60% at 37 °C, the order of the lipid bilayer appearing as the main parameter probed by fluorescence labels as temperature decreases (Jähnig, 1979).

ESR Experiments. Two probes (Syva Corp., Palo Alto, CA) specific of the membrane phospholipids were used. With its reporter group close to the surface of the bilayer, 5-doxylstearate was used to assess the order of the phospholipids by measuring the experimental hyperfine splitting $2T'_{\parallel}$. This probe gives reliable results with relative errors on $2T'_{\parallel}$ of the order of 0.4% and may report changes in the order as small as 0.6% (Pang et al., 1980).

ESR spectra of 16-doxylstearate were interpreted in terms of the rotation frequency, ν (and thus internal microviscosity), according to (Likhtenstein, 1976)

$$\nu = \frac{2.10^8}{[(h_0/h_{+1})^{1/2} - 1]\Delta h_0}$$

where h_{+1} and h_0 are the amplitudes of the low and central field lines and Δh_0 is the width of the central field line. Relative errors on $\log \nu$ were of about 1%. Rotation correlation time was taken as $1/\nu$.

Both probes were kept frozen at a concentration of 10 mM in dimethyl sulfoxide solution. To 110 μL of packed erythrocytes (5000g; 5 min) or packed ghosts or membranes (12000g; 9 min) was added 12 μL of drug to the desired final concentration. After 20 min of incubation at 25 °C, we added 2 μL of the ESR probe solution (final concentration 0.16 mM). The sample was then aspirated in a quartz cell and placed in the thermostated cavity of a Varian E-3 spectrometer. ESR spectra were recorded every 4–5 °C from 0 to 50 °C and plots of $2T'_{\parallel}$ vs. temperature or $\log \nu$ vs. $1/T$ (T = absolute temperature) were drawn. Contamination with free-rotating probes was negligible. Representation of $2T'_{\parallel}$ vs. temperature showed slight slope changes around 6 and 37 °C, and those of $\log (1/\nu)$ vs. $1/T$ were usually only faintly incurved.

Kinetic experiments were performed only at 30 or 37 °C. The time of mixing of the cell preparations with the drug was taken as zero time.

Adenylate Cyclase Assays and [^3H]Alprenolol Binding Measurements. In both cases, 50 μL of intact cells or ghosts was preincubated for 20 min at 37° C with 50 μL of drug in isotonic or hypotonic buffer, respectively.

Adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] was assayed in mixtures containing ~10^8 cells, 2 mM [α-^{32}P]ATP (2 × 10^6 dpm), 4 mM theophylline, 10 mM phosphocreatine, 0.5 g/L creatine kinase, 7.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4). Depending on the experiment, 0.1 mM GTP or Gpp(NH)p, 10 mM NaF, 0.05 mM isoproterenol, and the drug to the desired final concentration were added (Salesse & Garnier, 1979). For measurement of the catalytic unit activity, $MgCl_2$ was replaced by $MnCl_2$ at the same concentration.

[^{3}H]Alprenolol binding was assayed as previously described (Salesse & Garnier, 1979) either on ghosts in hypotonic buffer or on intact cells in isotonic buffer. Total binding (TB) was assayed in 75-μL mixtures containing ~10^8 cells and up to 100 nM [^{3}H]alprenolol and nonspecific binding (NS) in tubes containing the same reagents and 50 μM cold alprenolol. Specific binding was taken as TB − NS. [^{3}H]Alprenolol was purchased from Amersham.

Results

Fluorescence Anisotropy. Figure 1 shows that CPZ, a cationic drug, and octanol, a neutral one, had the same polyphasic effect on the fluorescence anisotropy of perylene, though at different concentrations: the fluorescence anisotropy value reached a minimum at 0.2–0.6 mM CPZ and 2–5 mM octanol; it then increased up to or even above the control level at 1 and 10 mM, respectively, whatever the temperature was. Below 26 °C, the two drugs raised the anisotropy at low concentrations (ca. 0.01 and 0.1 mM, respectively). Addition of 1 μM Ca^{2+} also increased the anisotropy, but the anisotropy increase by the drugs at low concentrations was still observed (not shown). In contrast to the above drugs, the anionic octanoic acid provoked a monotonous decrease of the anisotropy from 10 mM on, whatever the temperature was. At the highest concentration of drug compounds, membrane fragments tended to irreversibly aggregate and the anisotropy value dropped, indicating a collapse of the membrane structure.

Preliminary results with the Lure synchrotron light source in Orsay (Salesse et al., 1981) revealed two fluorescence lifetimes of perylene in pigeon erythrocyte membranes that decreased continuously with increasing concentrations of the

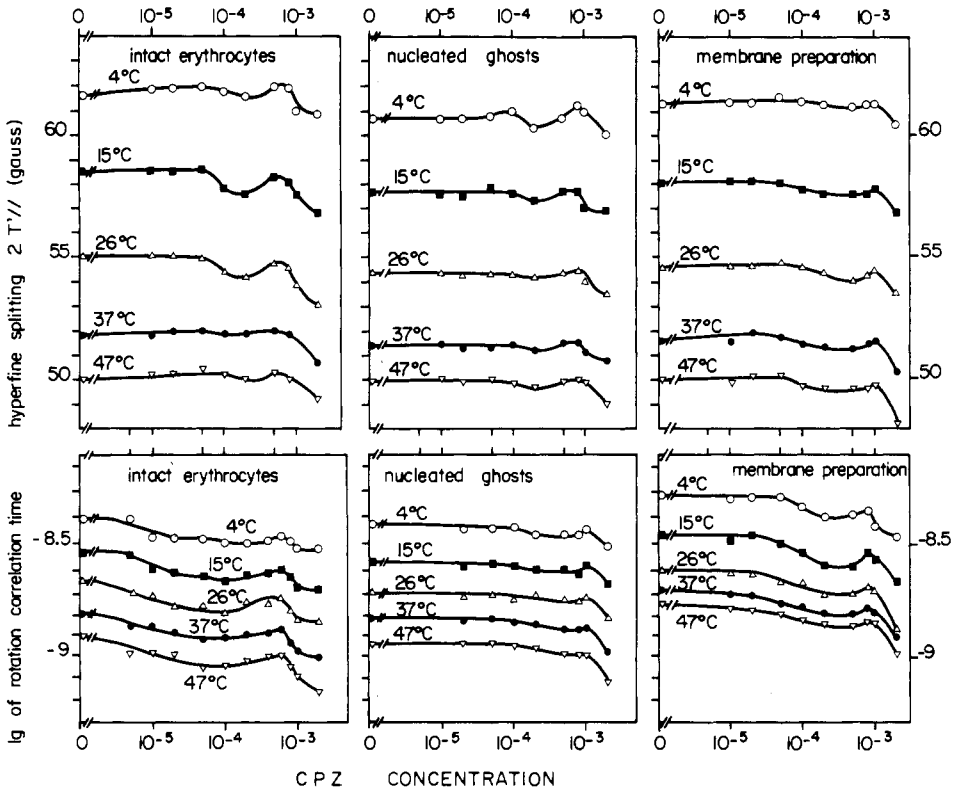

FIGURE 2: Effect of CPZ and temperature on intact erythrocyte (left), nucleated ghosts (middle), and membrane preparation (right). (Top) Hyperfine splitting, $2T'_{\parallel}$, of 5-doxylstearate; (bottom) rotation correlation time of 16-doxylstearate.

drugs, which explains the already reported quenching effect of CPZ (Römer & Bickel, 1979). Thus, the monotonous changes of lifetime will not account for the polyphasic variation of fluorescence anisotropy induced by cationic and neutral drugs.

ESR Experiments. Whereas the light absorption and diffusion by intact erythrocyte suspensions prevent fluorescence measurements, ESR experiments can be performed on intact cells, and comparison between nucleated ghosts and membrane preparation gave very similar results, as illustrated in Figure 2. One may, however, notice that the peak at high concentration is slightly shifted from 1 mM in purified membranes to 0.6 mM of CPZ in intact erythrocytes with either probe. We checked that the isolated nuclei only displayed a monotonous decrease in $2T'_{\parallel}$ and rotation correlation time with increasing CPZ concentration above 0.1–0.5 mM, ruling out a possible interference from probes in contaminating nuclear envelopes.

5-Doxylstearate reported essentially the same polyphasic variations as perylene did (Figures 2 and 3) with increasing amphipathic drug concentrations. The raise in lipid order at low temperature and low concentration of CPZ was also detectable (Figure 2). The internal microviscosity as probed by 16-doxylstearate tended to diminish prior to lipid order. Noticeably, the two maxima of internal microviscosity and hyperfine splitting, $2T'_{\parallel}$, coincide (Figures 2 and 3).

The anionic octanoic acid provoked a monotonous decrease of both lipid order and internal microviscosity at concentration higher than 2–5 mM (Figure 3). We observed with 2–5 mM tetracaine a transient increase of internal microviscosity and lipid order for about 10 min at 37 °C which was parallel to changes in fluorescence anisotropy and adenylate cyclase activity (Figure 4).

Sidedness of Action of the Amphipathic Drugs. Intact erythrocytes incubated for 1 h at 37 °C with MCPZ did not exhibit any change of the order parameter. However, during

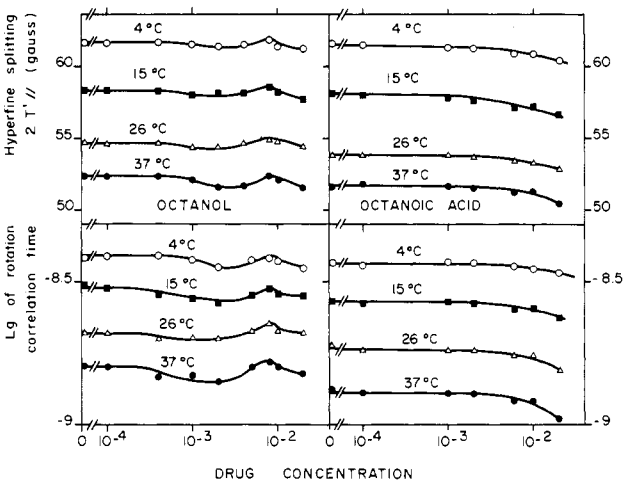

FIGURE 3: Effect of octanol (left) and octanoic acid (right) on the physical state of the membrane of intact erythrocyte at different temperatures. (Top) Hyperfine splitting, $2T'_{\parallel}$, of 5-doxylstearate; (bottom) rotation correlation time of 16-doxylstearate.

the same incubation time, a polyphasic fluidity change was observed in open cells (Figure 5). If intact cells were incubated 2 h or more with MCPZ, a polyphasic fluidity change can then be observed (Figure 5, top). A similar difference between intact and open ghosts is found in the inhibition of [^{3}H]alprenolol binding by MCPZ: 50% binding inhibition was achieved at 2.5 mM MCPZ in intact cells and at 0.5 mM in ghosts (Figure 5). This is not likely related to an alteration of the membrane properties during ghost preparation since CPZ achieved the same alprenolol binding inhibition at the same concentration with intact cells and ghosts (Figure 6).

Correlation between Changes of the Membrane Physical State and the Adenylate Cyclase Activity. Figure 6 displays the parallel effect of the drugs at 37 °C on the membrane microviscosity, lipid order, and adenylate cyclase activity

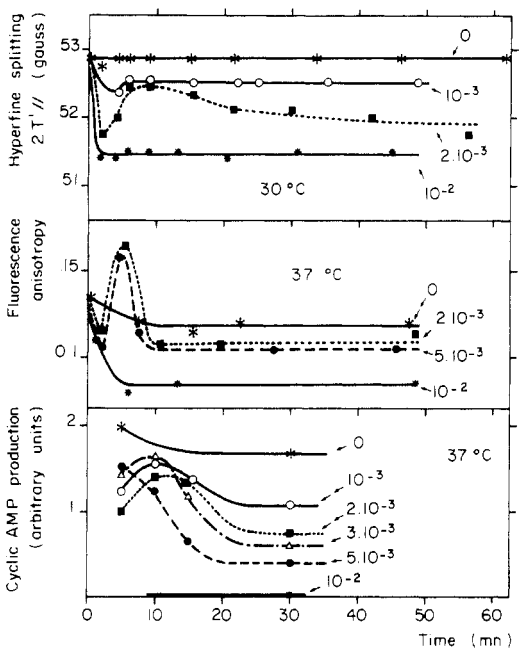

FIGURE 4: Dependence on time and temperature of the effect of different concentrations of tetracaine on (top) hyperfine splitting, $2T'_{\parallel}$, of 5-doxylstearate in the membrane of intact erythrocytes at 30 °C, (middle) fluorescence anisotropy of perylene in membrane preparation at 37 °C, and (bottom) cyclic AMP production by the adenylate cyclase in nucleated ghosts upon 10-min fluoride stimulation at 37 °C. Enzyme assays were performed for 5 min after 0-, 5-, 10-, and 25-min preincubation with the drug.

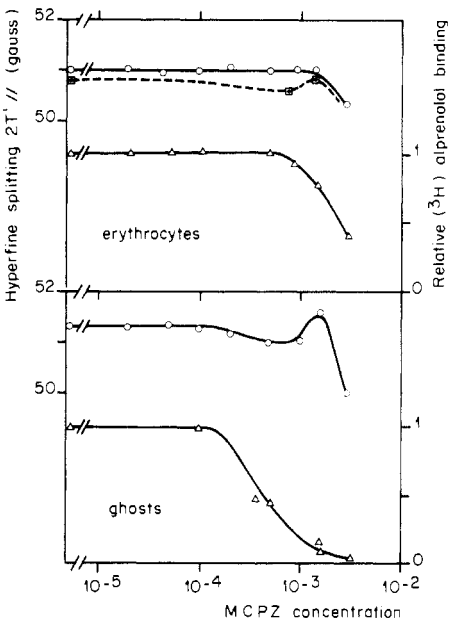

FIGURE 5: Effect of MCPZ at 37 °C on the hyperfine splitting of 5-doxylstearate after 1 h (○) and 2 h (□) of contact with the drug and on the binding of [^{3}H]alprenolol (△) in intact erythrocytes (top) and open ghosts (bottom).

stimulated by Gpp(NH)p but not or to a lesser degree by isoproterenol. Hormone receptors were lost early with CPZ, later with octanol, but not with octanoic acid. The basal activity with Mn-ATP as the enzyme substrate was not affected by the drugs (Figure 6).

Figure 4 (bottom) shows the production of cyclic AMP in 5 min upon fluoride stimulation in the presence of different concentrations of tetracaine at 37 °C. Taking into account that this production integrated the rate of the enzyme over a 5-min period, it becomes obvious that the enzyme activity is strongly correlated to the physical state of the membrane.

Discussion

Most of the amphipathic compounds used in this study were already recognized as fluidity-perturbing agents (Seeman, 1972; Leterrier, 1978). Fluidity changes could stem from the perturbation of the interactions between the main membrane components: proteins, phospholipids, cholesterol, and even calcium ions. CPZ is known to bind to proteins (Chatelain et al., 1979; Leterrier et al., 1976; Testylier & Leterrier, 1980) and even to release them from some membranes (Leterrier et al., 1974; Ogiso et al., 1977; Lahrichi et al., 1977), and proteins are recognized as important modulators of the membrane fluidity (Cornell et al., 1978; Owicki & McConnell, 1979; Heyn et al., 1981). CPZ interacts also with phospholipids, sometimes increasing (Breton et al., 1977; Jones & Woodbury, 1978) and sometimes decreasing (Chatelain et al., 1979; Lee, 1977) order and internal microviscosity. In model systems, the drugs displayed a dual behavior, either fluidizing or rigidifying depending on the cholesterol/phospholipid ratio (Neal et al., 1976; Pang & Miller, 1978). Moreover, cholesterol-interacting substances have been reported to inhibit (Puchwein et al., 1974) or activate (Dipple & Houslay, 1979) the adenylate cyclase system.

Calcium ions added to membrane preparations are known to increase phospholipid order (Seeman, 1972), in a competitive manner with CPZ (Breton et al., 1977). On the other hand, local anesthetics, regardless of charge, including tetracaine and straight-chain alcohols, displace Ca^{2+} from the cytoplasmic face of membranes (Low et al., 1979).

All these perturbations can be understood as surface effects or intercalation of the drugs into the lipid bilayer, though this last possibility has been recently questioned (Conrad & Singer, 1981). We can propose that at low concentration (0.01 mM CPZ, 0.1 mM MCPZ, and 5 mM octanoic acid), the drugs will begin to interact with the polar part of the membrane (Leterrier et al., 1976; Frenzel et al., 1978; Tenforde et al., 1978), thereby creating a fluid state where the interaction between the membrane components is progressively loosened. For cationic and neutral drugs, this loosening could facilitate the penetration of some drug into the membrane, up to a critical intramembrane concentration for which the drug molecules assume a restabilization of the membrane structure (at 1 mM CPZ or 10 mM octanol). Higher intramembrane concentrations of the drugs would lead to an extensive disordering of the membrane. This hypothesis implies that these drugs should have a time-dependent effect: this time dependence is found with tetracaine, a cationic drug whose effect is similar to that of CPZ or octanol, except that its peak of microviscosity and order is transient and lasts less than 10 min at 37 °C (Figure 4, middle) and approximately 20 min at 30 °C (Figure 4, top) for a concentration of 2–5 mM of the drug. With MCPZ, the rate of membrane fluidity perturbation was much slower (Figure 5).

The difference of action on intact erythrocyte between CPZ and its quaternary amine derivative, MCPZ, can be related to their very different p*K*s as already proposed for explaining their action on shape changes (Sheetz & Singer, 1976). Both compounds would have the ability to interact with the outer leaflet of the bilayer, but only CPZ in its neutral form could flip rapidly across the membrane when MCPZ would not (Sheetz & Singer, 1974; Elferink, 1977). This differential effect of MCPZ whether the inner leaflet of the membrane is attainable or not by this drug suggests that the cytoplasmic half-layer is closely linked to the observed multiphasic change of fluidity and to the loss of catecholamine receptors either

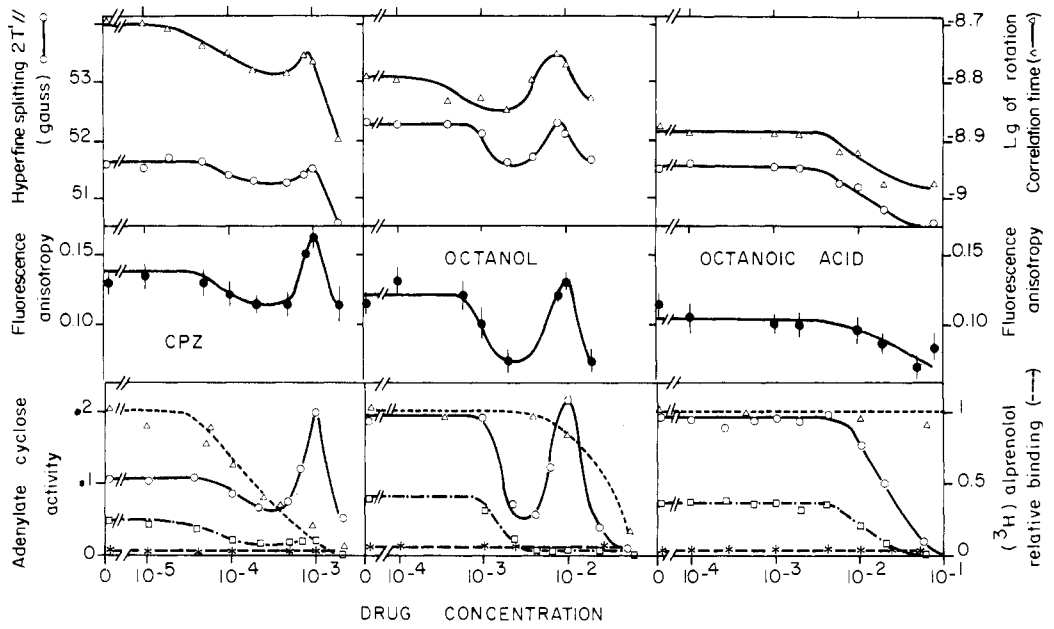

FIGURE 6: Comparison of the effects of CPZ (left), octanol (middle), and octanoic acid (right) at 37 °C on (top) hyperfine splitting of 5-doxylstearate and rotation correlation time of 16-doxylstearate in membrane preparation for CPZ and in the membrane of intact erythrocytes for octanol and octanoic acid, (middle) fluorescence anisotropy of perylene in purified membrane preparation, and (bottom) activity of adenylate cyclase (nanomoles of cAMP per minute per milligram of phospholipid) [basal (*), stimulated by Gpp(NH)p (○), and by isoproterenol plus GTP (□); number of [^{3}H]alprenolol binding sites (△); the "basal" assay was performed in the presence of $MnCl_2$ instead of $MgCl_2$].

because it is the seat of the perturbation itself by a preferential intercalation (Sheetz & Singer, 1974) or because in some way it controls the physical state of both layers.

In regard to the large variation in chemical structure, one may consider that the drug effect on the adenylate cyclase of pigeon erythrocyte membrane would be essentially indirect, mediated by the inner lipid layer physical state. The recently proposed models of the adenylate cyclase system (Tolkovsky & Levitzki, 1978; Rodbell, 1980) consider that two activating collisions or close associations are needed to fulfill the stimulation of the enzyme: once the hormone bound, the receptor must collide or closely associate with the resting GTP regulatory protein. This complex would then couple to the enzyme and turn it into its activated form.

Fluidization of the bilayer has been reported to increase the cyclic AMP production by enhancing the probability of activating encounters between the subunits of the system (Gordon et al., 1980; Dipple & Houslay, 1978; Rimon et al., 1978; Hirata et al., 1979). We, however, observed an opposite effect: fluidization reduced the activity of the system, except in the basal case (Figure 6). In fact, the change of lipid order might induce transconformation of proteins and lower enzyme rates (McMurphie & Raison, 1979) and/or alter the embedding of integral proteins (Borochov & Shinitzky, 1976); thereby, the interacting sites between the three subunits (receptor, regulatory protein, and enzyme) would not match any more (Levitzki & Helmreich, 1979; Bakardjieva et al., 1979) and their coupling would be impaired. Conversely, the restoration of the membrane organization brings the system back to activity and even hyperactivity if some enzyme control is lost [see Salesse et al. (1982)].

All these data lay emphasis on the importance of the maintenance in very narrow limits of acyl chain ordering and microviscosity for the basic functions supported by biological membranes. Recent results show that upon perturbation of this homeostatic control in acclimation of animals to temperature changes (Cossins, 1977; Augee et al., 1979), abetalipoproteinemia (Cooper et al., 1977), tumor cells (Shinitzky et al., 1979), and genetic obesity (Hyslop & York, 1980), membrane activities are affected together with membrane fluidity.

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