Modulation of the β-Receptor Adenylate Cyclase Interactions in Cultured Chang Liver Cells by Phospholipid Enrichment†

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ABSTRACT: Chang liver cells in culture fused with dimyristoylphosphatidylcholine or dioleoylphosphatidylcholine liposomes incorporate 20–30 mol % phospholipid into plasma membranes. In these membranes, the mobility of pyrenedecanoic acid, an excimer forming fluorescent probe, was increased. The broad thermotropic phase transition observed by EPR in untreated membranes between 20 and 30 °C with 5-nitroxystearic acid disappeared. The number of β receptors titrated with L-[^{3}H]dihydroalprenolol or [^{125}I]iodohydroxybenzylpindolol was reduced in these membranes at 37 °C above the gel–liquid crystalline phase transition, whereas the same number of β-receptor sites was titrated as in normal or dipalmitoylphosphatidylcholine enriched membranes at 17 °C below the phase transition. The 40–60% loss of receptors resulted in a comparable decrease in L(−)-isoproterenol activated adenylate cyclase activity [EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)], whereas Gpp(NH)p, NaF, and basal activities were much less reduced. There was little change in the various parameters of adenylate cyclase activity in membranes enriched with the same amount of several other phospholipids (including dipalmitoylphosphatidylcholine). An increase in the lateral mobility of β receptors in more fluid membranes is assumed to be responsible for the disappearance of receptors and its functional consequences. Apparently mobility is crucial for protein–protein interactions in enzyme systems which catalyze vectorial reactions in two-dimensional membranes; receptor–adenylate cyclase interactions and (Na^+, K^+)-ATPase exemplify this type of reactions.

The influence of lipids on the activity of membrane-bound enzymes and of hormone-stimulated adenylate cyclase, in particular, has been studied intensively in recent years (Cuatrecasas, 1974; Kimmelberg & Papahadjopoulos, 1974; Warren et al., 1974; Orly & Schramm, 1975; Engelhard et al., 1978; Houslay et al., 1976; Hesketh et al., 1976; Pohl et al., 1971; Rodbell et al., 1971; Rubalcava & Rodbell, 1973; Puchwein et al., 1974; Limbird & Lefkowitz, 1976). The recent findings that β receptor and adenylate cyclase are two separate and genetically distinct membrane proteins has revived the interest in the role of the membranous environment in receptor–adenylate cyclase coupling (Orly & Schramm, 1976; Ross et al., 1978; Tolkovsky & Levitzki, 1978). We have studied the effects of alterations of the phospholipid composition of plasma membranes on the function of the β-adrenergically stimulated adenylate cyclase system. Chang liver cells in stationary culture (Makman, 1971) were used and phospholipids were introduced by fusion of whole cells with liposomes. Chang liver cells contain a catecholamine sensitive adenylate cyclase and can be fused remarkably easily with liposomes. Moreover, plasma membranes are readily prepared from fused cells. The introduction into membranes of 20–30 mol % of dimyristoylphosphatidylcholine (DMPC)[1] and dioleoylphosphatidylcholine (DOPC) caused a drastic increase in the lateral mobility of pyrenedecanoic acid and of the spin label, 5-nitroxystearic acid. This could be correlated with a loss of β receptors and total β-receptor-mediated activity at 37 °C, whereas the number of binding sites and β-receptor activation of adenylate cyclase measured at 17 °C below the gel–liquid crystalline phase transition were the same as in normal membranes.

Materials and Methods

Cells. Chang liver cells were obtained from the American Type Culture Collection and grown as monolayers in Eagle's minimum essential medium plus Earle's salts (Eagle, 1959; Earle, 1943) obtained from LS-Labor, Munich, and 10% fetal calf serum (Boehringer, Mannheim). The cells were incubated at 37 °C under humidified air and 6% CO_2.

Liposomes and Fusion with Cells. Liposomes were prepared and fused with cells by a modification of the method of Martin & MacDonald (1976). Positively charged lipsosomes were prepared by dissolving phospholipids with 5% (w/w) stearylamine in chloroform. A trace of [^{14}C]DPPC was added as marker and the solvent was evaporated under N_2. One milliliter of 0.3 M sucrose in 10 mM Bicine–NaOH buffer, pH 7.8, was added to aliquots of 0.7 μmol of phospholipid and the lipid film ultrasonicated at 12 Hz for 5 min under nitrogen at a temperature above the phase transition temperature of the phospholipids. The vesicle suspension was added to about 5×10^5 cells/cm^2 and incubated for 15 min at 37 °C in Bicine–NaOH buffer, pH 7.8. The plates were then washed thoroughly with 0.15 M NaCl.

Plasma Membranes. Untreated and fused cells were harvested at a density of about 5×10^5 cells/cm^2. They were washed twice at room temperature with cold 0.15 M NaCl, scraped from the plates with a rubber policeman and transferred into 10 mM Tris-HCl, 1 mM EDTA, 0.25 M sucrose buffer, pH 7.4. Cells were counted in a Fuchs-Rosenthal chamber and membranes prepared by the method of McKeel & Jarett (1970) by sucrose density gradient centrifugation which was repeated if necessary. The fraction sedimenting at a sucrose density of 1.14 was collected and tested for 5′-nucleotidase (Ipata, 1967), (Na^+, K^+)-ATPase (Benson et al., 1975), and adenylate cyclase (Gilman, 1970), enzymes

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[1] Abbreviations used: DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; Bicine, *N*,*N*′-bis(2-hydroxyethyl)glycine; [^{125}I]IHYP, [^{125}I]iodohydroxybenzylpindolol; EPR, electron paramagnetic resonance; T_t, transition temperature; T_c, critical temperature where the "break" in Arrhenius plots occurs.

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considered typical for plasma membranes. The purity of the membrane preparation was checked by electron microscopy and contamination with endoplasmic, mitochondrial, and nuclear membranes was estimated by measuring NADPH-cytochrome *c* reductase (EC 1.6.2.4) (Dallner et al., 1966), cytochrome *c* oxidase (EC 1.9.3.1) (Smith, 1955), RNA (I-San Lin & Schjeide, 1969), and DNA (Giles & Myers, 1965). The plasma membranes contained no less than 3% and no more than 15% extrinsic material.

Membranes prepared from cells after fusion contained more total lipid since fusion did not deplete membrane lipids. Incorporation of phospholipids into membranes was followed under the electron microscope by using the freeze-etch technique, by gas–liquid chromatography, by measuring the phospholipid to protein ratio and the radioactivity of incorporated [^{14}C]DPPC. For gas–liquid chromatography, extracts were prepared by the method of Bligh & Dyer (1959) and phospholipids were separated on thin-layer chromatography in *n*-hexane–diethyl ether–methanol–acetic acid (90:20:2:3) and eluted three times with a 1:9 mixture of chloroform–methanol. Fatty acid methyl esters were prepared with BF_3/CH_3OH reagent as described by Morrison & Smith (1964). Radioactively labeled DPPC was extracted with chloroform–methanol and counted. Results from all three methods were in good agreement. [^{14}C]DPPC incorporated by fusion was found distributed to about 50% in the cytoplasm, to 25–30% in the plasma membrane and to 20–25% in the nuclear fraction. Incorporation was greatly influenced by cell density; therefore, fusion was always carried out with about 5×10^5 cells/cm^2 and membranes were used which contained comparable amounts, i.e., 20–30 mol % of incorporated phospholipids (see Table I). A random distribution of the incorporated phospholipids was ascertained by freeze-etch electron microscopy (see Figure 1) which was kindly carried out by Dr. W. Haase from the Max-Planck Institute for Biophysics in Frankfurt/M., F.R.G.

Enzyme Assays. The adenylate cyclase assay mixture contained 25 mM Tris-HCl, 3 mM ATP, 5 mM $MgCl_2$, 1 mM 1-methyl-3-isobutylxanthine, 20 mM creatine phosphate, 0.05 mg of creatine kinase, pH 8.0, and 30–60 μg of membrane protein in a total volume of 210 μL. Effects of a possible contamination of ATP with GTP were controlled by adding 1×10^{-5} M GTP to the assay mixture. The pH dependence of Tris-HCl buffer necessitated its replacement by 10 mM Bicine–NaOH buffer, pH 8.0, in the temperature–activity measurements (Figure 4). In all other cases, the pH of Tris-HCl buffer was carefully controlled. Reactions were stopped by heating to 100 °C for 2 min and cAMP was determined by the method of Gilman (1970). cAMP formation was linear up to 15 min at 37 °C and with respect to the protein concentrations under assay conditions.

Binding of L-[^{3}*H*]*Dihydroalprenolol and* [^{125}*I*]*Iodohydroxybenzylpindolol and Displacement by* L(−)-*Isoproterenol.* The cells were detached from the plates by incubation with 10 mM Tris-HCl, 1.5 mM Na_2EDTA, and 140 mM NaCl buffer, pH 7.4, at 37 °C for 20 min (Atlas et al., 1977). Cells (8×10^5 to 3×10^6) in a total volume of 500 μL were usually collected and incubated with 5–60 nM L-[^{3}H]dihydroalprenolol at 37 °C for 5 min (Lefkowitz et al., 1976). For binding of [^{125}I]iodohydroxybenzylpindolol and displacement by unlabeled L(−)-isoproterenol, membranes (150 μg of protein) were incubated with 30 000–40 000 cpm corresponding to about 100 pM [^{125}I]iodohydroxybenzylpindolol in 250 μL of 25 mM Tris-HCl, 5 mM $MgCl_2$ buffer, pH 7.5, for 30 min at 30 °C (Brown et al., 1976; Maguire et al., 1976). The samples containing varying concentrations of L(−)-isoproterenol were filtered through a 25-mm Whatman GF/C filter. The filter was washed five times with 5 mL of 0.15 M NaCl, containing 10^{-5} M DL-propranolol, dried, and counted. Unspecific binding was determined by measuring the residual binding of the radioactive ligands in the presence of 1×10^{-5} M DL-propranolol.

Fluorescence Measurements. The lateral mobility of pyrenedecanoic acid was determined in membranes of liposome-fused and normal cells. Liposomes containing pyrenedecanoic acid were prepared from chloroform solutions. After evaporation, the film was sonicated in Bicine–NaOH buffer, pH 7.8, for about 5 min. The final lipid concentration was 7×10^{-4} M and that of pyrenedecanoic acid, 2×10^{-5} M. In controls, 2×10^{-5} M pyrenedecanoic acid in buffer was added directly to membranes from untreated cells. Pyrenedecanoic acid is an excimer forming probe and the formation of excited complexes between molecules in the ground state and the first excited singlet state is measured. Since the excimer–monomer intensity ratio, I'/I, is a measure of the mobility of the probe, one should be able to derive from it a collision rate constant, k_a, and from it the lateral diffusion coefficient of the probe. Theoretically it is possible to calculate $k_a \sim (I'/I)(1/c)(1/\tau_0')$, where τ_0' is the lifetime of the excited dimer and c is the concentration of the probe. But, since the lifetime of the monomer is long, $\tau_0 \sim 400$ ns, nearly all the monomer must be converted into excimer in order to make measurements of the excimer lifetime feasible. Future efforts are directed to make such measurements possible. Excimer–monomer ratios were determined with a Schoeffel RRS 1000 fluorospectrometer equipped with dual monochromators on the emission side. The monomer fluorescence, I, was well separated from the excimer emission, I', and I and I' could be recorded simultaneously. Both intensities were divided by each other with an analogue computer. The method is described in detail by Galla & Sackmann (1974) and Hartmann & Galla (1978).

EPR Measurements. 5-Nitroxystearic acid with the nitroxide group at carbon atom 5 ($I(12,3)$) (2×10^{-5} M corresponding to about 3 mol % of the liposomes) was introduced into membranes as described for pyrenedecanoic acid. The first derivative EPR spectra were recorded with a Bruker ER 420 spectrometer equipped with a temperature control accurate to ±0.1 K. The spectra are composed of a central line and outer and inner pairs of lines separated by $T_{\parallel}$ and $2T_{\perp}$, respectively. The line shape is characteristic for a spin probe having a rapid anisotropic motion. $T_{ZZ} = 30.8$ G and $T_{XX} = 5.8$ G are the maximum and the minimum possible splittings of the outer and the inner pair of lines, respectively. $T_{ZZ} - T_{XX} = 25$ G. These parameters were used to characterize the gel–liquid crystalline phase transition in the cell membrane with 5-nitroxystearic acid and to calculate the order degree $s = (T_{\parallel} - T_{\perp})(T_{ZZ} - T_{XX})$ (Sackmann et al., 1973).

Phospholipids. Highest purity DOPC, DMPC, DPPC, and DPPE, as well as [^{14}C]DPPC (specific activity, 20 mCi/mmol), were obtained from A. Nattermann & Co., GmbH, Köln. DMPE and stearylamine were from Fluka AG, Buchs SG. The purity was controlled by thin-layer chromatography.

Other Chemicals. ATP, creatine phosphate, creatine kinase, and Gpp(NH)p were obtained from Boehringer, Mannheim, and 1-methyl-3-isobutylxanthine was from EGA-Chemie, Steinheim. cAMP binding protein was prepared by the method of Gilman (1970). Bicine was purchased from Serva, Heidelberg, and DL-propranolol hydrochloride from Sigma Chemical Co. [8-^{3}H]Adenosine 3′,5′-phosphate ammonium salt (specific activity, 27 Ci/mmol) was obtained from Radio

Table I: Phospholipid Composition of Membranes from Lipid-Fused Cells[a]

suppl phospholipid	phospholipid/ protein ratio	amount of phospholipid incorp (mol %)	
none	0.43 ± 0.03		
DOPC	0.58 ± 0.04	32 ± 3[b]	33 ± 3[c]
DMPC	0.52 ± 0.02	24 ± 3[b]	21 ± 5[c]
DPPC	0.57 ± 0.03	27 ± 5[b]	30 ± 5[c]
DMPE	nd[d]	22 ± 4[b]	nd[d]
DPPE	0.52 ± 0.09	21 ± 3[b]	21 ± 4[c]

[a] Mean ± SD of four experiments. For techniques see Materials and Methods. [b] From radioactivity measurements. [c] From gas–liquid chromatography. [d] Not determined.

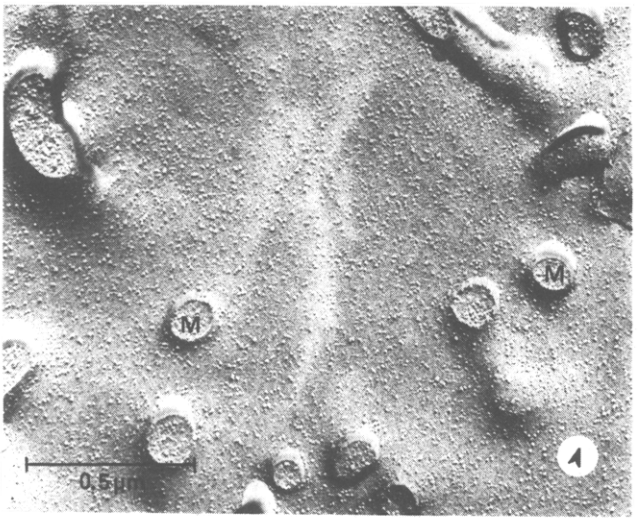

FIGURE 1: Freeze-fracture electron micrograph. The picture was taken 30 min after fusion of Chang liver cells with DMPC liposomes. Cytoplasmic face of plasma membrane. Bar, 0.5 μm × 30 000. M is microvillus.

Chemical Centre, Amersham, England. L-[^{3}H]Dihydroalprenolol (specific activity, 58.5 Ci/mmol) and [^{125}I]HYP (specific activity, 2200 Ci/mmol) were from New England Nuclear Co. 5-Nitroxystearic acid was purchased from Syva, Palo Alto. Pyrenedecanoic acid was synthesized according to Galla & Hartmann (1979). All other chemicals were from Merck, Darmstadt, and of the highest purity available.

Results

Phospholipid Composition. The lipid to protein ratio in Chang liver cell plasma membranes is 0.62 (w/w). The lipid fraction is composed of 71% phospholipid, 10% cholesterol, and 19% other lipids. The membrane phospholipids of fused cells increased by 1/3–1/5 (i.e., 20–30 mol %) over the phospholipids present in normal cells (Table I). Membrane cholesterol did not change substantially. The freeze-etch electron micrograph (Figure 1) of the membrane was taken 30 min after fusion. Only the cytoplasmic fracture face is shown, but micrographs were also taken from the external fracture face and at different times after fusion. They all were compatible with the assumption of randomization of the phospholipid incorporated.

Specific Activities. Table II summarizes specific activities at 37 °C of basal, NaF, L(−)-isoproterenol, and L(−)-isoproterenol and Gpp(NH)p stimulated adenylate cyclase in nonmanipulated membranes and in membranes enriched with DOPC, DMPC, DPPC, and DMPE. Only in membranes treated with DOPC and DMPC were all activities reduced, more for the isoproterenol stimulated enzyme than for the NaF stimulated and basal activities. Incorporation of 10^{-4} M Gpp(NH)p with liposomes increased the isoproterenol stimulated activity in the DPPC enriched membranes by about 1.8-fold. This should be compared with a 4-fold stimulation of the isoproterenol-activated enzyme in the DMPC enriched membranes. GTP, 1×10^{-5} M, had little effect. The Gpp(NH)p stimulated adenylate cyclase activity in the absence of isoproterenol in DMPC membranes was reduced by only about 1/4–1/5 of that measured in normal or DPPC-treated membranes. Here it needs to be emphasized that, in the experiments at 37 °C in Table II, specific activities were calculated from initial rates during the first 10 min, which at 3 mM ATP approach V_{max} (the same applies to the experiments in Figures 2 and 5 and Table III). The K_m value for

Table II: Adenylate Cyclase Activities in Membranes from Liposome Fused Cells at 37 °C[a]

lipid suppl	basal, non-stimulated	adenylate cyclase act.[b] (pmol of cAMP mg^{-1} min^{-1}) NaF	iso-proterenol	iso-proterenol + Gpp(NH)p
none	30 ± 3.0	82 ± 9.0	191 ± 25	nd[c]
DPPE	25 ± 1.9	89 ± 6.0	169 ± 20	nd[c]
DMPE	28 ± 3.5	75 ± 8.3	193 ± 21	nd[c]
DPPC	32 ± 2.8	80 ± 7.0	195 ± 32	357 ± 41
DMPC	10 ± 5.1	50 ± 9.5	71 ± 11	280 ± 34
DOPC	15 ± 2.7	46 ± 8.9	49 ± 7.4	nd[c]

[a] Values are mean ± SD of four experiments. [b] Assays were carried out at 1×10^{-2} M NaF, 1×10^{-5} M L(−)-isoproterenol, and 1×10^{-4} M Gpp(NH)p. For details see Materials and Methods. [c] Not determined.

Table III: (Na^+, K^+)ATPase and Adenylate Cyclase Activities in Membranes from Liposome Fused Cells[a]

temp (°C)	(Na^+, K^+)-ATPase in membranes treated with[b] (nmol of P_i mg^{-1} min^{-1}) none	DMPE	DPPC	DMPC	DOPC
37	68 ± 3.8	56 ± 5	nd[f]	12 ± 2.2	nd[f]
17	10 ± 1.9	9 ± 2.9	nd[f]	8 ± 3.1	nd[f]

temp (°C)	adenylate cyclase in membranes treated with[b] (pmol of cAMP mg^{-1} min^{-1}) none	DMPE	DPPC	DMPC	DOPC
17	6 ± 0.9[c]	nd[f]	5.5 ± 1.1[c]	5.5 ± 1.0[c]	4 ± 0.7[c]
17	34 ± 2.5[d]	nd[f]	32.5 ± 2.9[d]	28 ± 3.4[d]	8.5 ± 2[d]
17	21 ± 2.7[e]	nd[f]	20 ± 3.5[e]	18 ± 2[e]	12 ± 1.5[e]

[a] Mean ± SD of three experiments. [b] For details, see Materials and Methods. [c] Nonstimulated (basal) activities. [d] Stimulated with 1×10^{-5} M L(−)-isoproterenol. [e] Stimulated with 1×10^{-2} M NaF. [f] Not determined.

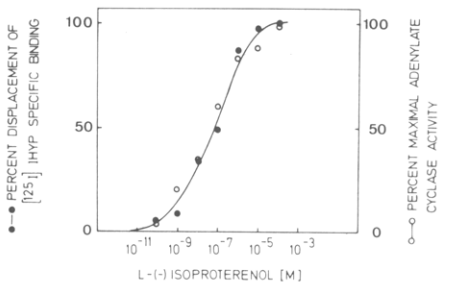

FIGURE 2: Displacement of [^{125}I]IHYP binding and activation of adenylate cyclase by L(−)-isoproterenol in Chang liver cell membranes. Data are plotted as percentage of specific binding displaced; nonspecific binding was subtracted. Adenylate cyclase activity measured in membranes is given in percent of activity mg^{-1} min^{-1}. (○—○) Adenylate cyclase activity; (●—●) [^{125}I]IHYP binding. The experiment is a representative example of several experiments.

ATP and adenylate cyclase at 37 °C under these conditions was 0.35 mM and was the same in normal and phospholipid-treated membranes. When adenylate cyclase activities were measured in the presence of Gpp(NH)p and L(−)-isoproterenol in prolonged incubations (up to 60 min) at 37 °C, a final level of activity was eventually obtained in the DMPC-treated cells which was not much different from that in normal membranes under identical conditions. This suggests that, in the presence of Gpp(NH)p even in DMPC-treated membranes, all the adenylate cyclase becomes hormonally activated, although at a slower rate. This is in agreement with the collision coupling model of Tolkovsky & Levitzki (1978) which predicts that the first-order rate constant for the coupling of β receptors to adenylate cyclase is directly proportional to the concentration of receptors, whereas the maximal number of catalytic units that can be activated is independent of the receptor concentration. These observations provoked us to see whether the reduction of catecholamine-stimulated activity in the DOPC- and DMPC-enriched membranes was due to a decrease in the number of β-receptor sites.

β-Receptor Sites. Figure 2 allows one to calculate and compare K_D for binding and K_a for adenylate cyclase activation by L(−)-isoproterenol. Displacement of the antagonist [^{125}I]HYP by L(−)-isoproterenol results in activation and the concentrations of L(−)-isoproterenol required for half-maximal displacement of [^{125}I]IHYP binding and activation were the same. Thus under the conditions of the experiments in Figure 2, $K_{D_{iso}} = K_{a_{iso}} = 1.4 \times 10^{-7}$ M. $K_{D_{iso}}$ was calculated according to $C_{50} = ((1 + S)/K_{D_{HYP}})K_{D_{iso}}$, where C_{50} is the L(−)-isoproterenol concentration ($\sim 10^{-7}$ M) required for 50% displacement of [^{125}I]IHYP, $K_{D_{HYP}}$ is the dissociation constant of the HYP–receptor complex ($\sim 3 \times 10^{-10}$ M), and S is the [^{125}I]IHYP concentration used (1×10^{-10} M) (cf. Lefkowitz et al., 1976). $C_{50} \approx K_{D_{iso}}$, when $S \leq K_{D_{HYP}}$. Thus, the correspondence between receptor occupancy and hormonal activation holds only for a rather narrow range of concentrations and under specified conditions.

Similar correspondence was found with turkey erythrocytes (Brown et al., 1976) and glioma C_6–TG1A cells (Maguire et al., 1976).

Binding of the radioactive antagonists [^{3}H]dihydroalprenolol and [^{125}I]iodohydroxybenzylpindolol to intact cells and membranes was complete in 5 min at 37 °C or 30 min at 30 °C, respectively. Specific binding was saturable and hyperbolic (see Figure 3). K_D of [^{3}H]dihydroalprenolol was $1–2 \times 10^{-8}$ M, K_D of [^{125}I]IHYP was $2.5–3.5 \times 10^{-10}$ M and the same in untreated and lipid-treated membranes. Although there was no change in the affinity of the receptor for the [^{3}H]-dihydroalprenolol in DMPC- and DOPC-enriched cells, the number of binding sites per cell (or per mg of membrane protein) was reduced by 40–60%, whereas the number of receptors titrated in cells enriched with DPPC (or with DPPE or DMPE, not shown) remained the same as in untreated cells (see Figure 3A). This was also shown by using [^{125}I]IHYP as the radioactive ligand (experiments not shown).

Incidentally, the loss of receptors does not appear to be a peculiar property of DMPC- or DOPC-enriched Chang liver cells; a similar decline of titratable β receptor was observed in DMPC-fused glioma C_6 cells in culture (A. Bakardjieva and E. J. M. Helmreich, unpublished experiments, 1978). The results with cells shown in Figure 3 were verified by binding studies with isolated membranes. These experiments showed moreover that addition of Gpp(NH)p could neither prevent nor reverse the loss of β receptors in DMPC-enriched

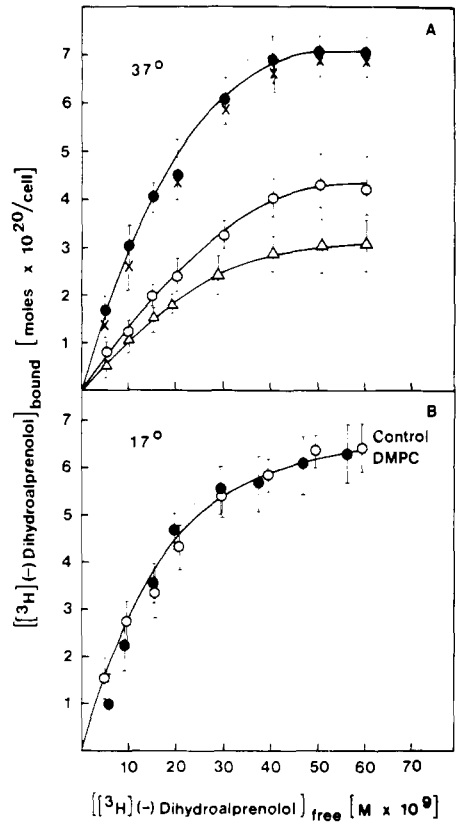

FIGURE 3: Specific binding of (−)-[^{3}H]dihydroalprenolol to Chang liver cells. (A) At 37 °C; (B) 17 °C; for experimental details, see Materials and Methods. (●—●) Normal cells; (X—X) DPPC; (○—○) DMPC; (△—△) DOPC enriched cells. Each point represents the mean of three and five experiments ± SD, respectively.

membranes. We have also considered the possibility that receptors are exchanged with liposomes but no protein and/or [^{3}H]dihydroalprenolol binding component was recovered in liposomes separated after cell contact. The least ambiguous controls were, however, provided by cells enriched to a comparable extent by fusion with other phospholipids, DPPC, for example, which were indistinguishable from normal cells (see Table II and Figure 3). The effect of DOPC was concentration dependent; treating cells with liposomes containing DOPC:DPPC at a ratio of 27:73 had little or no effect but treatment with an equal mixture of DOPC:DPPC already decreased [^{3}H]dihydroalprenolol binding. The cells regained in part binding sites and catecholamine activated adenylate cyclase when membrane phospholipid composition reverted to that before manipulation. This occurred spontaneously within 48 h. Regain of β receptors and adenylate cyclase activity did not appear to require protein synthesis because appreciable recovery, up to 80% of normal, occurred in the presence of 1 μg/mL cycloheximide. Assuming that the decrease in receptors in the DMPC- and DOPC-enriched membranes might be related to a change in membrane viscosity, we measured receptor binding at a temperature where the "fluidized" membranes would be expected to gel again. And indeed when binding (and activation) was studied at 17 °C below the gel–liquid crystalline transition of the DMPC-enriched membrane (21 °C) the number of receptors was the same as in nonmanipulated and DPPC-treated cells (Figure 3B). Moreover, catecholamine-stimulated activity at 17 °C, although low as to be expected, was likewise the same in normal and DPPC- and DMPC-treated cells but was still decreased in the DOPC-treated cells (Table III). Included in Table III are data for (Na^+, K^+)-activated ATPase. At

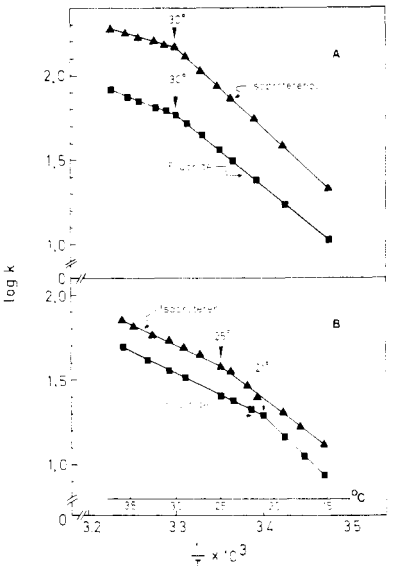

FIGURE 4: Temperature dependence of adenylate cyclase activity. Adenylate cyclase activity was determined in Bicine–NaOH buffer, pH 8.0, in (A) normal and DPPC-enriched membranes and (B) DMPC-enriched membranes, with 10^{-2} M NaF (■—■) and 1×10^{-5} M L(−)-isoproterenol (▲—▲). Assay conditions are described under Materials and Methods. k is expressed in pmol of cAMP min^{-1} mg^{-1}. Each point represents the mean of three separate experiments.

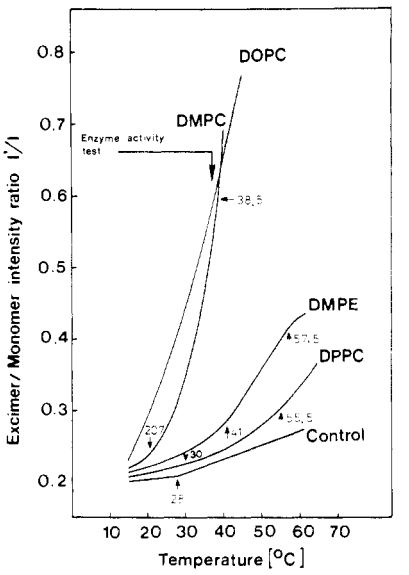

FIGURE 5: Temperature dependence of the excimer to monomer intensity ratio I'/I of pyrenedecanoic acid incorporated into normal and lipid-enriched membranes. Arrows indicate the temperatures (°C) where a lipid phase transition begins and ends. The temperature (37 °C) where enzyme activity was tested is also indicated. The data are representative examples of several experiments.

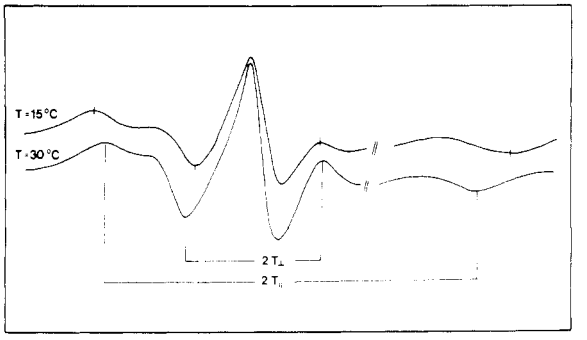

FIGURE 6: Typical EPR signal of 5-nitroxystearic acid incorporated in Chang liver cell membranes at T = 15 °C and T = 30 °C. The degree of motional anisotropy is expressed in terms of the order degree which reflects the flexibility of the fatty acid chain as a function of the lipid matrix viscosity. T_{ZZ} and T_{XX} correspond to the two outer hyperfine and to the two inner hyperfine maxima, respectively, of the first derivative spectra. Typical values for $2T_{\parallel}$ = 50.6 G, $2T_{\perp}$ = 18.8 G at 30 °C. Note that the high field minimum is amplified to correspond to the rest of the spectrum.

37 °C, the activity of the ATP-driven ion pump in DMPC-manipulated membranes was less than 15% of the activity in normal membranes whereas it was 80% of that in normal membranes at 17 °C.

Temperature–Activity Profiles. Temperature–activity profiles of DL-isoproterenol- and fluoride-stimulated adenylate cyclase in normal and DPPC-treated cells have a discontinuity around 30 °C (Figure 4A, top). Similar results have been reported before with liposome-fused rat liver membranes and glucagon by Houslay et al. (1976). Adenylate cyclase activity solubilized with Lubrol PX (not shown) was linearly dependent on temperature between 15 and 37 °C, suggesting that the discontinuities may reflect the lipid environment of the enzyme. In DOPC-treated membranes (not shown), the lines were likewise straight between 15 and 37 °C. In Figure 4B, bottom, are compared temperature–activity profiles of isoproterenol- and NaF-activated adenylate cyclase in DMPC-enriched membranes. The DMPC-enriched membranes show like normal or DPPC-treated membranes breaks in the activity profiles in the temperature range where adenylate cyclase activities can be measured. For the agonist L(−)-isoproterenol, the discontinuity shifted from about 30 °C in normal cells to about 25 °C in DMPC-enriched membranes. However, when the receptor was unoccupied as with the NaF-stimulated enzyme (or the nonstimulated, basal activity) the break occurred at 21 °C. Thus, in the DMPC-enriched membranes, the enzyme seemed to sense a different environment with the β receptor occupied than without liganded receptor.

The State of the Membrane. To obtain information on the dynamic state[2] of the membrane after fusion, fluorescence and EPR measurements were carried out with normal and manipulated membranes. Figure 5 gives excimer formation of pyrenedecanoic acid as a function of temperature. At low temperatures, the excimer yield is low, suggesting a nearly rigid lipid phase. For the nonmanipulated membrane, a faint discontinuity in the I'/I plot around 28 °C may suggest a lipid phase transition, although at 37 °C where activity measurements were carried out the mobility of the probe was little changed. The too low excimer yield precluded calculation of the diffusion coefficient of the probe. This was discussed under Materials and Methods. In the DPPC- or DMPE-enriched membranes, the break in the I'/I vs. temperature plot was shifted to higher temperatures. For pure DPPC or DMPE vesicles, the transition temperatures were 41 and 49 °C, respectively, as measured with pyrenedecanoic acid. In the DMPC- and DOPC-enriched membranes, the probe mobility increased by about a factor of three over the range between 17 and 37 °C. A broad temperature break is discernible beginning around 21 °C in the DMPC-treated membrane, whereas the curve for the DOPC-enriched membranes rose nearly linearly over the range of temperature tested. The observation that the probe was more mobile in DPPC- and DMPE-enriched membranes compared with the normal membrane is discussed later.

The order parameters of control and manipulated membranes were measured by EPR with 5-nitroxystearic acid.

[2] We follow Seelig & Seelig (1977) and distinguish between structural and dynamic changes in membranes.

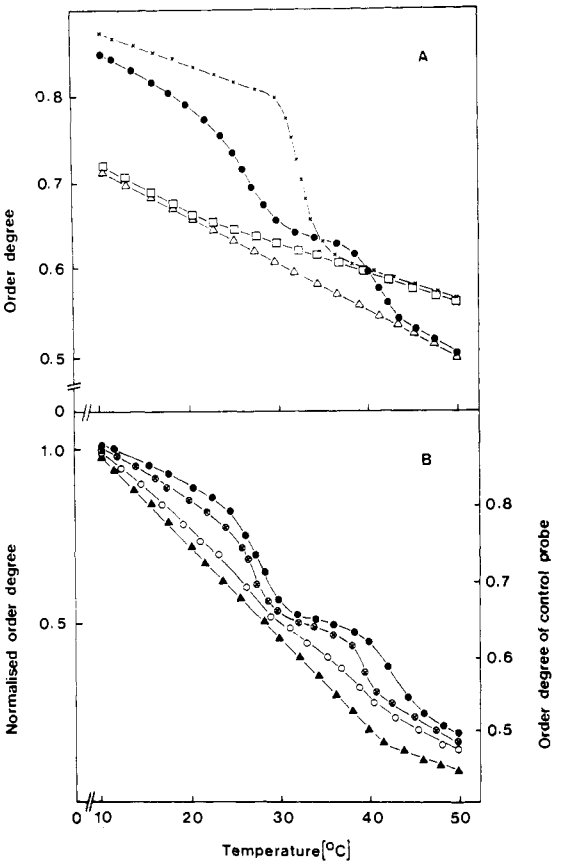

FIGURE 7: Temperature dependence of the order degree of 5-nitroxystearic acid incorporated into bilayers and cell membranes. (A) (●—●) Chang liver membranes; (x—x) DPPC liposomes; (△—△) DOPC liposomes; (□—□) DPPC:DOPC (3:1) liposomes. (B) (●—●) Control, Chang liver membranes; (⊗—⊗) DPPC-manipulated membranes; (○—○) DMPC-manipulated membranes; (▲—▲) DOPC-manipulated membranes. T_t is 32 °C for the phase transition curve (x—x) of DPPC vesicles, in A, which is characteristic for this bilayer and 5-nitroxystearic acid. The right-hand scale in B only applies to the normal membrane. All measurements with modified membranes in B were normalized with respect to the whole phase transition of the normal membrane. This was done by adjusting the height of the phase transition step of the lipid modified samples to the curve for the unmodified membranes. For that purpose, the height of the transition step in the unmodified membrane was recorded on a scale ranging from 0 to 1 which was used as a ruler to adjust the amplitudes of the modified samples on the Y axis.

Typical spectra for normal membranes are presented in Figure 6. Degrees of order(s) were 0.79 and 0.64 for 15 and 30 °C, respectively. Similar values were obtained with *E. coli* membranes and the same spin label (Sackmann et al., 1973). The similarity may be related to the low (10% of total lipids) cholesterol content of the Chang liver cell membranes. The temperature dependence of the order degree for Chang liver cell membranes is compared with that for pure DPPC and DOPC bilayers, and for a mixed DPPC-DOPC bilayer in Figure 7A and data for Chang liver cell membranes enriched with DPPC, DMPC, and DOPC are shown in Figure 7B. The phase transition in pure DPPC bilayers measured with the spin label occurs around 32 °C and about 9 °C below the endothermic phase transition (41 °C). This is probably a consequence of the introduction of the spin label (Sackmann et al., 1973). A first transition could be discerned in normal and DPPC-treated liver cell membranes at about the same temperatures (28–30 °C) but in cell membranes a second change occurred at temperatures >40 °C which was missing in the lipid extract and the bilayers. This second discontinuity could be due to denaturation of membrane proteins which might be expected to perturb the lipids. Denaturation of membrane-bound adenylate cyclase actually occurs at temperatures above 40 °C in rat liver plasma membranes (Renē et al., 1978). DOPC bilayers as expected do not exhibit phase transitions in the temperature range measured and the introduction of about 30 mol % DOPC into DPPC bilayers suppressed the phase transition characteristic for the DPPC bilayer but which is absent in DOPC liposomes (Figure 7A). The same effect was observed in Chang liver cells on incorporation of DMPC or DOPC (Figure 7B), whereas incorporation of DPPC did not change the phase transition behavior markedly, except that the width of the curve was somewhat narrower. One major difficulty which we encountered with the spin label incorporated in cell membranes was its reduction, which varied from preparation to preparation and made it necessary to reject all samples where the EPR signal faded during measurements.

Discussion

Receptor mobility is generally assumed to be a prerequisite for coupling reactions involving receptor and adenylate cyclase. The floating receptor hypothesis may serve as an example for this view (Cuatrecasas, 1974; Swillens & Dumont, 1977). From kinetic data a diffusion constant for the β receptor in turkey erythrocyte membranes of $D = 4 \times 10^{-11}$ cm^2 s^{-1} at 25 °C was estimated by Levitzki (Rimon et al., 1978) (cf. Bakardjieva et al., 1979). Therefore, the observations reported above that incorporation of only 20–30 mol % of DMPC or DOPC into membranes interferes with rather than facilitates receptor coupled hormonal activation are quite unexpected. In order to explain this phenomenon, we shall critically scrutinize the importance of each experimental variable for the disappearance of receptors.

As indicated in Table I, the enrichment of Chang liver cells with phospholipids changed the phospholipid to protein ratio in favor of the lipid. This "dilution" effect is quite likely responsible for the paradoxical increase in the mobility of pyrenedecanoic acid in the DPPC- and the DMPE-enriched membranes shown in Figure 5 and is also suggested by the reduced width of the phase transition curve observed with the spin label and the DPPC-enriched membrane in Figure 7B. This interpretation is based on the experiments of Cherry et al. (1977) who have shown that membrane viscosity in phospholipid bilayers is dependent on protein concentration. But since we have used membranes containing comparable amounts of incorporated phospholipids (See Table I), a dilution effect alone due to a change in the phospholipid to protein ratio cannot explain the more drastic changes observed with DMPC- and DOPC-enriched membranes (cf. Figures 5 and 7). For example, the decrease in the order degree of the membranes from Chang liver cells (cf. Figure 7) with increasing temperature between 20 and 32 °C was still seen in lipid extracts from Chang liver cells devoid of proteins and incorporation of DMPC or DOPC into liver cell membranes (cf. Figure 7B) broadened and smeared out the phase transition. The assumption of an overall fluidized membrane would account for all these observations. This assumption is also supported by the increase in the I'/I ratio in the DMPC- and DOPC-enriched membranes, resulting from the increased mobility of the probe (cf. Galla & Sackmann, 1974). We therefore propose on the basis of the concordant results obtained with two independent physical probes, an excimer forming probe and a spin label, that introduction of DMPC and DOPC makes Chang liver cell membranes more fluid in a random fashion.

To appraise structural parameters[2] in complex natural membranes with spin and fluorescent probes of the type used in this work is even more difficult than to analyze dynamic

changes,[2] such as fluidity. One of the uncertainties relates to a heterogeneous distribution of the probe, which, for example, might result from binding to proteins and/or from restricted access to different lipid domains. The freeze-etch electron microscopic pictures (see Figure 1) indicated randomization of the phospholipid introduced by fusion, but they do not tell us whether the phospholipids incorporated are distributed symmetrically or asymmetrically in the membrane. The different response of receptor-mediated and receptor-independent adenylate cyclase activities (Table II) could be indicative of an asymmetric distribution in the membrane (cf. Bretscher, 1973; Houslay et al., 1976) of the incorporated phospholipids. The Arrhenius plots of adenylate cyclase activity have discontinuities even in the "fluid" DMPC-treated membranes at T_c 25 and 21 °C (Figure 4B). These could reflect domains differing in viscosity (Schroeder, 1978). But, neither the fluorescent probe nor the spin-labeled probe sensed different lipid domains in the DMPC-enriched membranes (compare Figures 4B with Figures 5 and 7B). Equally disappointing is the lack of information regarding the "nearest-neighbor lipids" of the enzyme under study. Seelig & Seelig (1978) and Oldfield et al. (1978) have shown by ^2H quadrupole splitting in contrast to previous reports (Dahlquist et al., 1977) that incorporation of cytochrome *c* oxidase, a typical membrane protein into a bilayer, leads to a more disordered state of the lipids, characterized by rapid exchange (rate $> 10^4$ Hz) between lipids in contact with the protein and lipids further away. The rapid rate of exchange makes the existence of boundary lipids,[3] in this case, at least, doubtful. It should be recalled that two enzymes catalyzing vectorial reactions, adenylate cyclase and (Na^+, K^+)-ATPase, although they are present in different concentrations in Chang liver cell membranes and differ structurally and functionally, behaved alike, above and below the phase transitions in the DMPC-enriched membrane (Table II and III) and in Arrhenius plots (not shown). If each enzyme were to have its own specific boundary layer lipids, this uniform behavior would be difficult to explain. Therefore, one should consider other factors aside from chemically defined boundary layer lipids which could restrict the mobility of oligomeric enzymes in membranes in order to guarantee normal functions (Edidin, 1974).

Recent work of Zweig & Singer (1979) suggests that in membranes of reticulocytes "mobile" domains exist or are formed within a larger framework of "immobile" membrane. In these domains concanavalin A receptors are laterally mobile and can be clustered and endocytized. Along these lines and in analogy to the behavior of pyrenedecanoic acid (Figure 5), we wish to suggest that lateral diffusion rate and collision frequency of the β receptors are increased in the DMPC- and DOPC-enriched membranes and that the greater mobility of the receptors is responsible for their disappearance. What might happen to receptors which collide is at present a matter of speculation, as is the possible relevance of the observations reported here for receptor cryptization and for a biological control of membrane fluidity, but the elegant direct visualization by Schlessinger et al. (1978) of binding, aggregation, and internalization of insulin and epidermal growth factor on 3T3 fibroblasts shows a way how to search for lost receptors. Experiments in that direction have been initiated.

Acknowledgments

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[3] The recent evidence concerning boundary layer lipids is discussed in a critical and lucid manner by Chapman et al. (1979).

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Conformational and Immunochemical Analysis of the Cyanogen Bromide Fragments of Thermolysin†

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ABSTRACT: In order to explore the possible existence of folding domains predicted on the basis of protein morphology, we carried out conformational studies for the three cyanogen bromide fragments of thermolysin containing residues 1–120 (FIII), 121–205 (FI), and 206–316 (FII). As judged by far-ultraviolet circular dichroism, FII retains a relative content of α-helical structure similar to that exhibited by the corresponding region in native protein. The conformation of the fragment exhibits significant thermostability. Fragment FI, for which the corresponding region in the native protein contains all of the residues involved in the binding of both the functional zinc and three of the four calcium ions, exhibits a calcium-induced α-helical structure, again as judged by circular dichroism. In order to relate the spectroscopically defined conformations of fragments FI and FII to those of the corresponding regions in native protein, we prepared antisera in rabbits by using thermolysin, FI, and FII as immunogens. As indicated mainly by immunodiffusion, and for FI gel filtration radioimmunoassay, a significant amount of cross-reactivity exists between each of the fragments, especially FII, and native but not denatured (heat-treated, N^{ϵ}-succinylated) thermolysin. The immunochemical data obtained indicate that both FI and FII have the propensity to form stable conformations in solution that are comparable to those required for antigenicity in native thermolysin. The overall conformational data for fragments FI and FII agree with the view that peptides corresponding to domains in globular proteins can form native-like structures independently of the remainder of the molecule.

Physicochemical, functional, and immunological properties of fragments of well-characterized proteins often have been used as a means of elucidating the nature of the forces that direct the folding of sequences of amino acids into specific, biologically active conformations. Fragments obtained from various proteins generally have been found to contain less secondary structure than they exhibit in corresponding regions of intact proteins (Crumpton & Small, 1967; Epand & Scheraga, 1968; Scatturin et al., 1967; Hermans & Puett, 1971; Toniolo et al., 1975). Hence, it is likely that there is a critical set of information required to obtain the necessary medium- and long-range interactions that stabilize the local

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