Adenylate cyclase and a fatty acid spin probe detect changes in plasma membrane lipid phase separations induced by dietary manipulation of the cholesterol:phospholipid ratio

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Rats fed with a cholesterol supplement to their diet exhibited an increase in their plasma membrane cholesterol phospholipid (*C/P*)-lipid molar ratio from 0.72 to 0.98, whereas those fed the hypocholesterolaemic drug clofibrate in their diet exhibited a decrease in this ratio to 0.62. The properties of these membranes were analysed with regard to ligand-stimulated adenylate cyclase activity and the mobility of a fatty acid spin probe which allowed lipid phase separations to be identified. Membranes with elevated *C/P* ratios exhibited two distinct lipid phase separations, one at around 36°C that was attributed to the external half of the bilayer and one at around 22°C which was attributed to the inner half of the bilayer. Membranes with lowered *C/P* ratios exhibited a single lipid phase separation occurring at around 21°C which was attributed to the lipids of the inner half of the bilayer. These results were compared with those obtained by manipulation of *C/P* ratios in vitro using liposome-cholesterol exchange techniques. Dietary manipulation of the *C/P* ratio of plasma membranes in vivo led to alterations in the fold stimulation of adenylate cyclase by various stimulatory ligands.

Adenylate cyclase *Cholesterol* *Membrane fluidity* *Glucagon* *Dietary manipulation* *Clofibrate*

1. INTRODUCTION

Glucagon-stimulated adenylate cyclase is a multicomponent system consisting of at least 3 distinct integral proteins distributed asymmetrically in the plasma membrane. These are the receptor for glucagon, the guanine nucleotide regulatory protein N_s and the catalytic unit of adenylate cyclase [1,2]. We have presented considerable evidence that changes in membrane fluidity can influence both the activity of this enzyme and the degree of stimulation achieved by glucagon and ligands such as fluoride and guanine nucleotide analogues which function through N_s [3]. In vitro studies have demonstrated that cholesterol [3–10] and specific phospholipids [3] can influence the functioning of this enzyme and in vivo studies [11] have shown that dietary fat influences rat liver plasma membrane lipid composition and also leads to alterations in the activity of glucagon-stimulated adenylate cyclase.

Cholesterol is a ubiquitous and essential constituent of the plasma membrane of eukaryotic cells. Although its functional significance is unclear, it has the ability to alter bilayer fluidity, suppress non-specific leakage of polar substances across membranes and to create discrete domains in the bilayer by virtue of its interaction with specific phospholipid species (see [3,5,12]). Manipulation of the cholesterol content of rat liver plasma membrane in vitro using liposome-cholesterol exchange techniques leads to pronounced changes in the physical properties of the membrane and in adenylate cyclase activity [4,5]. Here we use

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dietary manipulations to show that functionally significant changes in the cholesterol:phospholipid molar ratio of rat liver plasma membranes can be achieved in vivo. In this study a cholesterol supplement to the diet was used to elevate plasma membrane cholesterol concentrations and the drug clofibrate, which is an effective hypolipidemic and hypocholesterolemic agent in both man and rat [13,14], to decrease plasma membrane cholesterol concentrations.

2. MATERIALS AND METHODS

Adenylate cyclase was assayed as described [15]; incubations were performed at 30°C unless stated otherwise with initial rates taken from linear time courses under all conditions. Stimulating ligands were used at concentrations that were saturating under all conditions and these are specified in the legends [15].

Total lipids were extracted from liver plasma membranes using the method of Johnson [16]. Phospholipid phosphorus was determined by the method of Petitou et al. [17] by using the mineralisation procedure of Morrison [18]. Cholesterol was determined colourimetrically using a horseradish peroxidase assay as detailed by us [4].

Liver plasma membranes were prepared from male Sprague-Dawley rats as described by us [19]. Twelve week old rats were allowed to feed and drink ad libitum, for a 4 week period prior to killing. Control rats were given the standard laboratory food which we have used in all of our previous studies (review [3]); this was Rat Breeding Diet (Oxoid 41B) from Pilsbury, Birmingham, England. The 'high' cholesterol animals had a 10% (w/w) supplement of cholesterol in their food and 'low' cholesterol animals had a 0.01% (w/w) supplement of clofibrate (ethyl 2-*p*-chlorophenoxyisobutyrate) in their food [13,14]. 5′-Nucleotidase, glucose-6-phosphatase and succinate dehydrogenase were assayed as in [19].

ESR procedures were performed as described by us [20–23]. Briefly the *N*-oxyl 4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid, I(3,12) was used as a spin probe at experimentally determined 'low probe' concentrations (9 μg/mg membrane protein). The outer and inner hyperfine splittings, $2T_{\parallel}$ and $2T_{\perp}$ were used to calculate the polarity corrected order parameter S. Arrhenius plots were constructed using S, which has been shown to be a useful parameter for following changes in fluidity in these membranes [20,21]. A Varian E9 EPR spectrometer was used on 2.0 G modulation amplitude, 0.30 s time constant, $\leqslant 5 \times 10^3$ receiver gain, 10 mW microwave power, 9.24 GHz microwave frequency all set on a scan range of 100 G run on a scan time of 4 min.

Clofibrate (Atromid S) was a gift from ICI Pharmaceuticals, Cheshire, England. Creatine kinase, ATP, GTP guanine nucleotide analogues, cyclic AMP and horseradish peroxidase were from Boehringer (England). Phosphocreatine and cholesterol were from Sigma (England). Benzyl alcohol and Norit GS charcoal were from Hopkin and Williams, Essex. Glucagon was a gift from Dr W.W. Bromer, Eli Lilly, IN, USA. [^{3}H]cyclic AMP was from Amersham International, Amersham, England. The spin probe I(12,3) was obtained from Syva, Palo Alto, CA.

3. RESULTS

The cholesterol/phospholipid molar ratio of the liver plasma membranes from animals fed on the standard diet was 0.72 ± 0.06, on the cholesterol-supplemented diet, 0.98 ± 0.03 and on the clofibrate-treated diet, 0.62 ± 0.02 (SE, n = 6 animals). The plasma membrane fractions recovered in all cases contained over 70% of the total adenylate cyclase and 5′-nucleotidase activities and were separated from endoplasmic reticulum and mitochondria as indicated by the fact that they contained less than 1% of the total glucose-6-phosphatase and succinate dehydrogenase activities.

Dietary manipulation of cholesterol concentrations clearly gives rise to a marked change in the fold activation of liver plasma membrane adenylate cyclase to stimulatory ligands (table 1). An enhanced stimulatory effect was seen with the high cholesterol membranes and a decreased effect with the low cholesterol membranes from clofibrate-treated animals. This contrasts with experiments [4,5] where the cholesterol content of the isolated plasma membranes was manipulated in vitro. Under such conditions little alteration in the fold activation, elicited by either fluoride or glucagon, was apparent (table 1).

Table 1

Fold stimulation of plasma membrane adenylate cyclase by various ligands

Stimulating ligand	Control membranes	High cholesterol membranes	Low cholesterol membranes
p[NH]ppG	×12 ± 2	×17 ± 4	× 6 ± 2
NaF	×16 ± 2 (×14 ± 3)[a]	×55 ± 11 (×15 ± 3)[a]	× 4 ± 1 (×16 ± 3)[a]
Forskolin	×20 ± 3	×33 ± 4	×10 ± 2
Glucagon	×18 ± 3 (×22 ± 2)[a]	×45 ± 7 (×16 ± 2)[a]	× 6 ± 2 (×20 ± 3)[a]
Glucagon + GTP	×37 ± 6	×90 ± 15	× 9 ± 2

[a] Data adapted from [4,5] where cholesterol manipulations of comparable magnitude were achieved in vitro using isolated plasma membranes with no changes in other membrane lipid components occurring

All assays were performed at 30°C. The specific activities of basal adenylate cyclase were 12.8 ± 2.0 pmol/min per mg protein (control membranes, C/P = 0.72), 2.7 ± 0.5 pmol/min per mg protein (high cholesterol membranes, C/P = 0.98) and 39.4 ± 3.0 pmol/min per mg protein (low cholesterol membranes, C/P = 0.62) (n = 6 animals, SE). Fold activation is based upon the net increase in activity over the corresponding basal activity. Concentrations of stimulatory ligands were NaF (15 mM), p[NH]ppG (1 mM), glucagon (1 μM) and glucagon (1 μM) + GTP (10 μM). p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate

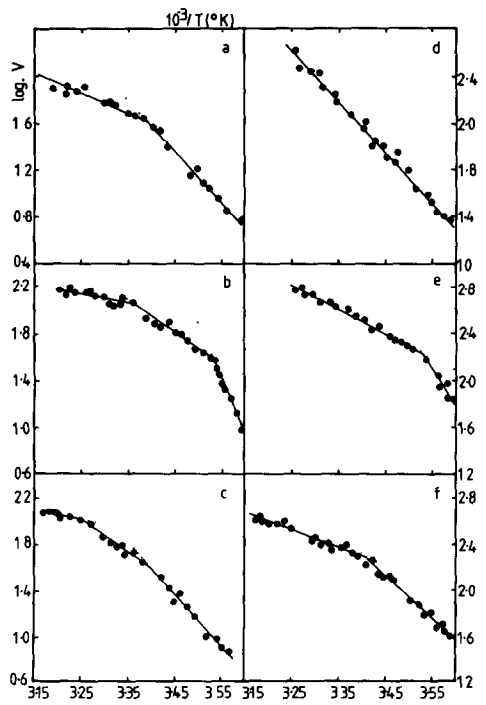

Fig.1. Arrhenius plots of adenylate cyclase activity in rat liver plasma membranes with dietary altered cholesterol content. Plots of adenylate cyclase activity (log V) in pmol/min per mg protein vs $1/T$ (K), for high cholesterol membranes (a,b,c) and low cholesterol membranes (d,e,f). Stimulating ligands were 15 mM NaF (a,d), 10^{-6} M glucagon + 50 mM benzyl alcohol (b,e) and 10^{-6} M glucagon alone (c,f).

In the presence of 50 mM benzyl alcohol the glucagon stimulated activity was increased some 1.7 ± 0.2 fold for control membranes, 1.7 ± 0.3 fold for high cholesterol membranes and 1.6 ± 0.2 fold for low cholesterol membranes. The fluoride-stimulated activity was increased some 2.4 ± 0.3 fold for control membranes, 2.4 ± 0.5 fold for high cholesterol membranes and 2.1 ± 0.4 fold for low cholesterol membranes.

Arrhenius plots of both fluoride- and glucagon-stimulated adenylate cyclase activity from high cholesterol membranes were non-linear (fig.1). The glucagon-stimulated activity showed two distinct breaks at 36 ± 3 and 22 ± 2°C, whereas the

fluoride-stimulated activity showed a single one at 21 ± 2°C. The presence of 50 mM benzyl alcohol caused the temperature at which the breaks occurred in the Arrhenius plot of the glucagon-stimulated adenylate cyclase activity to be depressed by some 10°C, occurring now at 26 ± 2 and 11 ± 2°C (fig.1). In low cholesterol membranes a single break at 21 ± 2°C occurred in Arrhenius plots of the glucagon-stimulated activity. This was decreased by some 9°C, to 12 ± 3°C, when assays were performed in the presence of 50 mM benzyl alcohol. The Arrhenius plot of the fluoride-stimulated activity was linear (fig.2) (SE, $n = 6$ animals).

In ESR experiments using the I(12,3) spin probe, then, in high cholesterol membranes, lipid phase separations occurring at both 34 ± 2 and 22 ± 3°C were evident from Arrhenius-type plots of the polarity corrected order parameter, S. In contrast, there was evidence for but a single lipid phase separation occurring at 20 ± 3°C in low cholesterol membranes (fig.2) (SE, $n = 6$ animals).

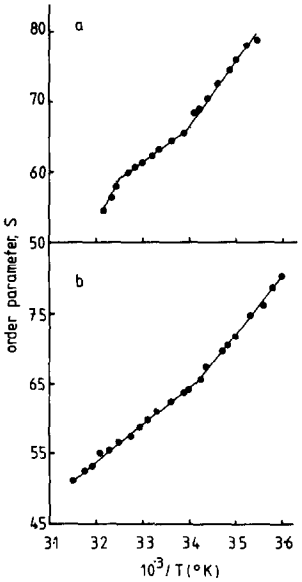

Fig.2. Identification of lipid phase separations occurring in rat liver plasma membranes with dietary manipulated cholesterol content. High cholesterol (a) and low cholesterol (b) liver plasma membranes were loaded with experimentally determined [20,21] 'low probe' concentrations of the spin label I(12,3). This shows Arrhenius plots of the polarity corrected order parameter S vs $1/T$ (K).

4. DISCUSSION

A number of independent physical techniques used by various workers have demonstrated that a lipid phase separation occurs at around 28°C in rat liver plasma membranes (see [3]). A number of criteria have been used to localise this lipid phase separation to the lipids in the external half of the plasma membrane bilayer (see [3]). Thus the activity of adenylate cyclase, when stimulated through the guanine nucleotide regulatory protein N_s is controlled solely by the lipids of the inner half of the bilayer where these components reside [3,22]. Arrhenius plots of NaF-stimulated adenylate cyclase activity thus monitor the inner half of the bilayer and are linear in rat liver plasma membranes [3,5,24]. In contrast, when stimulated by glucagon, a transmembrane complex forms between the glucagon receptor and these units whose activity is modulated by lipids of both halves of the bilayer [25]. Thus the Arrhenius plots of the glucagon-stimulated activity of rat liver plasma membranes exhibit a well-defined break at around 28°C [5,15,24]. The forms of the Arrhenius plots of these two ligand-stimulated states of adenylate cyclase have thus provided useful tools for dissecting out asymmetric changes in the liver plasma membrane [3].

Here we see that high cholesterol feeding elevated plasma membrane cholesterol whereas treatment with the hypocholesterolaemic drug clofibrate, decreased plasma membrane cholesterol. Such actions led to dramatic changes in the forms of the Arrhenius plots of adenylate cyclase activity in liver plasma membrane (fig.1). These are lipid-mediated effects, due to lipid phase separations, as corresponding effects were observed with a fatty acid spin probe inserted into the plasma membranes (fig.2). Also, benzyl alcohol, which increases membrane fluidity [3,20], activates adenylate cyclase [3,20] and decreases the onset of lipid phase transitions/separations [20], elicits an appropriate depression in the temperature at which break points occurred in the Arrhenius plots.

In high cholesterol membranes, Arrhenius plots of both the order parameter S (fig.2) and glucagon-stimulated adenylate cyclase (fig.1) exhibited two distinct breaks at around 36 and 22°C. However, Arrhenius plots of the fluoride-stimulated activity, which were linear using control

membranes, now exhibited a single break at around 22°C (fig.1). This suggests that the break occurring at around 36°C is located in the external half of the bilayer and that at around 22°C can be attributed to the lipids of the inner/cytosol half of the bilayer.

In low cholesterol membranes, Arrhenius plots of both the order parameter *S* (fig.2) and glucagon-stimulated activity (fig.1) exhibited a single break at around 21°C. As Arrhenius plots of the fluoride-stimulated activity were linear then the lipid phase separation occurring at around 21°C may be attributed to the lipids of the external half of the bilayer.

It is of interest to compare these results with those obtained from liver plasma membranes whose cholesterol content was manipulated to a comparable extent in vitro using liposome-cholesterol exchange techniques [4,5]. Comparable high cholesterol concentrations both in vitro [26] and in vivo led to the temperature at which the lipid phase separation occurred in the outer half of the bilayer to be increased from around 28 to around 36°C. However, in vivo manipulation led to a new lipid phase separation occurring in the inner half of the bilayer. Cholesterol depletion by comparable amounts both in vivo and in vitro by either liposome-cholesterol exchange [5] or by the polyene antibiotic amphotericin B [27] led to the temperature at which the lipid phase separation occurred in the outer half of the bilayer to be decreased from around 28 to around 22°C. However, in vitro a new lipid phase separation occurring at around 13°C was apparent [5]. Thus both in vitro and in vivo manipulations of the cholesterol: phospholipid ratio lead to very similar effects on the lipid phase separation occurring in the outer half of the bilayer. This is not true, however, for the inner half of the bilayer where distinctly different effects are exerted for comparable changes in the cholesterol:phospholipid molar ratio. The explanation for this is, however, undoubtedly due to the fact that both high cholesterol and clofibrate treatments have been shown to trigger changes in membrane phospholipid content in rat liver [28–30] and other tissues [31–33]. In the case of clofibrate, part of this effect may be due to its ability to perturb fatty acid metabolism [26,27]. However, in both instances changes in membrane cholesterol content might be expected to trigger the adaptive or homeoviscous mechanism present in mammalian and other cells (see [12,34]). This attempts to normalise the physical properties of membranes under a particular set of environmental conditions. Indeed, we see here that in vivo, despite a dramatic manipulation of membrane cholesterol content, the ability of benzyl alcohol to activate adenylate cyclase was unchanged, suggesting that the fluidity of the environment of this enzyme was similar in all cases. In contrast, alteration of cholesterol content in vitro led to marked differences in sensitivity of the enzyme to activation by benzyl alcohol [4,5]. A further indication that the membrane phospholipid environment of the enzyme might be altered during the in vivo dietary manipulations comes from our observations of the sensitivity of adenylate cyclase to stimulation by various ligands (table 1) where cholesterol-depletion reduced stimulation, whereas high cholesterol enhanced stimulation. This does not appear to be a direct action of cholesterol as its manipulation in vitro [4,5] did not lead to these profound changes (table 1). The precise reasons for these changes in response in vivo, be they lipid or protein-mediated, remain to be determined. Clearly, such alterations in the functioning of a key regulatory enzyme might contribute part of the explanation for the widespread metabolic and other changes that occur in hepatic tissues where cholesterol:phospholipid ratios are perturbed by diet, drugs or disease (see [12,34–36]).

Our study clearly shows that dietary manipulation of the cholesterol:phospholipid ratio in rat liver plasma membranes can lead to alterations in lipid phase separations and the functioning of an integral membrane protein, adenylate cyclase. The changes in lipid phase separations indicate that liver cells have the ability to elicit asymmetric changes in the lipid bilayer. This is consistent with asymmetric changes in the lipid phase separations that are triggered in hamster liver plasma membranes during hibernation [37].

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