Elevated membrane cholesterol concentrations inhibit glucagon-stimulated adenylate cyclase

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A method was devised which increases the cholesterol concentration of rat liver plasma membranes by exchange from cholesterol-rich liposomes at low temperature (4°C). When the cholesterol concentration of liver plasma membranes is increased, there is an increase in lipid order as detected by a decrease in mobility of an incorporated fatty acid spin probe. This is accompanied by an inhibition of adenylate cyclase activity. The various ligand-stimulated adenylate cyclase activities exhibit different sensitivities to inhibition by cholesterol, with inhibition of glucagon-stimulated > fluoride-stimulated > basal activity. The bilayer-fluidizing agent benzyl alcohol is able to reverse the inhibitory effect of cholesterol on adenylate cyclase activity in full. The thermostability of fluoride-stimulated cyclase is increased in the cholesterol-rich membranes. Elevated cholesterol concentrations abolish the lipid-phase separation occurring at 28°C in native membranes as detected by an incorporated fatty acid spin probe. This causes Arrhenius plots of glucagon-stimulated adenylate cyclase activity to become linear, rather than exhibiting a break at 28°C. It is suggested that the cholesterol contents of both halves of the bilayer are increased by the method used and that inhibition of adenylate cyclase ensues, owing to the increase in lipid order and promotion of protein–protein and specific cholesterol–phospholipid interactions.

Cholesterol is an ubiquitous and essential constituent of the plasma membrane of eukaryotic cells. Although its functional significance is far from clear (see, e.g., Kimelberg, 1977), it has the ability to alter bilayer fluidity (Kimelberg, 1977; Lee, 1977; Ladbroke *et al.*, 1968), to suppress non-specific leakage of polar substances across it (Blok *et al.*, 1977), and to create discrete domains within the bilayer (Andrews & Cohen, 1979; Montesano, 1979; Hui *et al.*, 1980; Orci *et al.*, 1981), presumably by virtue of its preferential interaction with specific types of phospholipids (Demel *et al.*, 1977; Van Dijk, 1979). For any particular membrane there is usually a characteristic cholesterol content (cholesterol/phospholipid molar ratio), which is only significantly altered in various disease states and dietary regimens, upon transformation and in aging [see, e.g., Kimelberg (1977) for review].

Hormone-stimulated adenylate cyclases are key regulatory enzymes, which consist of at least three

components, a receptor to recognize hormone, a guanine nucleotide regulatory protein and a catalytic unit, all of which appear to be integral plasma-membrane proteins (Limbird, 1981; Rodbell, 1980; Ross & Gilman, 1980). The activity of this enzyme from various sources appears to be increased by elevations in membrane fluidity (see, e.g., Orly & Schramm, 1975; Dipple & Houslay, 1978; Brasitus & Schachter, 1980; Krall *et al.*, 1981; Needham & Houslay, 1982), although little attempt has been made to assess the effect of decreased lipid fluidity on the activity of this enzyme (see Houslay & Gordon, 1983). Cholesterol can be expected to exert a profound effect on membrane fluidity (Kimelberg, 1977; Houslay & Gordon, 1983), and we have thus investigated the effects of elevated cholesterol concentrations on the glucagon-stimulated adenylate cyclase activity in rat liver plasma membranes and the mobility of a membrane-incorporated spin probe. In order to do this, we have developed a technique for increasing the cholesterol content by using the isolated membranes *in vitro* rather than

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employing either whole animals *in vivo* or intact cells, where adaptive changes in other plasma membrane lipid constituents can be expected to result (Kimelberg, 1977).

Materials and methods

Liver plasma membranes were isolated from male Sprague-Dawley rats weighing 200–300 g as described previously (Houslay *et al.*, 1976a; Marchmont *et al.*, 1981).

Protein was determined by a modification (Houslay & Palmer, 1978) of the micro-biuret method (Goa, 1953), with bovine serum albumin as a standard.

Adenylate cyclase was assayed as previously described (Houslay *et al.*, 1976a); initial rates were taken from linear time-courses under all conditions. Specific binding of ^{125}I -labelled glucagon to rat liver plasma membranes was performed as described by Houslay *et al.* (1977). Thermal-inactivation experiments were performed as described in Needham *et al.* (1982).

Total lipids were extracted from liver plasma membranes and liposomes by using the method of Johnson (1979). Phospholipid phosphorus was determined by the method of Petitou *et al.* (1978) by using the mineralization procedure of Morrison (1964). Cholesterol was assayed by a modification of the clinical assay kit supplied by BDH, Poole, Dorset, U.K. Briefly, dried lipid samples in 100 μl of propan-2-ol were added to 2.5 ml of 'cholesterol-assay solution'. This resulted in a mixture containing (final concns.) 223 mM-Tris/HCl, final pH 7.2, 0.5 mM-4-aminophenazone, 7.5 mM-phenol, 6.0 mM-sodium cholate and horseradish peroxidase (0.02 mg/ml). To this was added 10 μl of cholesterol oxidase (1 mg/ml), and after vortex-mixing the solution was incubated for 15 min at 37°C. The tubes were then allowed to attain room temperature and their A_{500} was read against a reagent blank. A standard curve was constructed for every set of assays with a freshly prepared cholesterol solution in propan-2-ol.

Preparation of cholesterol-rich liposomes

A chloroform/methanol (2:1 v/v) solution containing between 80 and 340 μmol of dipalmitoyl phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), but in some instances either dimyristoyl phosphatidylcholine or dioleoyl phosphatidylcholine, was prepared containing cholesterol at a cholesterol/phospholipid molar ratio of 3:1. Also added were radiolabelled cholesterol (either 2–5 μCi of [^{14}C]cholesterol or 20–40 μCi of [^3H]cholesterol) and either 4–10 μCi of [^3H]phospholipid or 2–10 μCi of glycerol tri[^{14}C]oleate. The solution was evaporated under a stream of N_2 and

then kept under vacuum for 2.5 h. After this, 15 ml of a N_2 -saturated solution containing 240 mM-KCl and 10 mM-MgSO₄ in 80 mM-Tris/HCl buffer, final pH 7.2, was added to the lipid film and the flask shaken vigorously for 20 min under a N_2 atmosphere at 20°C. The flask was then placed in a Megason ultrasonic cleaning bath containing water, at a temperature above the phase transition of the phospholipid, and sonicated for 2–3 h. The lipid dispersion was then centrifuged for 10 min at 15 000 g_{av} . The supernatant, which contained $37 \pm 10\%$ (s.d., $n = 4$) of the phospholipid and $28 \pm 9\%$ of the cholesterol, had a cholesterol/phospholipid ratio between 2.1 and 2.6 and was used immediately for incubation with rat liver plasma membranes.

Liposomes with a cholesterol/phospholipid ratio of 0.7 were prepared in a similar fashion, except that the original chloroform/methanol solution contained cholesterol and phospholipid in the ratio 0.7:1.

Preparation of plasma membranes with elevated cholesterol/phospholipid ratios

Plasma membranes (10–20 mg of protein) were incubated with 10 ml of liposomes, at 4°C, in a solution of total volume 15 ml containing (final concns.) 160 mM-KCl, 6.7 mM-MgSO₄, 16 mM-2-mercaptoethanol, 1 mM-ATP, 5.6% (w/v) sucrose, 21 mM-phosphocreatine, creatine kinase (1 mg/ml) and 53 mM-Tris/HCl, final pH 7.2. After set time intervals over a 6 h period, 200–400 μl samples were withdrawn from the incubation mixture and mixed with 1 ml of 'wash buffer' containing 240 mM-KCl, 10 mM-MgSO₄, 6% sucrose and 80 mM-Tris/HCl buffer, final pH 7.2, in Microfuge tubes. The tubes were then centrifuged at 8500 g for 6 min on a Jobling model 320 micro-centrifuge at 4°C. The supernatant was discarded and the plasma-membrane pellet resuspended in 1 ml of wash buffer and vigorously vortex-mixed. This washing procedure was repeated three times.

Before assay of adenylate cyclase activity or cholesterol determination, samples were resuspended in 100 μl of 50 mM-Tris/HCl buffer, pH 7.2.

When washed samples were taken for scintillation counting, the final pellet was resuspended in 100 μl of sodium cholate (10 mg/ml), pH 7.2 (see Warren *et al.*, 1975, for purification of cholate), in the Microfuge tubes and, after vortex-mixing, left overnight at 4°C in order to stabilize. The resulting solution was transferred to a scintillation vial together with 2 $\times$ 150 μl of sodium cholate (10 mg/ml) used to wash out the Microfuge tube.

Preparation of cholesterol-enriched liposomes containing entrapped [^3H]inulin

Cholesterol-rich liposomes containing glycerol tri[^{14}C]oleate were prepared as described above,

except that only 10 ml of buffer mixture was used to resuspend the lipid film and the resuspension buffer contained 50 μ Ci of [^{3} H]inulin. After centrifugation as above, the cholesterol-rich liposomes were passed down an Ultrogel AcA 34 column (18 cm $\times$ 1 cm diam.), which had been packed under gravity and pre-equilibrated with 5 bed vol. of 240 mM-KCl/10 mM-MgSO₄/80 mM-Tris/HCl buffer, pH 7.2, at room temperature. After application of a 5 ml sample of liposomes, the column was run at 15 ml/h and 1.5 ml fractions were collected. The vesicles eluted at the void volume, which contained both glycerol tri[^{14} C]oleate and [^{3} H]inulin, were taken for incubation with plasma membranes.

Preparation of dipalmitoyl phosphatidylcholine liposomes containing entrapped inulin[^{14} C]carboxylic acid

Dipalmitoyl phosphatidylcholine liposomes containing the ^{3} H-labelled phospholipid (5 μ Ci) were prepared as described above, except that 20 μ Ci of inulin[^{14} C]carboxylic acid was added to the resuspension buffer. The liposomes were subjected to gel filtration as described above.

Sucrose-density-gradient treatment of cholesterol-enriched plasma membranes

After incubation of plasma membranes with cholesterol-enriched liposomes, the samples were washed twice, as detailed above. The resuspended pellet was then placed on a discontinuous sucrose-density gradient formed of equal volumes of 10, 20, 30 and 42.5% (w/v) sucrose in 240 mM-KCl/10 mM-MgSO₄/80 mM-Tris/HCl, pH 7.2, and then centrifuged at 100 000 g_{av} , for 5 h at 4°C in a 3 $\times$ 25 ml swing-out rotor on a MSE PrepSpin 65 centrifuge. Plasma membranes were collected at the 20–30% (w/v) sucrose interface, washed twice in 240 mM-KCl/10 mM-MgSO₄/80 mM-Tris/HCl buffer, pH 7.2, and stored at –80°C.

Materials

Creatine kinase, ATP, GTP, cyclic AMP and triethanolamine hydrochloride were from Boehringer. Phosphocreatine, dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine were from Sigma. Glucagon was kindly given by Dr. W. W. Bromer, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Benzyl alcohol and charcoal Norit GSC were from Hopkin and Williams, Chadwell Heath, U.K. Dioleoyl phosphatidylcholine and [^{3} H]dimyristoyl phosphatidylcholine were kindly given by Dr. G. A. Smith, University of Cambridge. [^{3} H]AMP, [^{3} H]cholesterol, [^{14} C]cholesterol, cyclic [^{3} H]AMP, glycerol tri[^{3} H]oleate, glycerol tri[^{14} C]oleate, [^{3} H]inulin, inulin[^{14} C]carboxylic acid and ^{125} I were

from The Radiochemical Centre, Amersham, Bucks., U.K. The *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-oxostearic acid, I(12,3), was obtained from Syva Co., Palo Alto, CA, U.S.A.

Spin-labelling and e.s.r. analysis of liver plasma membranes

Methods for e.s.r. studies were essentially as described previously in some detail (Gordon *et al.*, 1978, 1980). Native and cholesterol-enriched liver plasma membranes were each suspended in 50 mM-triethanolamine/HCl/8% (w/v) sucrose, final pH 7.6, at 4 mg of protein/ml. These samples were added to the I(12,3) spin probe that had initially been deposited on the side of the tube by evaporation from aqueous ethanol to yield an experimentally determined low probe concentration of 6 μ g of I(12,3)/mg of membrane protein (Sauerheber *et al.*, 1977) at 30°C. Spectra were recorded on a Varian E-104A e.s.r. spectrometer and the cavity temperature was calibrated as specified by Gordon *et al.* (1978).

The outer ($2T_{||}$) and inner ($2T_{\perp}$) hyperfine splittings were determined from 'unexpanded spectra with magnified wings' (Gordon *et al.*, 1978). The following order parameter expressions (Gordon & Sauerheber, 1977) may be used to evaluate the flexibility of the membrane-incorporated fatty acid spin probe:

$$S(T_{||}) = \frac{1}{2} \left[\frac{3(T_{||} - T_{xx})}{(T_{zz} - T_{xx})} - 1 \right] \quad (1)$$

$$S = \left(\frac{T_{||} - T_{\perp}}{T_{zz} - T_{xx}} \right) \left(\frac{a_N}{a_{N'}} \right) \quad (2)$$

Here, T_{xx} and T_{zz} are the hyperfine splitting elements of the static interaction tensor ($\mathbf{T}$) parallel to the static Hamiltonian ($\mathbf{H}$) principal nuclear hyperfine axes x and z respectively. The x axis is parallel to the N–O bond direction, and the z axis is parallel to the nitrogen $2p$ orbital. The elements of $\mathbf{T}$ used in this study were earlier determined by incorporating nitroxide derivatives into host crystals as substitutional impurities: $(T_{xx}, T_{zz}) = (6.1, 32.4)$ G (Seelig, 1970). $a_{N'}$ and a_N are the isotropic hyperfine coupling constants for the probe in the membrane and crystal states respectively [i.e. $a_{N'} = 1/3(T_{||} + 2T_{\perp})$ and $a_N = 1/3(T_{zz} + 2T_{xx})$].

The order parameters S and $S(T_{||})$ reflect the membrane fluidity (or, more accurately, the flexibility of the membrane-incorporated probe). S and $S(T_{||})$ may each assume values between 0 and 1; these extreme order parameters indicate that the probe samples fluid and immobilized environments respectively. The order parameter S , which requires both hyperfine splittings, corrects for small polarity differences between the membrane and reference

crystal. Although $S(T_H)$ does not include corrections for polarity contributions, this expression has been found to be a useful approximate measure of the 'fluidity' in those cases where only the outer hyperfine splitting is usable (Sauerheber *et al.*, 1977).

Results

Preparation of plasma membranes with an increased cholesterol/phospholipid ratio

Native rat liver plasma membranes have a cholesterol/phospholipid ratio of 0.71 ± 0.018 (mean $\pm$ S.E.M. of 11 determinations on different plasma-membrane preparations) and a cholesterol/protein ratio of 0.26 (μM -cholesterol/mg of protein). Incubation of native plasma membranes with cholesterol-rich liposomes at an endogenous-cholesterol/exogenous-cholesterol ratio of up to 1:40 could increase the plasma-membrane cholesterol/phospholipid ratio to 1.04 with a 6 h incubation at 4°C (see Fig. 1a). The increase in the cholesterol/phospholipid ratio of the plasma membranes was independent of the ATP concentration in the incubation medium, and rates of transfer were independent of the type of phospholipid used to form the carrier cholesterol-rich liposomes, whether dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine or dioleoyl phosphatidylcholine was used. The rate of cholesterol transfer varied over the 6 h incubation period, there being an initial fast rate over the first hour, followed by a slower, relatively linear, period occurring over the next 5 h (Fig. 1a). Membranes incubated with cholesterol for 2 h, then washed as detailed in the Materials and methods section before resuspension in fresh cholesterol-rich liposomes, did not exhibit a second initial fast rate of cholesterol transfer, but instead showed a rate of transfer corresponding to the slower second phase in Fig. 1(a). If, however, membranes were incubated with liposomes having a cholesterol/phospholipid ratio of 0.7 there was no alteration in the cholesterol/phospholipid ratio of the plasma membranes (Fig. 1a).

The transfer of cholesterol was normally followed by the radiometric determination of cholesterol and phospholipid associating with the membrane; however, identical results could be obtained by using enzymic/chemical analysis of cholesterol and phospholipid. The cholesterol-rich liposomes were stable under the incubation conditions for periods up to 12 h, and no cholesterol or phospholipid was precipitated by centrifugation, as used in the washing procedure, over this period.

When radiolabelled cholesterol and either radiolabelled glycerol trioleate or radiolabelled phospholipid were used, there was a marked alteration in $^3\text{H}/^{14}\text{C}$ ratio of the membranes compared with the

Fig. 1. Incubation of rat liver plasma membranes with cholesterol-rich liposomes

(a) Rat liver plasma membranes with a cholesterol/phospholipid (C/P) molar ratio of 0.72 were incubated at 4°C under the conditions described in the Materials and methods section with either cholesterol-enriched liposomes (●) (C/P = 2.10) or liposomes (○) with a C/P ratio (0.72) identical with that of the native membranes. After the indicated periods of incubation, samples were taken and washed to remove the liposomes. The membranes were then analysed for their cholesterol and phospholipid contents. The change in cholesterol/phospholipid molar ratio of the membranes is plotted as a function of time. (b) In the same experiment samples were taken for assay of glucagon-stimulated adenylate cyclase activity at 30°C . Data are for membranes incubated with cholesterol-enriched liposomes (●) (C/P = 2.10) or for membranes incubated with liposomes (○) with a C/P molar ratio (0.7) identical with that of the plasma membranes.

cholesterol-rich liposomes. This ratio altered progressively with time of incubation (results not shown), indicating that there was a selective transfer of cholesterol, rather than phospholipid, to the

membranes from the cholesterol-rich liposomes. Over the 6 h incubation period, the amount of liposomal phospholipid associated with the plasma membrane represented less than 6% of the total endogenous plasma-membrane phospholipid, whereas the liposomally derived cholesterol attained values equal to 40–50% of the endogenous membrane cholesterol.

The washing procedure employed to separate the cholesterol-rich liposomes from the plasma membrane was effective in removing about 95% of the liposomes taken in the sample volume after a 6 h incubation. It can be calculated that the remaining liposomes do not contain sufficient cholesterol to alter the apparent cholesterol/phospholipid ratio of the membranes, as determined by cholesterol analysis. Such liposomes with tracer ^3H -labelled phospholipids were made containing entrapped inulin- ^{14}C carboxylic acid and incubated with the plasma membranes under standard conditions. After washing, the $^3\text{H}/^{14}\text{C}$ ratio of the plasma membranes was identical with that of the liposomes, indicating that the small fraction of phospholipid associated with the plasma membrane after the incubation was in the form of intact, inulin-containing, liposomes rather than phospholipid fused into the membranes. Similar experiments were performed with labelled cholesterol and inulin. This residual liposomal contamination could be finally removed from the membrane by discontinuous sucrose-density-gradient centrifugation. Here, plasma membranes collected at the 20%/30%-sucrose interface, and liposomes collected at the 4.6%/10%-sucrose interface. After this treatment there appeared to be no appreciable contamination of the plasma membranes with exogenous phospholipid (<1% of endogenous phospholipid pool). For spin-label studies, membranes were routinely purified through to this stage, whereas for adenylate cyclase studies it sufficed to wash by centrifugation as detailed in the Materials and methods section.

Adenylate cyclase activity in cholesterol-enriched liver plasma membranes

Altered cholesterol/phospholipid ratios in rat liver plasma membranes have marked effects on the activities of basal and ligand-stimulated adenylate cyclase. Incubation of plasma membranes at 4°C with cholesterol-rich liposomes led to a progressive decrease in glucagon-stimulated adenylate cyclase activity over a 6 h period, whereas the presence of liposomes with a cholesterol/phospholipid ratio identical with that of the native membranes (0.72) had no effect on activity (Fig. 1b). Similar results were obtained for basal and fluoride-stimulated activities (results not shown). When the changes in activities are plotted as a function of the chol-

esterol/phospholipid ratio, we observe that basal and all ligand-stimulated activities were progressively inhibited as the cholesterol/phospholipid ratio was increased (Fig. 2). The glucagon-stimulated activity is clearly most sensitive to inhibition by increased cholesterol concentration, followed by the fluoride-stimulated and then the basal activity. If we assume that the inhibition of all these activities reaches a plateau, and defining ID_{50} as the cholesterol/phospholipid molar ratio at which 50% of this inhibitory effect is exerted, then the ID_{50} values are 0.81 for glucagon plus low GTP (50 nM), 0.81 for glucagon plus high GTP (0.1 mM), 0.91 for fluoride and 0.97 for basal.

The inhibitory effect on adenylate cyclase was only elicited if the cholesterol/phospholipid ratio of the membranes was raised. Incubation with liposomes with cholesterol/phospholipid ratio identical with that of the native membranes had no effect on the activities of the enzyme (Fig. 1b). This inhibitory effect was also independent of the carrier phospholipid used to form the cholesterol-rich liposomes, and for any particular cholesterol/phospholipid ratio achieved very similar adenylate cyclase activities were observed, even though incubation times, at 4°C , could be altered by varying the ratio of liposomes to plasma membranes. Finally, incubation of cholesterol-enriched plasma membranes, with either cholesterol-free liposomes or liposomes having a cholesterol/phospholipid ratio of 0.72, led to an increase in adenylate cyclase activity, such that at native cholesterol/phospholipid ratios greater than 90% of the original activity was attained. Thus adenylate cyclase was not being denatured through incubation at 4°C with liposomes.

The heightened sensitivity of glucagon-stimulated adenylate cyclase to inhibition by increased cholesterol content of the membranes is not due to an effect on glucagon binding to its receptor. The specific binding of glucagon to the membrane remained unaffected at cholesterol/phospholipid ratios of 1.0 (specific binding of ^{125}I -labelled glucagon was $104 \pm 3\%$ of the native value; means $\pm$ S.D., $n = 3$).

Thermostability of adenylate cyclase

The thermostability of fluoride-stimulated adenylate cyclase was examined in both native and cholesterol-enriched liver plasma membranes. As shown in Fig. 3, the thermostability of fluoride-stimulated adenylated cyclase in cholesterol-enriched membranes was markedly increased. The half-life for inactivation at 40°C was 3.4 ± 0.3 min in native membranes and 8.4 ± 1.7 min in membranes with a cholesterol/phospholipid ratio of 0.89 (S.D., $n = 3$).

Fig. 2. Response of ligand-stimulated adenylyl cyclase activity and the mobility of a fatty acid spin probe, in rat liver plasma membranes, to increased cholesterol concentrations

(a) Basal enzyme activity; (b) fluoride-stimulated activity; (c) activity with glucagon + low (50 nM) GTP, under which conditions the enzyme is activated as in a Mobile Receptor Model (Houslay *et al.*, 1980a); (d) activity with glucagon + high (100 μ M) GTP, under which conditions the enzyme is activated as in a Collision Coupling Model (Houslay *et al.*, 1980a); (e) the 'fold' activation elicited by glucagon + low GTP over the basal activity derived by dividing curve (c) by curve (a); data are shown where appropriate pairs are available to yield a true ratio; (f) the change in order parameter $S(T_{II})$ for membranes labelled with 6 μ g of I(12,3)/mg of membrane protein. In all instances the assay temperature was kept at 30°C and the data represent individual experiments on plasma samples which were incubated with cholesterol-enriched liposomes at 4°C, with subsequent washing and analysis as described in the Materials and methods section. Results are means $\pm$ s.d. ($n = 3$).

Spin-label analysis of native and cholesterol-enriched liver plasma membranes

Fig. 2(f) clearly demonstrates that augmenting the cholesterol content of rat liver plasma membranes leads to an increase in the value of the order parameter $S(T_{II})$. Native and cholesterol-enriched

membranes were each labelled with experimentally determined low concentrations of I(12,3) by the protocol of Sauerheber *et al.* (1977). As cholesterol enrichment of native plasma membranes induced similar increases in the polarity-corrected order parameter S (results not shown), the results in Fig. 4 indicate that the incorporation of cholesterol into the

Fig. 3. Thermostability of fluoride-stimulated adenylate cyclase in native and cholesterol-enriched membranes. Experiments were carried out as described previously (Needham *et al.*, 1982), except that the plasma membranes were incubated at 40°C before assay at 30°C. The data show a semi-logarithmic plot which describes the decay of activity in native membranes (O; cholesterol/phospholipid = 0.72) and cholesterol-enriched membranes (●; cholesterol/phospholipid = 0.97). For reasons discussed previously (Needham *et al.*, 1982), we believe that these apparently linear decay plots primarily reflect the lability of the catalytic unit.

bilayer decreases the flexibility of the probe and hence lowers the fluidity of the membrane environment of the probe. The increase in $S(T_{II})$ achieved by increasing the cholesterol/phospholipid molar ratio from approx. 0.62 to 0.95 is equivalent to the change in fluidity of native membranes effected by a 6°C decrease in temperature.

Effect of benzyl alcohol on adenylate cyclase activity and the order parameter $S(T_{II})$ of I(12,3)-labelled membranes

In native rat liver plasma membranes, benzyl alcohol has previously been shown to activate both glucagon- and fluoride-stimulated adenylate cyclase and to decrease the value of the order parameter $S(T_{II})$ for I(12,3)-labelled membranes (Gordon *et al.*, 1980). Such effects on adenylate cyclase activity were maximal over a concentration range of 40–60 mM (Dipple & Houslay, 1978; Gordon *et al.*, 1980). Table 1 illustrates the effects of 50 mM-benzyl alcohol on both the glucagon- and fluoride-stimulated adenylate cyclase activities, as well as the change in order parameter $S(T_{II})$ for the incorporated I(12,3) spin probe, in native plasma membranes and

Fig. 4. Temperature-dependence of fluoride- and glucagon-stimulated adenylate cyclase activities in cholesterol-enriched liver plasma membranes. These are Arrhenius plots of (■) fluoride-stimulated and (●) glucagon-plus-low-GTP-stimulated adenylate cyclase activities in cholesterol-enriched membranes. The data are representative of those obtained from four plots for each ligand using different membrane preparations with cholesterol/phospholipid ratios of 0.97 ± 0.03 (s.d., $n = 4$). In native membranes assayed under these conditions, the glucagon-stimulated activity, while obeying a Mobile Receptor Model of activation, yields Arrhenius plots with a well-defined break at around 28°C, whereas those for the fluoride-stimulated activity are linear (Houslay *et al.*, 1976a; Dipple & Houslay, 1978; Rene *et al.*, 1978; Houslay *et al.*, 1980a).

Table 1. Effect of benzyl alcohol on both ligand-stimulated adenylate cyclase activities and the order parameter $S(T_{II})$ of an incorporated fatty acid spin probe in native plasma membranes and plasma membranes with elevated cholesterol concentrations

Observations were made at 30°C as described in the Materials and methods section. Native membranes had a cholesterol/phospholipid ratio of 0.70, whereas the ratio for the elevated-cholesterol ones was 0.95. Results are means $\pm$ s.d. for four experiments.

Membrane sample	Elevation achieved by 50 mM-benzyl alcohol (%)		$\Delta S(T_{II})$ (%)
	Fluoride-stimulated adenylate cyclase	Glucagon-stimulated adenylate cyclase	
Native membranes	160 $\pm$ 5	155 $\pm$ 14	-5.7 $\pm$ 1.0
Elevated-cholesterol membranes	240 $\pm$ 20	241 $\pm$ 20	-6.2 $\pm$ 2.1

Fig. 5. Temperature-dependence of the order parameter $S(T_0)$ for I(12,3)-labelled rat liver plasma membranes enriched with cholesterol

$S(T_0)$ is plotted as a function of the reciprocal of the absolute temperature; here, the order parameter $S(T_0)$ was calculated as in the Materials and methods section for cholesterol-enriched membranes loaded with $6\mu\text{g}$ of I(12,3)/mg of protein. Results are means $\pm$ 1 S.D. for three different cholesterol-enriched preparations, having cholesterol/phospholipid molar ratios of 0.98, 0.97 and 0.94; the absence of an error bar indicates that the calculated s.d. is less than the size of the symbol denoting $S(T_0)$. Cholesterol-enriched membranes were prepared as in the Materials and methods section.

cholesterol-enriched plasma membranes. The degree of activation of adenylate cyclase elicited by optimal benzyl alcohol concentrations is greater in the cholesterol-enriched membranes than in the native membranes at 30°C . Indeed, exposure of both the native and cholesterol-enriched membranes to 50mM -benzyl alcohol yields very similar values for the final specific activities in each instance. This appears to be true for both the fluoride- and glucagon-stimulated activities in cholesterol-enriched membranes, which attain 104% and 93% respectively of that observed in the native membranes. This implies that this concentration of benzyl alcohol completely overcomes the inhibitory actions of cholesterol.

However, in contrast with these effects on adenylate cyclase activity, 50mM -benzyl alcohol exerts similar decreases in the value of the order parameter $S(T_0)$ for the spin probe inserted into either the cholesterol-enriched or native membranes (Table 1).

Arrhenius plots of adenylate cyclase activity and the order parameter $S(T_0)$ for cholesterol-enriched membranes

In membranes with cholesterol/phospholipid molar ratios of around 1.0, the Arrhenius plots for both glucagon- and fluoride-stimulated activities are linear over the temperature range studied (Fig. 4), with similar activation energies of 95.7 ± 8.9 and $105.9 \pm 17.4\text{ kJ/mol}$ respectively (s.d., $n = 4$). Elevation of the cholesterol/phospholipid ratio appears to induce similar actions on Arrhenius-type plots of the order parameter $S(T_0)$ for I(12,3)-labelled plasma membranes for the range $3\text{--}39^\circ\text{C}$ (Fig. 5). Certainly, the high-temperature onset occurring at 28°C in native membranes, and normally detected in Arrhenius-type plots of $S(T_0)$ over the temperature range studied, is not apparent. Nevertheless, there is an indication that the low-temperature onset at approx. 19°C (Gordon *et al.*, 1980) is not completely obliterated by these augmented cholesterol concentrations (Fig. 5).

Discussion

The values for the cholesterol/phospholipid molar ratio and the cholesterol/protein ratio found for our liver plasma membranes are very similar to those observed by other workers (Colbeau *et al.*, 1971; Emmelot & Van Hoeven, 1975; Luly & Shinitzky, 1979).

Incubation of rat liver plasma membranes with cholesterol-rich liposomes leads to the transfer of cholesterol from the liposomes to the plasma membrane. Such an interpretation is consistent with the selective association of radiolabelled cholesterol with the plasma membrane, whereas little if any radiolabelled phospholipid or radiolabelled glycerol trioleate, a non-exchangeable triacylglycerol used routinely as an indicator of liposome function with membranes (see, e.g., Johnson & Zilversmit, 1975; Crain & Zilversmit, 1980), resides with the washed membranes. The small amount of phospholipid contaminating the washed membrane fraction appears to be due to intact liposomes, as indicated by the continued association of, presumably entrapped, inulin at a ratio identical with that observed for the native inulin-containing liposomes. Such residual contaminating liposomes can be removed from the washed membranes by discontinuous-sucrose-density-gradient centrifugation. The data here do not, however, allow us to make any detailed speculation on the mechanism of cholesterol transfer to the plasma membrane from the liposomes.

Under the incubation conditions developed and specified here, this procedure allows for the manipulation of membrane cholesterol at low temperatures (4°C), which are essential for maintaining the stability of labile proteins, with negligible fusion of

the carrier liposomes with the membrane. Our method thus has marked advantages over using prolonged incubations of whole cells, where adaptive changes in the phospholipid components will occur (see Kimelberg, 1977), or employing crude lipoprotein fractions from serum, which again use elevated temperatures and also have been shown to influence adenylate cyclase activity (Pairault *et al.*, 1977; Ghiselli *et al.*, 1981). The extent to which carrier liposomes for cholesterol might fuse with the membrane is a major concern in the interpretation of such studies, as insertion of defined phospholipids into liver plasma membranes profoundly modulates both the membrane lipid structure and the activities of ligand-stimulated adenylate cyclase (Houslay *et al.*, 1976a,b; Bakardjieva *et al.*, 1979). Since incorporation of exogenous phospholipid is negligible with our technique *in vitro*, we are now able to analyse unambiguously the actions of cholesterol loading on membrane order and adenylate cyclase activity.

Increasing the cholesterol content of rat liver plasma membranes leads to an inhibition of both basal and ligand-stimulated adenylate cyclase activities. This effect is independent of the nature of the carrier phospholipid used to form the liposomes. It also does not appear to be due to denaturation over the incubation period. This is because treatment with liposomes with cholesterol/phospholipid molar ratio identical with that of native membranes has no effect on the adenylate cyclase activity, and also similar degrees of inhibition can be obtained by using different lengths of incubation with liposomes of various cholesterol/phospholipid ratios. The effect is reversible on removal of cholesterol from the membranes.

Concomitant with our observation that both glucagon- and fluoride-stimulated adenylate cyclase activities are inhibited by increasing the cholesterol content of the membranes, we note a parallel increase in the value of the order parameter $S(T_0)$, using a nitroxide-labelled fatty acid as a probe of membrane structure (Fig. 2). This signifies that, at 30°C, cholesterol exerts an ordering effect on the bilayer, decreasing the 'fluidity' of the liver plasma membrane. Such an observation is consistent with investigations on model lipid systems, where cholesterol has been shown to exert an ordering and condensing effect on lipid bilayers (Ladbroke *et al.*, 1968; Lee, 1977). Furthermore, in accord with its actions on model membrane systems (Ladbroke *et al.*, 1968; Kimelberg, 1977; Lee, 1977), cholesterol appears to abolish the lipid-phase separation occurring in liver plasma membranes (Fig. 5). Although native membranes exhibit a well-defined lipid-phase separation, with a high-temperature onset at 28°C and a low-temperature onset at 19°C (Hui & Parsons, 1976; Houslay *et al.*, 1976a; Dipple & Houslay, 1978; Gordon *et al.*, 1978, 1980; Luly &

Shinitzky, 1979; Livingstone & Schachter, 1980), that we have proposed is localized to the external half of the bilayer (Houslay *et al.*, 1976b, 1981; Whetton *et al.*, 1982), membranes with elevated cholesterol concentrations exhibit no such lipid-phase separation (Fig. 5).

We have previously demonstrated that increases in bilayer fluidity achieved by either neutral (Dipple & Houslay, 1978; Gordon *et al.*, 1980) or charged (Houslay *et al.*, 1980b, 1981) local anaesthetics leads to a pronounced activation of ligand-stimulated rat liver plasma-membrane adenylate cyclase activity. Similarly, ligand-stimulated adenylate cyclase from a variety of sources, such as turkey erythrocytes (Orly & Schramm, 1975), rat enterocytes (Brasitus & Schachter, 1980), uterine smooth muscle (Krall *et al.*, 1981), human thyroid (Amir *et al.*, 1981) and rat brain striatum (Needham & Houslay, 1982), have all been shown to be activated by agents that increase bilayer fluidity. Such activation has been presumed to be due to increased bilayer fluidity, as they exhibited the same concentration-dependence and the activation was observed for the membrane-bound, but not the solubilized, enzyme (Dipple & Houslay, 1978; Houslay & Gordon, 1983). Furthermore, shifts in the onset temperature of lipid-phase separation elicited by these agents were paralleled by similar shifts in the break temperatures observed in Arrhenius plots of glucagon-stimulated adenylate cyclase activity (see Houslay *et al.*, 1981; Houslay & Gordon, 1983). This again suggests that the activity of adenylate cyclase is modulated by the physical properties of its lipid environment. We therefore propose that the inhibition of adenylate cyclase achieved by elevated cholesterol concentrations is due, at least in part, to the decrease in bilayer fluidity mediated by cholesterol. That the perturbation of the bilayer by elevated cholesterol concentrations does indeed affect adenylate cyclase can be noted from observations of the Arrhenius plots of its activities (Fig. 4). In this instance the well-defined break at 28°C in Arrhenius plots of glucagon-stimulated adenylate cyclase from native membranes (Dipple & Houslay, 1978; Houslay *et al.*, 1976a) is abolished in membranes with elevated cholesterol concentrations (Fig. 4). Arrhenius plots of the fluoride-stimulated activity, which are linear for native membranes, as the activity of this enzyme when not coupled to the glucagon receptor is modulated only by the inner half of the bilayer (see Houslay *et al.*, 1981; Houslay & Gordon, 1983), remain linear when cholesterol-enriched membranes are used as a source of the enzyme. However, the activation energy for this reaction was elevated above that seen for the enzyme in native membranes (Dipple & Houslay, 1978). This is in contrast with the bilayer-fluidizing agent benzyl alcohol,

which decreases the activation energy for the reaction when activating the membrane-bound enzyme (Dipple & Houslay, 1978). These observations, together with those described below, also imply that cholesterol loading elevates cholesterol concentrations in both the internal- and external-facing halves of the bilayer.

Further evidence which suggests that the environment of the enzyme is more ordered and affects the structure and functioning of the protein comes from our observations on the thermostability of the enzyme. We have previously demonstrated (Needham *et al.*, 1982) that increased bilayer fluidity appears to decrease the thermostability of fluoride-stimulated adenylate cyclase. Conversely we note here that in cholesterol-enriched membranes the thermostability of the enzyme was greatly enhanced (Fig. 3), implying that the increase in bilayer order exerts a protective effect on the enzyme. This is consistent with other evidence (Cress & Gerner, 1980) which showed that the thermal sensitivity of cells was inversely proportional to their cholesterol content. It also appears that the effect of cholesterol in inhibiting the activity of both glucagon- and fluoride-stimulated adenylate cyclase can be fully reversed by the action of benzyl alcohol. Not only are these activities more potently activated by benzyl alcohol when cholesterol-enriched membranes are used as the enzyme source rather than native membranes (Table 1), but the specific activities achieved on maximal stimulation by benzyl alcohol for either ligand were very similar in both membranes. Thus under these conditions benzyl alcohol and cholesterol seem to exert opposing effects on both membrane structure and adenylate cyclase activity.

As well as modulating adenylate cyclase activity by increasing membrane order, cholesterol might also exert inhibitory effects on the enzyme through a variety of other means, such as direct interaction with the protein, promotion of protein clustering, change in the head-group conformation of phospholipids (Yeagle *et al.*, 1977), interaction with specific classes of phospholipids (Demel *et al.*, 1977; Van Dijck, 1979) that may be essential for the activity of the enzyme (Rubaclava & Rodbell, 1973), and alterations in bilayer thickness (Stockton & Smith, 1976). We view a direct interaction of cholesterol with the enzyme as unlikely, because integral proteins have been shown to segregate into cholesterol-poor domains (Kleemann & McConnell, 1976; Cherry *et al.*, 1980) and cholesterol, presumably because of its rigid structure, appears to be excluded from the annular lipid domain of a number of integral membrane proteins (Warren *et al.*, 1975). However, it is highly probable that cholesterol exerts significant inhibitory effects on adenylate cyclase, either by promoting protein-protein interactions or

by restricting the availability to the enzyme of phospholipids that are critical for maintaining maximal activity. In this context it is particularly noteworthy that, although adenylate cyclase activity is more potently activated by benzyl alcohol when the enzyme is in the cholesterol-enriched rather than in the native membranes, benzyl alcohol exerts similar fluidizing effects [decreases in $S(T_H)$] on both membranes (Table 1). This bears striking analogy with our observations on native membranes that, although benzyl alcohol increases the fluidity to a similar extent at temperatures both above and below the lipid-phase separation, benzyl alcohol is a far more potent activator of the enzyme at the lower temperatures (Gordon *et al.*, 1980). For example, 40 mM-benzyl alcohol activates the glucagon-stimulated activity by 2.2- and 9.2-fold at 30 and 10°C respectively (Dipple & Houslay, 1978; Gordon *et al.*, 1980). We previously suggested (Gordon *et al.*, 1980) that the potentiating effect noted at low temperatures may be due to benzyl alcohol disrupting pre-existing protein clusters or cholesterol-phospholipid adducts that are promoted at temperatures below the lipid-phase separation. As we are now able to mimic this potentiating effect, isothermally at 30°C, simply by raising the membrane cholesterol content, which would be expected to increase both protein clustering and cholesterol-phospholipid adducts (see Kimelberg, 1977; Van Dijck, 1979), this hypothesis may not be too unreasonable.

Alterations in bilayer thickness and in phospholipid head-group orientation might also be expected to affect adenylate cyclase activity. Although evidence has been presented which suggested that perturbation of the head-group region of the bilayer might affect fluoride-stimulated activity, there was no indication that the glucagon-stimulated activity was affected (Dipple & Houslay, 1978), making this an unlikely major cause of cholesterol's effect on adenylate cyclase. Changes in bilayer thickness have been shown to have dramatic effects on transmembrane ATPases inserted into model lipid bilayers (Johannsson *et al.*, 1981*a,b*), where 'over-thickening' of the bilayer can lead to poor interaction of proteins with bilayer lipids, leading to activity loss. This effect might be expected to be particularly acute for transmembrane enzymes, such as adenylate cyclase when stimulated by glucagon, which have a central hydrophobic domain separating two polar domains. However, partly penetrating integral proteins, such as the catalytic unit of adenylate cyclase and its regulatory protein, which are responsible for the fluoride-stimulated activity and are believed to interact only with the inner monolayer (see Houslay, 1980; Houslay & Gordon, 1983; Houslay *et al.*, 1980*a*; Whetton *et al.*, 1982), are unlikely to be particularly sensitive to such

changes (see Israelachvili, 1977). Again it is unlikely that a change in bilayer thickness is a major contributor to the inhibition of adenylate cyclase by cholesterol, as it is difficult to understand how such an effect might be simply reversed by benzyl alcohol.

Sensitivity to inhibition by elevated cholesterol concentrations occurs in the order glucagon-stimulated > fluoride-stimulated > basal adenylate cyclase activity. The acute sensitivity of the glucagon-stimulated response is not, however, due to cholesterol perturbing the binding of hormone to its receptor, which remained unaltered. It is possible that this order of sensitivity reflects the need for distinct mobile entities (Tolkovsky & Levitzki, 1978; Houslay, 1980; Houslay *et al.*, 1980a; Limbird, 1981), namely the catalytic unit, guanine nucleotide/fluoride regulatory unit and glucagon receptor, to interact in a fluid lipid membrane. Changes in cholesterol concentrations might influence these interactions by decreasing membrane fluidity and hence affecting the conformational flexibility of the proteins (Dipple & Houslay, 1978) and their ability to migrate and interact freely in the plane of the bilayer. However, it may be that the heightened sensitivity of the hormonal response to inhibition by elevated cholesterol concentrations reflects contributions accruing from alterations in bilayer thickness and the selective interaction of cholesterol with certain phospholipid species as described above. Clearly, because of the differential response to cholesterol small increases in cholesterol concentrations will have a dramatic effect on the degree of stimulation, above basal values, of the enzyme by hormone (Fig. 2e). Thus, if such increases were to occur *in vivo*, they might well have marked effects on the response of the tissue to glucagon, for only at very high cholesterol concentrations, when both hormone-stimulated and basal activities were equally inhibited, would the degree of stimulation of the enzyme by hormone be normalized.

We observe here that basal activity is apparently unaffected by the decrease in fluidity achieved by cholesterol (Fig. 2), for $S(T_{11})$ does not significantly increase, with increasing cholesterol/phospholipid molar ratios, above that observed at around 0.8, yet basal adenylate cyclase activity remains unaffected until this molar ratio is greater than about 0.97 (Fig. 2). This is consistent with our previous observations that basal adenylate cyclase activity, expressed in liver (Gordon *et al.*, 1980) and brain (Needham & Houslay, 1982), is relatively insensitive to the marked increases in fluidity elicited by benzyl alcohol. Indeed Arrhenius plots of basal adenylate cyclase activity from a variety of sources exhibit a break at around 20°C which is protein-, rather than lipid-, mediated. It has been suggested that this reflects two states of the enzyme, exhibiting different activation energies for their reaction, which

are in equilibrium (see Needham & Houslay, 1982; Houslay & Gordon, 1983). When the enzyme is in this 'ground' or 'basal' state, it is apparently relatively insensitive to changes in lipid fluidity and any lipid phase separation that might occur in the membrane. Indeed, a similar low-activity fluidity-insensitive state can be induced by Ca^{2+} liganding to the enzyme (Houslay & Gordon, 1983). On this basis it would appear to be very unlikely that the marked inhibition of basal activity was due to any change in membrane fluidity. Rather, some other effect such as enhanced protein-protein interactions or even a direct effect of cholesterol on the enzyme itself might be responsible for this sudden fall in activity. It seems that any such effect was peculiar for the basal activity state of the enzyme. This is because the precipitous fall in the basal activity, observed at cholesterol/phospholipid values greater than 0.97, was not paralleled by a similar fall in either the glucagon- or fluoride-stimulated activities (Fig. 2). Again this implies that the activity of adenylate cyclase, in its stimulated states, achieved either by hormones or fluoride, responds to changes in its lipid environment very differently to the enzyme in its basal/ground state.

Our suggestion is that the decrease in membrane order elicited by cholesterol is the major factor contributing to the inhibition of the glucagon-stimulated activity of liver plasma-membrane adenylate cyclase. This is in accord with a large number of observations that increased membrane fluidity leads to an activation of the enzyme (see Houslay & Gordon, 1983). The inhibition of enzyme activity by elevated cholesterol concentrations that we have observed with adenylate cyclase has also been seen for both Na^{+} - and K^{+} -dependent ATPase (Kimelberg & Papahadjopoulos, 1974) and mitochondrial ATPase (Bruni *et al.*, 1975). On the other hand, our findings are not entirely in agreement with results from studies examining the effect of increased cholesterol on the adenylate cyclase activity in other systems, namely fibroblasts (Klein *et al.*, 1978), human platelets (Sinha *et al.*, 1977; Insel *et al.*, 1978) and Chinese-hamster ovary cells (Sinensky *et al.*, 1979). In all three instances, intact cells were incubated for prolonged periods with either cholesterol-rich liposomes or cholesterol-supplemented media; however, no systematic attempts were made to assess the extent of cholesterol incorporation into the plasma membrane, the degree of fusion of liposomes with the membrane, and whether adaptive changes occurred in membrane phospholipids. The failure to perform such control experiments may explain why Sinha *et al.* (1977) and Insel *et al.* (1978) obtained conflicting results in human platelet studies. Initially, Sinha *et al.* (1977) reported that elevated cholesterol concentrations suppressed fluoride-, hormone- and guanine nucleotide-stimu-

lated activities. They now (Insel *et al.*, 1978) maintain that platelets incubated with cholesterol-rich liposomes exhibit an enhanced basal activity but similar hormone-stimulated activities (i.e. there is a net decrease in the degree of stimulation of basal activity achieved by hormones). Klein *et al.* (1978) demonstrated that incubation of fibroblasts with cholesterol-enriched liposomes mediated a loss of basal, fluoride- and prostaglandin E_1 -stimulated adenylated cyclase activity. Contrarily, the basal activity of the enzyme from Chinese-hamster ovary cells was actually activated by cholesterol enrichment, although the relative stimulation achieved by fluoride or prostaglandin E_1 was much smaller (Sinensky *et al.*, 1979). The rather disparate findings in these earlier studies are undoubtedly the result of a series of membrane perturbations that occur in addition to any manipulation of the surface membrane cholesterol/phospholipid molar ratios.

It would seem that elevated cholesterol concentrations lead to an inhibition of adenylate cyclase activity and, in particular, a loss of hormone-responsiveness. This effect may have relevance during transformation, diabetes, aging and as a result of certain diets, where elevated cholesterol concentrations in plasma membranes have been noted (see Kimelberg, 1977; Chandramouli & Carter, 1975; Nassar *et al.*, 1981).

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