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THE LIPID ENVIRONMENT OF THE GLUCAGON RECEPTOR REGULATES ADENYLATE CYCLASE ACTIVITY

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SUMMARY

1. The lipid composition of rat liver plasma membranes was substantially altered by introducing synthetic phosphatidylcholines into the membrane by the techniques of lipid substitution or lipid fusion. 40–60 % of the total lipid pool in the modified membranes consisted of a synthetic phosphatidylcholine.

2. Lipid substitution, using cholate to equilibrate the lipid pools, resulted in the irreversible loss of a major part of the adenylate cyclase activity stimulated by F^-, GMP-P(NH)P or glucagon. However, fusion with presonicated vesicles of the synthetic phosphatidylcholines causes only small losses in adenylate cyclase activity stimulated by the same ligands.

3. The linear form of the Arrhenius plots of adenylate cyclase activity stimulated by F^- or GMP-P(NH)P was unaltered in all of the membrane preparations modified by substitution or fusion, with very similar activation energies to those observed with the native membrane. The activity of the enzyme therefore appears to be very insensitive to its lipid environment when stimulated by F^- or GMP-P(NH)P.

4. In contrast, the break at 28.5 °C in the Arrhenius plot of adenylate cyclase activity stimulated by glucagon in the native membrane, was shifted upwards by dipalmitoyl phosphatidylcholine, downwards by dimyristoyl phosphatidylcholine, and was abolished by dioleoyl phosphatidylcholine. Very similar shifts in the break point were observed for stimulation by glucagon or des-His-glucagon in combination with F^- or GMP-P(NH)P. The break temperatures and activation energies for adenylate cyclase activity were the same in complexes prepared with a phosphatidylcholine by fusion or substitution.

5. The breaks in the Arrhenius plots of adenylate cyclase activity are attributed to lipid phase separations which are shifted in the modified membranes according to the transition temperature of the synthetic phosphatidylcholine. Coupling the receptor to the enzyme by glucagon or des-His-glucagon renders the enzyme sensitive to the lipid environment of the receptor. Spin-label experiments support this interpretation and suggest that the lipid phase separation at 28.5 °C in the native membrane may only occur in one half of the bilayer.

INTRODUCTION

The inflections in the Arrhenius plots of the activities of membrane transport proteins and membrane-bound enzymes have been widely attributed to lipid phase transitions and separations in the membrane [1, 2]. The Arrhenius plots for the activity of adenylate cyclase stimulated by various ligands described in the previous paper [3] were consistently linear for stimulation by fluoride or GMP-P(NH)P, but showed an inflection at 28.5 °C if glucagon or des-His-glucagon were present. This suggested that whereas the direct stimulation of the enzyme by fluoride or GMP-P(NH)P might be insensitive to any phase separations occurring in the bilayer, the inflection occurring at 28.5 °C might correspond to a phase separation sensed by the receptor, localised in the outer half of the bilayer. By coupling the receptor to the adenylate cyclase by glucagon or des-His-glucagon, the effect of the lipid phase separation could then be sensed by the enzyme. An alternative possibility is that the inflection represents a conformational change in the receptor or its interaction with the catalytic unit which leads to a change in reaction mechanism or rate-limiting step that is independent of the lipid environment.

We have attempted to resolve these alternatives by making a substantial change in the lipid environment of the receptor adenylate cyclase complex, to determine whether the activity profile of the enzyme or the receptor-enzyme complex is significantly altered. We have used the techniques of lipid-substitution and fusion to introduce up to 60 % of synthetic phosphatidylcholines into the lipid bilayer associated with the membrane. We demonstrate that the coupling of the receptor to the catalytic unit changes adenylate cyclase activity in a manner consistent with the phase properties of the exogenous phosphatidylcholines, whereas the stimulation of the adenylate cyclase by fluoride or GMP-P(NH)P is apparently insensitive to the phase separation which is shown to occur in the lipid bilayer. This is consistent with the hypothesis that by coupling the receptor to the catalytic unit, its activity becomes sensitive to the lipid environment of the receptor.

METHODS

The preparation of rat liver plasma membranes and the determination of adenylate cyclase activity is described in the previous paper [3]. The purification and determination of cholic acid, and the synthesis of lipids have also been described previously [4].

Preparation of lipid-substituted plasma membranes

The endogenous lipids of rat liver plasma membranes were replaced by added exogenous phospholipids using cholate equilibration followed by centrifugation as described in detail elsewhere for sarcoplasmic reticulum [4] and mitochondrial membranes [5]. The lipids used were dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, and dioleoyl phosphatidylcholine.

Plasma membranes (2.7 mg protein) were incubated for 2 h at 4 °C with 10 mg of phospholipid; 5 mg potassium cholate; 50 mM $MgSO_4$; 50 mM ATP; 0.2 mM dithiothreitol; 700 mM KCl; 170 mM sucrose and 35 mM potassium phosphate buffer at a final pH of 8.0 in a volume of 1.6 ml. This mixture was then loaded onto a dis-

continuous sucrose gradient of 0.25 ml 60 % sucrose, 10 mM triethanolamine/HCl buffer, pH 7.2, and 1.7 ml of 25 % sucrose, 10 mM triethanolamine/HCl buffer pH 7.2 and centrifuged for 16 h at 4 °C at 200 000 $\times g$. The particulate material at the boundary of the 60 and 25 % sucrose layers was collected, resuspended and washed in a buffer containing 1 M KCl, 250 mM sucrose and 50 mM triethanolamine/HCl buffer, pH 8.0. There was less than 0.005 mg cholate per mg of phospholipid in the substituted membranes.

Preparation of fused lipid-membrane complexes

Highly sonicated suspensions of dimyristoyl or dioleoyl phosphatidylcholine (50 mg/ml) were prepared in 10 % sucrose, 10 mM triethanolamine/HCl buffer, pH 7.2 as described previously [5]. Sonicated lipid (25 mg) was added to plasma membranes (4.5 mg protein) in a final volume of 2.0 ml containing 15 % sucrose and 10 mM triethanolamine/HCl buffer pH 7.2. This was incubated for 25 min at 25 °C, before cooling to 4 °C. The mixture could then be readily assayed by either diluting 5-fold into the assay cocktail, or by diluting 5-fold with 0.25 M sucrose; 1 M KCl; 50 mM triethanolamine/HCl buffer pH 7.2, and centrifuging for 1 h at 100 000 $\times g$ before resuspending the pellet in 1 mM $KHCO_3$ pH 7.2 for assay.

ESR

Electron spin resonance (ESR) spectra was obtained using a Varian E3 spectrometer with a variable temperature controller. The spin label used was the *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid (Synvar). The concentration of rat liver plasma membrane lipid was approximately 30 mg/ml in 1 mM $KHCO_3$, pH 7.2 and the molar ratio of spin label to lipid was 1 : 100. The order parameter S was obtained from the expression $S = T_{||} - T_{\perp}/T_{zz} - T_{xx}$ where T_{zz} and T_{xx} are the values of the hyperfine coupling tensors in the z and x directions (assuming axial symmetry) and $2T_{||}$ and $2T_{\perp}$ are the separations of the outer and inner hyperfine extrema respectively. A value of 26.3 G was used for $T_{zz} - T_{xx}$ [6].

RESULTS

Composition and activities of lipid-substituted and lipid-fused complexes

Rat liver plasma membrane preparations partially substituted or fused with synthetic phosphatidylcholines were prepared with dioleoyl phosphatidylcholine, dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine and the composition and activities of these complexes are given in Table I. The percentage of defined phosphatidylcholine in the lipid pool was between 48 and 60 %, and the lipid to protein ratio in the substituted membranes was 0.33–0.40 mg lipid per mg of protein, which was significantly less than in the native membranes (0.55 mg/mg). However the complexes prepared by fusion contained a substantially increased proportion of total lipid since the fusion process did not deplete the endogenous lipid pool.

In all of the substitution complexes the residual fluoride or glucagon stimulated activities were between 5 and 20 % of the corresponding activities in the native membrane. Since it was not possible to restore the activity of the adenylate cyclase by back-titration with endogenous lipid from rat liver plasma membranes, we conclude

TABLE I

PROPERTIES OF LIPID-SUBSTITUTED AND FUSED COMPLEXES

Defined lipid	Complex type	% defined lipid in total lipid pool*	Lipid protein ratio (mg/mg)*	Glucagon activity (μunits/mg)		Fluoride activity (μunits/mg)	
				30 °C	37.5 °C	30 °C	37.5 °C
–	Original membranes	– (4)	0.55±0.04	66.1	83.2	11.6	27.2
Dimyristoyl phosphatidylcholine	Substitution	48±5 (4)	0.33±0.03	4.2	8.6	1.7	4.3
Dimyristoyl phosphatidylcholine	Fusion	60±5 (2)	1.35±0.33	30.2	55	10	23
Dipalmitoyl phosphatidylcholine	Substitution	55±5 (3)	0.36±0.03	3.3	8.6	1.5	3.8
Dioleoyl phosphatidylcholine	Substitution	50±5 (4)	0.40±0.03	5.1	8.5	2	4.7
Dioleoyl phosphatidylcholine	Fusion	60±5 (2)	1.35±0.33	19.5	39.8	10.5	22.4

* Data is given ± standard deviation on the mean (no. of samples in brackets).

that the loss of activity was mainly due to the conditions used for the substitution and could be attributed to the use of cholate as the agent used to equilibrate the lipid pools. Addition of cholate alone to the membranes led to substantial and irreversible inactivation of the adenylate cyclase over the time required for substitution to occur.

The fusion technique caused little loss of hormone stimulated adenylate cyclase activity, and the effect on the fluoride stimulated activity was insignificant. This suggests that the defined phosphatidylcholines are structurally competent to support the catalytic activity of the enzyme, at least at proportions up to 60 % of the total lipid pool.

Arrhenius plots of lipid-substituted and lipid-fused complexes

(*i*) *Dioleoyl phosphatidylcholine.* After substitution or fusion with dioleoyl phosphatidylcholine the Arrhenius plots of adenylate cyclase activity stimulated by fluoride were linear (Figs. 1a, b) with the same activation energies as in the native membrane (Table II). Linear plots with similar activation energies (21–23 kcal · mol^{-1}) were obtained for fluoride stimulation of all complexes with defined phosphatidylcholines prepared either by lipid substitution or fusion (Table II, and Figs. 1, 2, 4). The Arrhenius plots of the glucagon-stimulated activity of membranes substituted or fused with dioleoyl phosphatidylcholine were also linear, in contrast to the break at 28.5 °C observed in the native membranes, and the activation energy of 13 kcal · mol^{-1} is between the values for temperatures above and below 28.5 °C for the native membranes. A linear plot with a similar activation energy was also obtained when adenylate cyclase activity was measured in the presence of both fluoride and des-His-glucagon (Fig. 1, Table II).

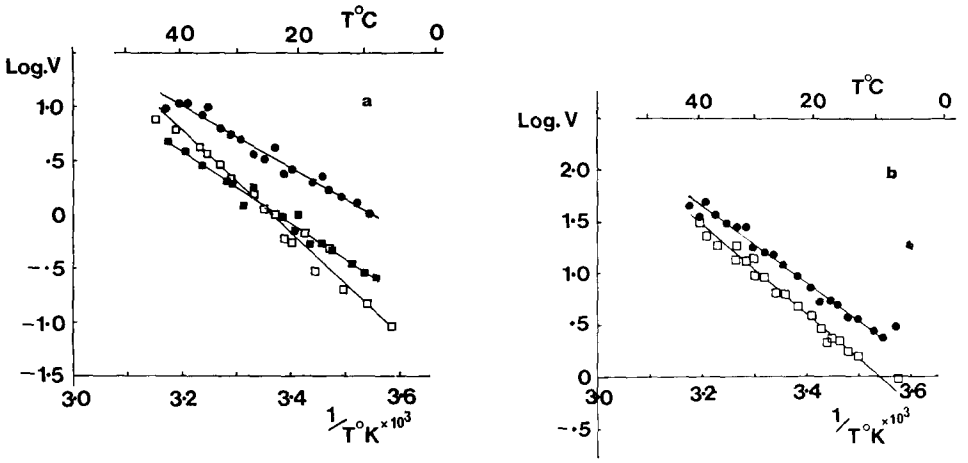

Fig. 1. Arrhenius plots of adenylate cyclase activity in dioleoyl phosphatidylcholine complexes. (a) Substitution complex. Adenylate cyclase activity determined in the presence of 10^{-6} M glucagon, (●); $1.5 \cdot 10^{-2}$ M fluoride, (□); 10^{-6} M des-His-glucagon + $1.5 \cdot 10^{-2}$ M fluoride, (■). (b) Fusion complex. Adenylate cyclase activity determined in the presence of 10^{-6} M glucagon, (●); $1.5 \cdot 10^{-2}$ M fluoride, (□). Reaction velocities in all figures are expressed in μunits/mg. protein.

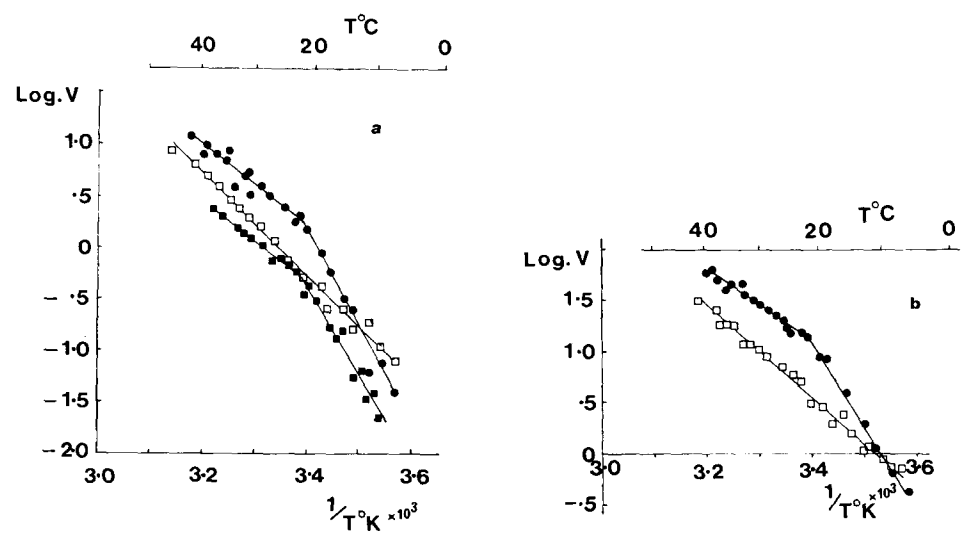

Fig. 2. Arrhenius plots of adenylate cyclase activity in dimyristoyl phosphatidylcholine complexes. (a) Substitution complex. Adenylate cyclase activity determined in the presence of 10^{-6} M glucagon, (●); $1.5 \cdot 10^{-2}$ M fluoride, (□); 10^{-6} M des-His-glucagon + $1.5 \cdot 10^{-2}$ M fluoride, (■). (b) Fusion complex. Adenylate cyclase activity determined in the presence of 10^{-6} M glucagon, (●); $1.5 \cdot 10^{-2}$ M fluoride, (□).

TABLE II

SUBSTITUTION AND FUSION COMPLEXES. ACTIVATION ENERGIES AND BREAK POINTS FOR ARRHENIUS PLOTS OF ADENYL CYCLASE ACTIVITY

Data is given ± standard deviation on the mean (no. of samples in brackets)

Complex	Ligand added	Break point (°C)		Activation energy (kcal/mol^{-1})		
				Above break		Below break
Dioleoyl phosphatidylcholine substitution	Glucagon	–	(6)		13 ±3	
	Fluoride	–	(6)		22 ±2	
	des-His-glucagon +fluoride	–	(2)		16 ±3	
Dioleoyl phosphatidylcholine fusion	Glucagon	–	(2)		17.3±2	
	Fluoride	–	(2)		20 ±3	
Dimyristoyl phosphatidylcholine substitution	Glucagon	22±1	(6)	18±2		43±4
	Fluoride	–	(6)		23 ±1	
	des-His-glucagon +fluoride	22±1	(2)	18±3		40±6
	GMP-P(NH)P	–	(3)		25 ±3	
	GMP-P(NH)P +glucagon	23±1	(2)	16±2		48±4
Dimyristoyl phosphatidylcholine fusion	Glucagon	22±0.5	(2)	16±2		38±2
	Fluoride	–	(2)		21 ±2	
Dipalmitoyl phosphatidylcholine substitution	Glucagon	32.5±1	(2)	20±2		43±6
	Fluoride	—	(2)		23 ±2	

(*ii*) *Dimyristoyl phosphatidylcholine.* Figs. 2a, b show the linear Arrhenius plots for the fluoride stimulated adenylate cyclase activity in complexes substituted or fused with dimyristoyl phosphatidylcholine whereas the glucagon stimulated activities have biphasic plots with breaks at 22 °C, substantially depressed below the break at 28.5 °C characteristic of the native membranes. The activation energies of both components of the plots were similar in both the substituted and fused dimyristoyl phosphatidylcholine complexes, and substantially higher than the corresponding activation energies for the native membrane by 2- to 3-fold. An Arrhenius plot of adenylate cyclase activity in dimyristoyl phosphatidylcholine-substituted membranes stimulated by fluoride and des-His-glucagon, is also shown in Fig. 2a. This plot exhibits a break at 22 °C and activation energies similar to those shown by glucagon alone (Table II) however the specific activity at any temperature is slightly less than that obtained with fluoride alone.

Consistent results are obtained for dimyristoyl phosphatidylcholine substituted membranes stimulated by GMP-P(NH)P alone or in combination with glucagon (Fig. 3). The Arrhenius plot for GMP-P(NH)P stimulated activity is linear with an activation energy of 25 kcal · mol^{-1}, which is only slightly higher than for the native membrane, while the addition of glucagon and GMP-P(NH)P produces a break at

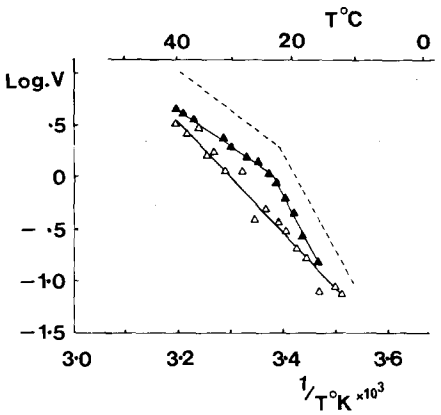

Fig. 3. The effect of GMP-P(NH)P or adenylate cyclase activity in dimyristoyl phosphatidylcholine complexes. Arrhenius plot of adenylate cyclase activity in the presence of 10^{-4} M GMP-P(NH)P, (△); 10^{-6} M glucagon+10^{-4} M GMP-P(NH)P, (▲); glucagon alone, (– – –).

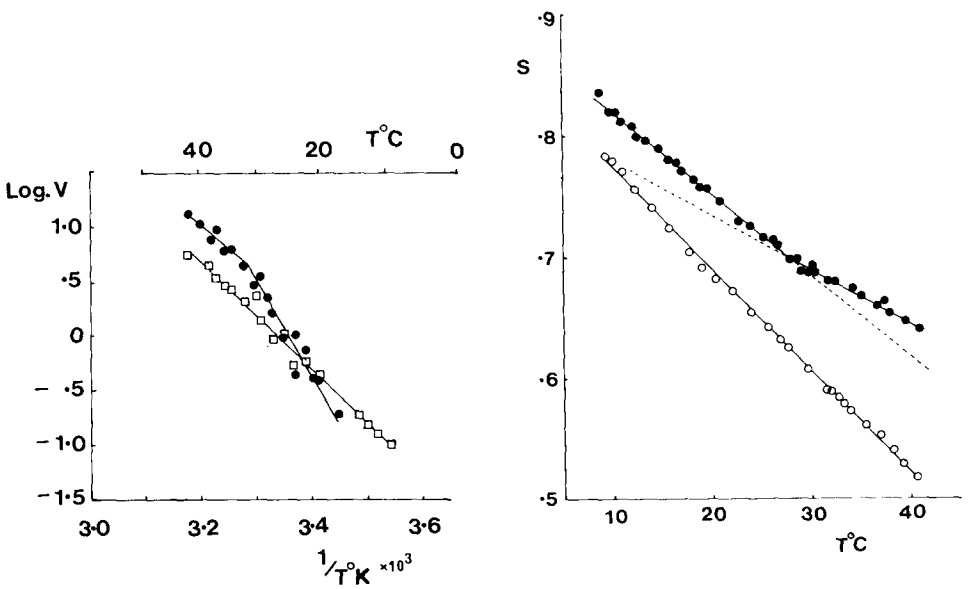

Fig. 4. Arrhenius plot of adenylate cyclase activity in a dipalmitoyl phosphatidylcholine substitution complex. Determined in the presence of 10^{-6} M glucagon, (●); $1.5 \cdot 10^{-2}$ M fluoride, (□).

Fig. 5. Order parameter for 5-doxyl stearic acid as a function of temperature in rat liver plasma membranes. Native membranes, (●); extracted lipids, (○).

23 °C close to that observed with glucagon alone (Fig. 3). The activity of the enzyme stimulated by GMP-P(NH)P and glucagon is significantly lower by 2- to 3-fold than in the presence of glucagon alone. This is qualitatively similar to the antagonistic action of GMP-P(NH)P on glucagon stimulation observed with the native membranes (see previous paper). This suggests that the residual adenylate cyclase activity in the lipid substituted complex remains functionally coupled to the receptor in a similar manner to the native membrane. This is confirmed by the very similar form of the Arrhenius plots in break temperatures and activation energies for complexes prepared by lipid substitution or fusion, although the fusion complexes retain much higher adenylate cyclase activities.

(*iii*) *Dipalmitoyl phosphatidylcholine.* The results obtained for dipalmitoyl phosphatidylcholine-substituted membranes are very similar to those for dimyristoyl phosphatidylcholine substitution, except that the break temperature for glucagon stimulated activity is increased from 28.5 °C to 32.5 °C (Fig. 4). The activation energies for the high and low temperature ranges are very similar to those for the dimyristoyl phosphatidylcholine complexes (Table II). A further similarity is that the activities of fluoride and glucagon stimulated complexes with both dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine converge and cross over at low temperatures (Figs. 2, 4). This is in contrast to the divergence of the Arrhenius plots at low temperatures for fluoride and glucagon stimulated dioleoyl phosphatidylcholine complexes (Fig. 1).

To determine whether the break in the Arrhenius plot of glucagon stimulated adenylate cyclase activity in the native membranes is mediated by the lipids, the order parameter for 5-doxyl stearic acid was measured as a function of temperature. Fig. 5 shows the change in order parameter as the temperature was increased, with a break at about 28 °C. However no break in the corresponding plot for the extracted lipid was observed. These results suggest that if the break in the plot for the native membranes depends on the lipid, then it must be attributed to an asymmetric lipid distribution which is lost in vesicles of the extracted lipid.

DISCUSSION

The adenylate cyclase activity stimulated by fluoride, or GMP-P(NH)P, was virtually insensitive to major changes in the composition of the lipid pool. Thus the activation energies for fluoride stimulated activities were the same for the native membrane in all complexes, whether prepared by fusion or substitution, and were constant over the temperature range 5–45 °C. In contrast, the break in the Arrhenius plot for glucagon stimulated activity disappeared in dioleoyl phosphatidylcholine complexes, was depressed in temperature by dimyristoyl phosphatidylcholine and elevated by dipalmitoyl phosphatidylcholine. These results are consistent with a lipid phase separation in the native membrane at 28.5 °C which is shifted by the insertion of defined phosphatidylcholines into the lipid pool, according to their phase transition temperatures. Dioleoyl phosphatidylcholine has a phase transition at about −22 °C, and we suggest that the linear Arrhenius plot for glucagon-stimulated activity in dioleoyl phosphatidylcholine-complexes can be attributed to a large depression of the phase separation temperature in the mixed lipid pool to below 5 °C. Dimyristoyl phosphatidylcholine, which has a transition temperature at 23.5 °C causes a much

smaller depression of the phase separation temperature, whereas dipalmitoyl phosphatidylcholine raises the temperature because its transition is at 41 °C. The large increase in activation energies observed with the saturated phosphatidylcholines below the phase separation temperatures, in contrast to the reduced activation energy observed in the dioleoyl phosphatidylcholine complexes, is consistent with this interpretation. In particular, the glucagon stimulated activities of the dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine complexes become lower than the fluoride stimulated activities at low temperature because of the sharp drop in activity below the phase separation temperatures in these complexes, which does not significantly influence the fluoride stimulated activity.

There are at least two explanations for the insensitivity of the fluoride stimulated response to lipid substitution. It may be that the catalytic unit does not interact substantially with the lipid chain region of the bilayer and is to be classed as an extrinsic protein attached primarily to the membrane through its interaction with phospholipid headgroups. Alternatively the catalytic unit may be located in the inner half of the bilayer in which no significant phase separations occur to which the catalytic unit is sensitive. For example if the catalytic unit is able to select its immediate lipid environment from the total lipid pool, its temperature activity profile may be determined by the composition of this segregated lipid environment. The fluoride stimulated activities of the samples fused with phosphatidylcholine are within 20 % of the values observed for the native membranes. This suggests that either the phosphatidylcholine headgroup fully supports this activity of the enzyme, or that the enzyme is able to select for its immediate environment those endogenous lipids from the original pool which maintain its temperature-activity profile unaltered. However the larger inhibitions observed in the glucagon stimulated activities of the membranes fused with dioleoyl phosphatidylcholine or dimyristoyl phosphatidylcholine indicate lipid specificity for the headgroup in coupling the receptor to the catalytic unit, in addition to the sensitivity to the lipid chain structure which is caused by coupling the receptor to the catalytic unit. This would be consistent with other work which has implicated specificity of the lipid headgroup in coupling receptors to adenylate cyclase activity [7–9].

The ESR data suggests that in the native membrane, a lipid phase separation occurs at the same temperature as the break in the Arrhenius plot of the adenylate cyclase activity stimulated by glucagon. In the vesicles from the lipid extracted from the membranes no phase separation was detected. We can rationalise these observations by assuming that the lipid phase separation occurs in the native membranes in the outer half of the bilayer which is responsible for modulating the glucagon stimulated activity. Randomising the lipids from the inner and outer halves of the bilayer in the vesicle prevents this phase separation occurring. Taken together, the simplest interpretation of the biochemical and physical evidence is that the catalytic unit is situated in or on the inner half of the bilayer and does not sense phase separations occurring in the outer half of the bilayer when it is physically uncoupled from the receptor.

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