Phenobarbital selectively modulates the glucagon-stimulated activity of adenylate cyclase by depressing the lipid phase separation occurring in the outer half of the bilayer of liver plasma membranes

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The glucagon-stimulated (coupled) activity of rat liver plasma-membrane adenylate cyclase could be selectively modulated by the anionic drug phenobarbital, whereas the fluoride-stimulated (uncoupled) activity remained unaffected. It is suggested that the cationic drug phenobarbital preferentially interacts with the external half of the bilayer, as the negatively charged phospholipids are found at the cytosol-facing side. This results in a selective fluidization of the external half of the bilayer, leading to a depression in the high-temperature onset of the lipid phase transition (from 28° to 16°C) occurring there. This was detected both by e.s.r. analysis, using a fatty acid spin probe, and also by Arrhenius plots of glucagon-stimulated activity, where the enzyme forms a trans-membrane complex with the receptor and is sensitive to the lipid environment of both halves of the bilayer. However, in the absence of hormone, adenylate cyclase only senses the lipid environment of the inner (cytosol) half of the bilayer. Thus its fluoride-stimulated activity and Arrhenius plots of this activity remained unaffected by the presence of phenobarbital (less than 12 mM) in the assay. These results support the view that independent modulation of the fluidity or chemical constituents of each half of the bilayer can selectively affect the receptor-coupled and uncoupled activities of adenylate cyclase.

The lipid bilayers of liver plasma membranes from both the rat and hamster are asymmetric with respect to their fluidity (Houslay *et al.*, 1976*b,c*; Houslay & Palmer, 1978; Houslay, 1979) and chemical composition (Higgins & Evans, 1978). Similar to the surface membranes of other eukaryotic cells, the negatively charged phospholipids predominate at the cytosol surface of the bilayer of rat liver plasma membranes (Higgins & Evans, 1978).

Sheetz & Singer (1974) have suggested that, owing to the asymmetric distribution of charged phospholipids, bilayer-perturbing drugs of opposite charge may act preferentially at one or other side of the bilayer. In this manner they may selectively modulate the activity of functioning proteins that are themselves asymmetrically orientated within the bilayer. The glucagon-stimulated adenylate cyclase activity of rat liver plasma membranes provides us with an asymmetric system, where the receptor is

exposed at the external surface of the plasma membrane and the catalytic unit is at the internal surface (Houslay *et al.*, 1980*a*). In the absence of glucagon, the receptor and catalytic units are able to undergo free lateral diffusion as independent entities (Houslay *et al.*, 1977; Houslay, 1981), and the activity of the free catalytic unit is sensitive to the lipid environment of the cytosol side of the bilayer only (Houslay & Palmer, 1978; Houslay, 1979). However, in the presence of glucagon, the occupied receptor and catalytic unit interact to form a transmembrane complex spanning the lipid bilayer (Houslay *et al.*, 1977; Martin *et al.*, 1979; Houslay, 1981). This complex allows adenylate cyclase to respond to the lipid environment of both halves of the bilayer (Houslay & Palmer, 1978; Houslay, 1979, 1981). Under conditions when adenylate cyclase obeys such a Mobile Receptor Model (see Houslay, 1981), it provides a useful system for probing the lipid environment of both halves of the bilayer. However, when excess GTP is added to the reaction, the mode of coupling changes to a

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Collision Coupling Mechanism. In this instance the complex formed between the receptor, guanine nucleotide regulatory protein and catalytic unit dissociates, releasing an active catalytic unit which is sensitive only to the lipid environment of the inner (cytosol) half of the bilayer (see Houslay *et al.*, 1980a for detailed discussion).

We have previously shown that the cationic local anaesthetic prilocaine can selectively fluidize the inner half of the bilayer of liver plasma membranes (Houslay *et al.*, 1980b). The anionic drug phenobarbital can act as a local anaesthetic and has been shown to be capable of fluidizing model membranes (Papahadjopoulos *et al.*, 1975; Lee, 1976; Pang *et al.*, 1980). The present study uses adenylate cyclase under conditions where it obeys a Mobile Receptor Model (Houslay *et al.*, 1977, 1980a; Martin *et al.*, 1979) to investigate the effect of the anionic drug phenobarbital on liver plasma membrane.

Materials and methods

The I(12,3) spin label (*N*-oxyl-4',4'-dimethyl-oxazolidine derivative of 5-oxostearic acid) was obtained from Syva Co., Palo Alto, CA, U.S.A. Creatine kinase, phosphocreatine, ATP, cyclic AMP and triethanolamine hydrochloride were from Boehringer (U.K.) Ltd. Phenobarbital was from Sigma. Glucagon was kindly given by Dr. W. W. Bromer, Lilly Research Laboratories, Indianapolis, IN, U.S.A.

Liver plasma membranes were isolated from male Sprague-Dawley rats weighing 200–300 g as described previously (Houslay *et al.*, 1976a). Adenylate cyclase activity was measured as described by Houslay *et al.* (1976a), and phenobarbital was shown not to affect the method (Brown *et al.*, 1972) of determination of cyclic AMP. Enzyme assays were carried out in the presence of either 1 μ M-glucagon or 15 mM-NaF, at protein concentrations of 0.4 mg of membrane protein/ml. Phenobarbital was made up as a 40 mM stock solution and the pH adjusted to 8.5 before dilution into the assay. The final assay pH for both enzyme and spin-label experiments was 7.6. Determination of enzyme activity was done from linear initial rates at all temperatures (see Houslay & Palmer, 1978). Fluoride-activated adenylate cyclase was solubilized with Lubrol PX as described by Swislocki *et al.* (1975).

Methods for e.s.r. studies were as described previously by us in some detail (Gordon *et al.*, 1978, 1980a). Liver plasma membranes were suspended in 50 mM-triethanolamine/HCl/8% (w/v) sucrose, final pH 7.6, at 1.4 mg of protein/ml. These samples were added to the I(12,3) spin label 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 9 μ g of I(12,3)/mg of membrane protein (Sauerheber *et al.*, 1977). Spectra were recorded on a Varian E-104A Century Series e.s.r. spectrometer and the cavity temperature was calibrated as described by Gordon *et al.* (1978).

The outer hyperfine splittings, $2T_{||}$, of labelled liver plasma membranes were determined from 'un-expanded spectra with magnified wings' (Gordon *et al.*, 1978). The order parameter $S(T_{||})$ may then be calculated (Gordon & Sauerheber, 1977) and used to evaluate the flexibility of the liver plasma-membrane-incorporated fatty acid spin label:

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

Here, T_{xx} and T_{zz} are the hyperfine splitting elements of the static interaction tensor (T) parallel to the static Hamiltonian (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 $2p\pi$ orbital. The elements of T used in this study were previously determined by incorporating nitroxide derivatives into host crystals as substitutional impurities:

$$(T_{xx}, T_{zz}) = (6.1, 32.4) \text{ G} \quad (1 \text{ G} = 0.1 \text{ mT})$$

(Seelig, 1970).

The order parameter $S(T_{||})$ reflects the membrane fluidity (or, more accurately, the flexibility of the membrane-incorporated probe). $S(T_{||})$ may assume values between 0 and 1, with the extreme order parameters indicating that the probe samples fluid and immobilized environments respectively. Although $S(T_{||})$ does not correct for small polarity differences between the membrane and reference crystal, this expression has been found to be a useful approximate measure of the fluidity in those cases where only the outer hyperfine splitting is available (Gordon & Sauerheber, 1977; Sauerheber *et al.*, 1980). Only $S(T_{||})$ was employed to assess the effects of phenobarbital on the fluidity of rat liver plasma membranes, because the overlap of 'liquid-lines' occurring under these experimental conditions precluded an accurate determination of the inner hyperfine splitting, $2T_{\perp}$. This spectral interference prevented us from calculating the polarity-corrected order parameter, S (Sauerheber *et al.*, 1977; Gordon & Sauerheber, 1977).

Results

Action of phenobarbital on the glucagon- and fluoride-stimulated activities of adenylate cyclase

Phenobarbital initially inhibited the glucagon-stimulated (coupled) activity at concentrations of

about 1 mM, above which concentrations a dramatic activation ensued, which reached a maximum between 4 and 6 mM-phenobarbital. Any further increase in phenobarbital concentration led to a progressive loss in enzyme activity (Fig. 1). All of these changes in activity were readily reversed upon removal of the phenobarbital by washing (three times) the membranes in 1 mM-KHCO₃, pH 7.4, at 4°C by repeated centrifugation for 6 min at 14 000 *g*_{av}.

No effect of phenobarbital was observed on the fluoride-stimulated (uncoupled) activity of either native membranes or a Lubrol-solubilized enzyme.

Arrhenius plots of the glucagon- and fluoride-stimulated adenylate cyclase activity in the presence of phenobarbital

In the absence of phenobarbital, the glucagon-stimulated activity exhibited a well-defined break at 28°C, owing to its sensitivity to a lipid phase separation occurring in the bilayer, whereas the fluoride-stimulated activity yielded a linear plot (Houslay *et al.*, 1976a; Dipple & Houslay, 1978; and Fig. 2).

Fig. 1. Sensitivity of the adenylate cyclase activity of rat liver plasma membranes to phenobarbital

Phenobarbital was added to adenylate cyclase assays containing 0.4 mg of plasma membranes/ml. The percentage change in activity is shown for the glucagon-stimulated (coupled) enzyme (●), the fluoride-stimulated (uncoupled) enzyme (■) and the Lubrol-solubilized enzyme (□). The Lubrol-solubilized enzyme is pre-activated with fluoride and exhibits, like other solubilized preparations, no sensitivity to glucagon. It is used as an indicator of effects that may be exerted directly on the protein rather than through the bilayer. All assays were performed at 30°C.

The addition of optimum (4 mM) phenobarbital concentrations to the assays had a dramatic effect on the form of the Arrhenius plots for the glucagon-stimulated (coupled) activity. The break in the plots was depressed to 16.1°C (14.6–17.1°C; 95% confidence limits, *n* = 8), with activation energies of 43.7 ± 7.1 and 63.8 ± 11.8 kJ·mol⁻¹ (s.d., *n* = 8) above and below the break respectively. Such activation energies are considerably less than those exhibited in the absence of phenobarbital (Houslay *et al.*, 1976a; Dipple & Houslay, 1978). However, the form of the Arrhenius plot of the fluoride-stimulated (uncoupled) activity was apparently unaffected by the presence of phenobarbital in the assays (Fig. 2). It remained linear with an activation

Fig. 2. Arrhenius plots of coupled and uncoupled adenylate cyclase activity (*v*) in the presence of phenobarbital. Arrhenius plots of the glucagon-stimulated (coupled) activity (●) and the fluoride-stimulated (uncoupled) activity (■) in the presence of 4 mM-phenobarbital are given. The break point occurred at a similar temperature with 12 mM-phenobarbital. The upper and lower broken lines indicate the coupled and uncoupled activities respectively in the absence of phenobarbital.

energy [$75 \pm 15 \text{ kJ} \cdot \text{mol}^{-1}$ (s.d., $n = 7$)] similar to that exhibited by the enzyme in the absence of phenobarbital (Houslay *et al.*, 1976a; Dipple & Houslay, 1978).

Effect of phenobarbital on Arrhenius plots of $S(T_{\parallel})$ obtained from I(12,3)-spin-labelled rat liver plasma membranes

In native plasma membranes there is a well-defined lipid phase separation with a high-temperature onset occurring at 28°C that can be detected by the I(12,3) spin probe (Houslay *et al.*, 1976b; Gordon *et al.*, 1980a), by light-scattering (Dipple & Houslay, 1978), by fluorescent probes (Houslay, 1979; Luly & Shinitzky, 1979) and by differential scanning calorimetry (Livingstone & Schachter, 1980). The addition of phenobarbital to the membranes caused a marked depression of the high-temperature onset of this lipid phase separation to 15.8°C ($13.5\text{--}16.5^{\circ}\text{C}$; 95% confidence limits, $n = 3$) as indicated by Arrhenius plots (Fig. 3) of $S(T_{\parallel})$. At temperatures greater than 30°C or less than 10°C , phenobarbital had no effect or even slightly decreased the flexibility of the I(12,3) probe.

Fig. 3. *Effect of phenobarbital on the temperature-dependence of the order parameter, $S(T_{\parallel})$, for the I(12,3) probe incorporated into rat liver plasma membranes* The order parameter $S(T_{\parallel})$ for the I(12,3) spin-probe is plotted as a function of the reciprocal of the absolute temperature. In this instance the protein concentration was 1.4 mg/ml , and thus a phenobarbital concentration of 12 mM was employed so as to keep the phenobarbital/protein ratio the same as in the functional studies with adenylate cyclase (see legend to Fig. 2). Similar results could, however, be obtained with 4 mM -phenobarbital. Data are shown for $S(T_{\parallel})$ with membranes treated with phenobarbital (●), together with reference points taken in the absence of phenobarbital (Δ) in order to demonstrate the fluidity increase. Arrow indicates the high-temperature onset of the lipid phase separation.

However, between 10 and 30°C there was a significant and consistent fluidization of the bilayer by phenobarbital, with the low-temperature onset of the lipid phase separation presumably being depressed to a temperature at or below 10°C .

Discussion

The lipid phase separation with a high-temperature onset occurring at 28°C in rat liver plasma membranes is believed to be due to the formation of 'quasi-crystalline' clusters of lipid (Gordon *et al.*, 1978, 1980a) within the bulk fluid domain of the external half of the lipid bilayer (Houslay, 1979). This lipid phase separation appears to occur in cholesterol-poor regions of the external half of the bilayer (Houslay & Palmer, 1978; Dipple & Houslay, 1979), where the activity of integral membrane proteins which have functional globular domains associated with them are sensitive to the high-temperature onset (Houslay, 1979).

In the absence of glucagon, the uncoupled catalytic unit of adenylate cyclase may be stimulated through an associated guanine nucleotide regulatory unit (G/F) by fluoride ions (see Ross & Gilman, 1980; Limbird, 1981), and is able to undergo free lateral diffusion in the plane of the bilayer (Houslay *et al.*, 1977), where it is sensitive to the lipid environment of the inner half of the bilayer only (Houslay & Palmer, 1978; Houslay, 1979). However, in the presence of glucagon, the receptor and catalytic unit couple to form a transmembrane complex (Houslay *et al.*, 1977; Martin *et al.*, 1979), which is sensitive to the lipid environment of both halves of the bilayer (Houslay, 1979, 1981; Houslay & Palmer, 1978; Houslay *et al.*, 1976b,c). We have previously demonstrated that, in the basal, glucagon-, fluoride- and guanine nucleotide-stimulated states, adenylate cyclase is stimulated (Dipple & Houslay, 1978; Houslay, 1981) by increases in bilayer fluidity that can be achieved by the local anaesthetic benzyl alcohol (Gordon *et al.*, 1980a). Benzyl alcohol is a neutral drug, and this precludes any selective effects owing to charge interactions with bilayer phospholipids that may be asymmetrically disposed. However, phenobarbital is an anionic compound that has been demonstrated to increase bilayer fluidity efficaciously in neutral or positively charged liposomes, but not in negatively charged ones (Papahadjopoulos *et al.*, 1975; Lee, 1976). As the negatively charged phospholipids are found almost exclusively at the inner surface of the liver plasma membrane (Higgins & Evans, 1978), we might well expect to observe selective actions of this anionic drug at the external half of the bilayer.

Observations of the glucagon-stimulated activity (Fig. 1) show that after an initial inhibition there is a marked activation, which reaches a maximum at about $4\text{--}6 \text{ mM}$ -phenobarbital, at concentrations

above which the activity is inhibited. We would like to suggest that the activation observed is due to the preferential fluidization of the external half of the bilayer by phenobarbital. Consistent with an effect of phenobarbital on the external half of the bilayer are our observations that the temperature at which the break occurs in the Arrhenius plot of the glucagon-stimulated activity is depressed from 28° to 16°C. At the same time there is a concomitant decrease in the activation energies for the reactions both above and below the break. This would be expected for an increase in bilayer fluidity relieving a constraint on the protein, increasing its conformational flexibility and hence its activity (Dipple & Houslay, 1978). That this decrease in the break temperature is due to a lipid phase separation is indicated from our observations of the Arrhenius plot of the order parameter $S(T_{II})$ for the I(12,3) spin-label incorporated into the membrane. This shows that a break occurs at about 16°C in the presence of phenobarbital and that there is a significant increase in the fluidity of the bilayer (Fig. 3). These effects seem to be exerted selectively at the external half of the bilayer, as the activity of the fluoride-stimulated (uncoupled) enzyme was unaffected at all concentrations of phenobarbital that were tested (Fig. 1). Furthermore, Arrhenius plots of the fluoride-stimulated activity were linear and gave activation energies that were very similar to those found in the absence of phenobarbital (Dipple & Houslay, 1978).

It is significant that phenobarbital is much more effective at activating glucagon-stimulated (coupled) adenylate cyclase activity at temperatures below about 28°C than above (Fig. 2). Indeed, a similar effect has been noted with the fluidizing agent benzyl alcohol (Dipple & Houslay, 1978). We have suggested that, in order for lipid-phase separations to occur and be sensed by integral liver plasma-membrane proteins, they must be localized to relatively cholesterol-poor regions of the membrane (Houslay & Palmer, 1978). Thus as the temperature is lowered below that of the lipid phase separation we can expect both the phospholipid composition of the fluid lipid pool to alter, and protein-protein interactions to be promoted by a restriction in the amount of fluid-phase lipid. Indeed, the ability of phenobarbital to increase the fluidity of liposomes has been shown to be dependent on their cholesterol content (Pang & Miller, 1978), where it was suggested that phenobarbital could disrupt lipid associations. If protein-protein interactions were inhibitory and lipid cluster formation selectively removed certain lipid species that promoted coupled adenylate cyclase activity, then phenobarbital at temperatures below the lipid phase separation may exert activating effects by disrupting protein-protein interactions and by affecting the species of

phospholipids recruited into clusters. Thus phenobarbital might be expected to exert a variety of effects on integral proteins through perturbing the bilayer in ways other than a simple fluidizing effect achieved by virtue of its acting as an impurity and depressing the temperature at which the lipid phase separation occurs.

This study complements that carried out by us with the cationic local anaesthetic prilocaine (Houslay *et al.*, 1980b), for prilocaine preferentially fluidizes the inner half of the bilayer, where it induces a lipid phase separation while leaving the lipid phase separation at 28°C in the outer half of the bilayer unaffected. Fluidization of the inner half of the bilayer, however, affects both the glucagon- (coupled) and fluoride-stimulated (uncoupled) activities, as they are both expressed through the catalytic unit. This is in contrast with the anionic drug phenobarbital, which by preferentially acting on the external half of the bilayer can selectively perturb the glucagon-stimulated activity. These effects are summarized schematically in Fig. 4. Indeed, our approach (Houslay & Palmer, 1978) of using Arrhenius plots of various ligand-stimulated activities of adenylate cyclase, when the enzyme either forms a transmembrane complex with the receptor or diffuses in the membrane independently of the receptor, has been used successfully with turkey erythrocyte membranes (Rimon *et al.*, 1980).

The above results suggest only that charged drugs exhibit a greater tendency to interact with one or the other half of the bilayer of liver plasma membranes, and do not prove that any of these agents exclusively reside in only one half of the bilayer. It is likely, for example, that the anionic fatty acid spin probe used in this study, I(12,3), distributes between both halves of the bilayer. Clearly, some fraction of the label samples the outer half of the bilayer, since I(12,3) detects the lipid phase separation in the outer leaflet (Houslay *et al.*, 1976b; Gordon *et al.*, 1980a) and is acutely sensitive to phenobarbital, which depresses the phase separation (Figs. 2 and 3). Inasmuch as the incorporated I(12,3) probably retains a partial negative charge, it would not be unexpected for this probe to reside in the outer half of the bilayer, since that is where positive and neutral lipids are concentrated. Nevertheless, there is evidence suggesting that I(12,3) also partitions into the inner half of the bilayer (Houslay *et al.*, 1980b). Since the cationic local anaesthetics nupercaine, carbocaine and prilocaine each begin to stimulate fluoride-stimulated adenylate cyclase (i.e. an enzyme localized in the cytosol-facing leaflet) at the same concentrations that decrease $S(T_{II})$, a certain fraction of I(12,3) must be restricted to the inner half of the bilayer to report on this fluidization (Houslay *et al.*, 1980b; Gordon *et al.*, 1980b). That phenobarbital induces such dramatic effects on the lipid

Fig. 4. Sensitivity of ligand-stimulated adenylate cyclase to phase separations occurring in the inner and outer halves of the rat liver plasma-membrane bilayer

Key: R, receptor; C, catalytic unit; g, G/F regulatory protein conveying fluoride and guanine nucleotide sensitivity; h, hormone. These components are asymmetrically disposed within the lipid bilayer (Houslay *et al.*, 1980a). All of them are integral proteins, although the G/F protein has only a small hydrophobic domain (see Limbird, 1981). In the presence of fluoride or guanine nucleotides the enzyme is not associated with the receptor and only senses the lipid environment of the inner half of the bilayer. However, in the presence of glucagon, when a Mobile Receptor Model is obeyed, the transmembrane complex formed between these units conveys a sensitivity to lipid phase separations occurring in the outer half of the bilayer upon the enzyme (Houslay & Palmer, 1978; Houslay *et al.*, 1980a). Charged drugs can thus be expected to have selective effects on the various ligand-stimulated activities. T_s is the lipid-phase-separation temperature that can be identified from both Arrhenius plots of the appropriate ligand-stimulated adenylate cyclase activity and also from e.s.r. studies of the temperature-dependence of $2T_{1\rho}$.

phase separation monitored by I(12,3) (Fig. 3) indicates that the proportion of probe may be somewhat greater in the outer than in the inner half of the bilayer, perhaps owing to selective charge interactions.

The inhibition of the coupled activity that is observed at low concentrations of phenobarbital and other anionic drugs (Gordon *et al.*, 1980b) may be due to these drugs having a direct effect on the receptor or at the coupling interface (see Houslay & Palmer, 1979; Whetton & Houslay, 1980), although it is possible that they could disrupt the interaction with specific bilayer lipids that are required for activity (see Rubaclava & Rodbell, 1973). The inhibition of activity that is seen at high phenobarbital concentrations and is characteristic of a number of neutral and charged local anaesthetics (Gordon *et al.*, 1980a,b; Houslay *et al.*, 1980b) has been proposed to be due to the displacement of annular lipid from around the enzyme (Gordon *et al.*, 1980a,b).

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