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Regulation of adenylate cyclase (*EC* 4.6.1.1) activity by its lipid environment

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Many hormones and neurotransmitters exert their effects on target cells by altering the intracellular concentrations of cyclic AMP. In most instances they achieve this by regulating the activity of the ubiquitous, multicomponent, membrane-bound enzyme, adenylate cyclase (*EC* 4.6.1.1; see Ross & Gilman, 1980; Houslay, 1984). Like many integral membrane-bound enzymes the activity of adenylate cyclase is modulated by the nature of its membrane lipid environment (for review, see Houslay & Gordon, 1983).

We have studied in some detail the sensitivity of the activity of the glucagon-stimulated adenylate cyclase of rat liver plasma membranes to changes in its lipid environment. This is a key regulatory enzyme through which the peptide hormone glucagon exerts effects on glycolysis and gluconeogenesis in the liver. Indeed, glucagon causes a dramatic increase (approximately forty fold) in the activity of liver adenylate cyclase activity (Houslay *et al.* 1980*a*). Here we showed that the activity of this enzyme is particularly sensitive to changes in membrane fluidity (Houslay *et al.* 1980*a*). Thus increases in membrane fluidity augment enzyme activity whereas activity is reduced when membranes become more rigid. In this instance we envisage fluidity as encompassing the overall degree of disorder–order in the membrane-lipid environment. Clearly this has some complexity due to the wide variety of molecular motions available to lipids within the plane of the bilayer (see Houslay & Stanley, 1982). Moreover, due to the asymmetric nature of this system, selective alterations on basal and hormone-stimulated responses can be elicited. Alterations in the activity of this enzyme and its response to hormonal activation undoubtedly have important consequences for the functioning of the liver. This is likely to have relevance under conditions where alterations in membrane properties ensue, caused by drugs, diet, disease and transformation.

Hormone-stimulated adenylate cyclase is a multicomponent, asymmetrically-disposed membrane system

This information-transfer system consists of three distinct protein components. These are a receptor, for binding the hormone, a catalytic unit, for producing cyclic AMP from ATP, and a guanine nucleotide regulatory protein (N_s) which acts as a transducer between these two units (see Ross & Gilman, 1980). In the liver it has

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been demonstrated that the glucagon receptor, N_s and the catalytic unit of adenylate cyclase are asymmetrically disposed within the plasma membrane (Fig. 1; Houslay *et al.* 1980*a*). Moreover, in the absence of glucagon these components appear to be able to undergo free lateral diffusion, independently of each other, within the plane of the membrane (Houslay *et al.* 1977, 1980*a*; Houslay, 1980). Only when glucagon is present do they structurally and functionally interact. In this instance the interaction of an occupied glucagon receptor with N_s leads to the release of the β-subunit of N_s, which allows activation of the α-subunit. It is this α-subunit of N_s which has binding sites for the receptor, the catalytic unit of adenylate cyclase, for divalent cations and for GTP (for review, see Houslay, 1984). Under conditions where GTP concentrations are low (<100 nM), all three units form a transmembrane complex and activation ensues via a mobile receptor mechanism (Houslay *et al.* 1980*a*). However, in the presence of higher concentrations of GTP (>1 μM), activation ensues via a collision coupling mechanism. In this instance the complex dissociates to yield a free, activated

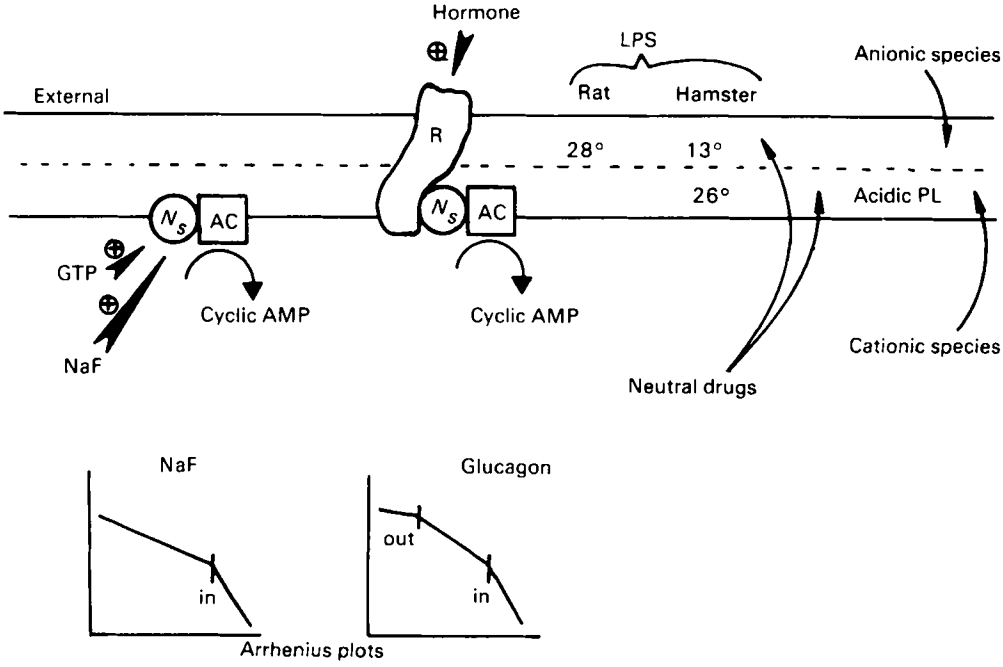

Fig. 1. Adenylate cyclase (AC; *EC* 4.6.1.1) is a structurally and functionally asymmetric system. It is shown in both its coupled, hormone-stimulated form and in the uncoupled state, when either unstimulated (basal) or stimulated through the guanine nucleotide regulatory protein, N_s, by ligands such as sodium fluoride or non-hydrolysable analogues of GTP.

The site of action of 'fluidizing' or 'rigidizing' agents is shown, i.e. drugs which interact with the bilayer. Neutral species act on both halves, whereas selective effects can be elicited with charged species. Note that the acidic phospholipids (PL) are almost exclusively associated with the inner (cytosol) half of the bilayer.

The lipid-phase separation temperatures (LPS) are given for hamster and rat liver plasma membranes (lpm). In hamster lpm there are two LPS which cause distinctive Arrhenius plots of fluoride- and glucagon-stimulated adenylate cyclase activity (shown schematically here).

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catalytic unit (Martin *et al.* 1979; Houslay *et al.* 1980*a*). This effect of high-GTP concentrations in changing the kinetics of hormone activation can, however, be negated if Mn^{2+} replaces Mg^{2+} in the assay (Houslay *et al.* 1983).

The activity of adenylate cyclase is modulated by the fluidity of the membrane-lipid bilayer

A simple non-perturbing method of altering membrane fluidity is to vary the temperature. In biological membranes this invariably leads to the occurrence of lipid-phase separations. Thus a fall in temperature might cause the formation of 'rafts' of solid-phase lipid to occur within a 'sea' of fluid-phase lipid. As lipids with high 'melting points' (transition temperatures) tend to cluster–solidify first, then clearly the lipid composition of these two environments will be very different. Eventually, of course, as the temperature falls sufficiently it will lead to the total solidification of the bilayer. As integral membrane proteins tend to cluster within fluid-phase lipid then their lipid environment will alter during a lipid-phase separation. Such dramatic changes in temperature and, of course, lipid-phase separations are not going to be experienced physiologically. However, they provide a useful indicator of the potential of changes in the lipid environment to alter the activity of a functioning integral protein. In such instances, Arrhenius plots of the activity of these proteins display a distinct break or discontinuity at the lipid-phase-separation temperature (for detailed discussion see Houslay & Stanley, 1982 and Houslay & Gordon, 1983).

In rat-liver plasma membranes a distinct lipid-phase separation occurs at around 28° which can be detected by a variety of physical probes (Houslay & Gordon, 1983). Arrhenius plots of glucagon-stimulated adenylate cyclase activity and a variety of other integral liver plasma membrane enzymes do indeed exhibit a well-defined break at this temperature (Whetton *et al.* 1982). This implies a modulating effect of the membrane environment on the activity of these enzymes.

Another independent means of altering bilayer fluidity is to employ a local anaesthetic. We have employed benzyl alcohol for this purpose. Our reason for this is: (1) benzyl alcohol is a neutral molecule, which precludes any charge interaction with either the enzyme or any other membrane components, (2) it is water soluble, which allows for easy addition and testing of reversibility, (3) its interaction with biological membranes has been well-characterized at a molecular level (Houslay & Gordon, 1983). Treatment of liver plasma membranes with benzyl alcohol leads to an increase in lipid fluidity as detected using a fatty acid spin probe (Gordon *et al.* 1980) with concomitant elevation of adenylate cyclase activity (Dipple & Houslay, 1978). This ability of benzyl alcohol to stimulate adenylate cyclase activity is only seen using the membrane-bound enzyme and so is presumed not to be due to any direct effect of benzyl alcohol on the protein. Indeed, there is close correlation between the activity of adenylate cyclase and the fluidity of the bilayer (Fig. 2). High concentrations of benzyl alcohol are, however, inhibitory. This is believed to be due to the alcohol competing for lipid-binding sites on the enzyme that are essential for its correct functioning (Gordon *et al.* 1980).

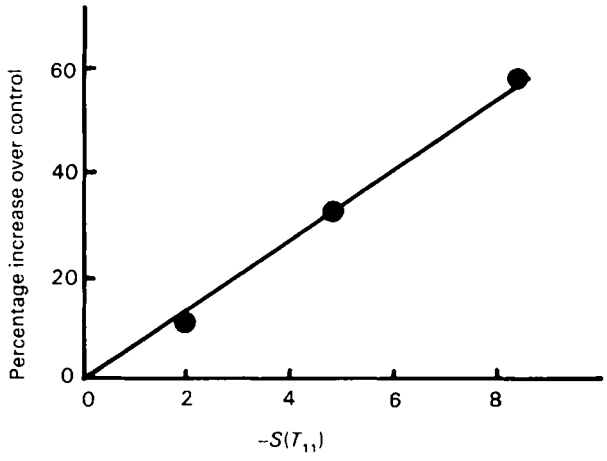

Fig. 2. The activity of adenylate cyclase (*EC* 4.6.1.1) is regulated by membrane fluidity. 'Fluidity' is measured as a change (%) in the order index $S(T_{11})$ for the spin label 5-nitroxide stearate incorporated into rat liver plasma membranes. This shows that the activity of glucagon-stimulated adenylate cyclase is influenced in proportion to the increase in bilayer 'fluidity'. Similar effects are seen for other ligand-stimulated activities. Values are adapted from Gordon *et al.* (1980). Decreases in activity have been shown to occur in proportion to falls in bilayer fluidity also (Whetton *et al.* 1984).

One important observation is that the basal activity of the enzyme is much less sensitive to alterations in membrane fluidity, caused by benzyl alcohol, than the hormone-stimulated enzyme (Gordon *et al.* 1980). This leads to a relatively greater degree of stimulation of the enzyme by glucagon as bilayer fluidity is enhanced.

Benzyl alcohol also decreases the temperature at which the lipid-phase separation occurs in liver plasma membranes. This causes a parallel decrease in the temperature at which the break occurs in Arrhenius plots of glucagon-stimulated adenylate cyclase activity (Dipple & Houslay, 1978). This provides further support for the notion (Dipple & Houslay, 1978) that the activity of adenylate cyclase is regulated by the nature of its lipid environment.

Such actions, of the effect of increased membrane fluidity, on the activity of adenylate cyclase from rat liver are also observed for the enzyme from a number of other sources (Houslay & Gordon, 1983) including the dopamine-stimulated enzyme from brain (Needham & Houslay, 1982).

Does a decrease in bilayer fluidity inhibit adenylate cyclase activity? The answer is yes. We have used two methods to ascertain this. The first employed the liver carcinogen, dimethylnitrosamine (DMN). This we found actually to decrease the fluidity of liver plasma membranes in a reversible fashion. Although DMN exerted somewhat complex effects on adenylate cyclase activity, its potent inhibitory effect showed a strong correlation with its ability to decrease bilayer fluidity. DMN also caused a small increase in the temperature at which the lipid-phase separation occurred in these membranes. This again led to a corresponding shift in the

temperature of the break in the Arrhenius plot of glucagon-stimulated adenylate cyclase activity (Whetton *et al.* 1984).

An alternative means of altering the fluidity of membranes is to change their cholesterol content. In model membrane systems, consisting of a single species of phospholipid, cholesterol abolishes the lipid-phase transition. Thus, as the temperature rises the lipid goes from a relatively-ordered state to a relatively-disordered state. The net effect of this is that at temperatures below the lipid-phase transition, bilayer fluidity is increased by cholesterol whereas, at temperatures above the lipid-phase transition, bilayer fluidity is decreased. However, in biological membranes consisting of mixtures of phospholipids, a considerably more complex situation arises with cholesterol. In such instances the bilayer exhibits lateral asymmetry with domains of lipid rich in cholesterol and domains of lipid poor in cholesterol. The presence of 'cholesterol-poor' lipid domains explains why lipid-phase separations can be observed even in membranes with relatively-high cholesterol contents. That such lipid-phase separations exert effects on the activity of functioning membrane proteins would appear to be because globular membrane proteins occur preferentially in the cholesterol-poor domains of the membrane. This situation exhibits further complexity as the lipid domains themselves will exhibit distinct phospholipid compositions. This is because cholesterol interacts preferentially with certain phospholipid species (for detailed discussion, see Houslay & Stanley, 1982; Whetton *et al.* 1982; Houslay & Gordon, 1983).

We have developed methods for manipulating the cholesterol content of isolated membranes in vitro (Whetton *et al.* 1983*a*,*b*). This prevents any adaptive (homeoviscous) changes in other membrane-lipid constituents occurring, as would be expected if cholesterol were administered to either whole cells or given in the diet. Under conditions where we increased the cholesterol content of the plasma membrane in vitro, this led to a reversible inhibition of adenylate cyclase activity. This was shown to be caused by the decreased fluidity of the membrane that occurred when its cholesterol content was raised. As before, the hormone-stimulated activity was affected more than basal adenylate cyclase activity. On decreasing the cholesterol content of the membrane, adenylate cyclase activity was also inhibited. Under such conditions complex changes in the lipid domain structure of the membrane were shown to have occurred (Whetton *et al.* 1983*a*,*b*). It was suggested that the main cause of this inhibition was the release of an inhibitory species of phospholipid, from the cholesterol-rich domains, that was now able to interact with the enzyme.

What is clear is that cholesterol optimizes the functioning of adenylate cyclase in these membranes (Fig. 3). This suggests that any alteration in membrane cholesterol content is likely to have a profound effect on this system.

The ability of membrane fluidity to affect adenylate cyclase activity is envisaged to be due to effects on the conformational flexibility of the protein itself. In other words one envisages this enzyme, like other integral proteins, to be sealed-in and constrained by the lipids of the bilayer. Thus changes in the physical properties of

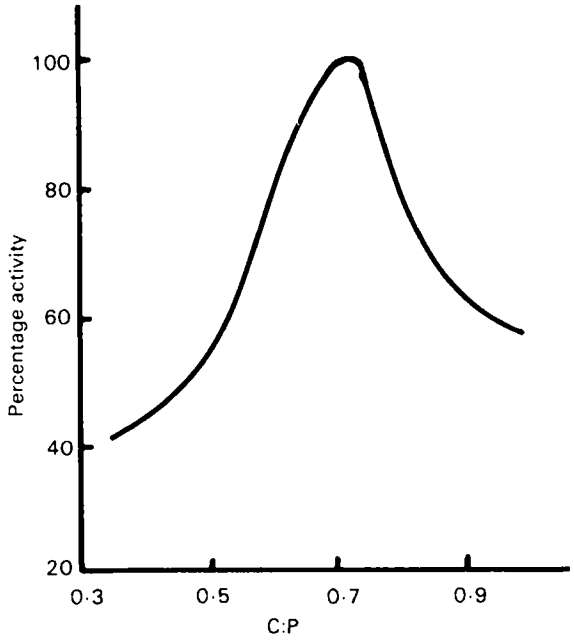

Fig. 3. Cholesterol optimizes the functioning of glucagon-stimulated adenylate cyclase (*EC* 4.6.1.1). Native liver plasma membranes from rat have a cholesterol: phospholipid (C:P) value of about 0·72. Here we see, schematically, that if the cholesterol content of these membranes is either reduced or increased, under conditions where no other lipid changes occur, then the activity of adenylate cyclase is reduced. The activity of glucagon-stimulated adenylate cyclase in native membranes with a C:P value of 0·72 is taken as 100% in order to allow comparison. This effect is reversible. These values are adapted from Whetton *et al.* (1983*a*,*b*).

these lipids would be expected to alter the constraining effect of the bilayer on the internal motions of the peptide chain of these entities and hence their activity.

Changes in bilayer fluidity might also be expected to alter the efficacy of interaction between the various components of this enzyme system. However, the rate of lateral diffusion between these components, in a fluid lipid bilayer, is so very fast, relative to the turnover number of this enzyme system, that any increase in lateral mobility would be unlikely to exert any significant effect on activity.

Changes in membrane fluidity can also exert effects on the thermostability of integral membrane proteins. In this instance increases in membrane fluidity appear to decrease the thermostability of adenylate cyclase (Needham *et al.* 1982) and vice versa (Whetton *et al.* 1983*a*).

Charged ligands which interact with the asymmetric bilayer can exert selective effects on glucagon-stimulated adenylate cyclase

As discussed previously, glucagon-stimulated adenylate cyclase is an asymmetric membrane system. Similarly, the lipid bilayer has been shown to be asymmetric

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(Higgins & Evans, 1978). Under conditions where adenylate cyclase is activated by glucagon through a mobile receptor mechanism, the activity of the transmembrane complex so formed senses the lipid-phase separation occurring in this membrane. However, if the enzyme is stimulated through N_s, using either fluoride or a non-hydrolysable analogue of GTP, then Arrhenius plots of adenylate cyclase activity are linear. From all the manipulations described previously we know that these activities are modulated by changes in the lipid environment. However, both the catalytic unit of adenylate cyclase and N_s have small hydrophobic domains. It is thus likely that their functional globular domains are only embedded in the inner (cytosol) half of the bilayer. We have thus proposed that the lipid-phase separation which occurs at around 28° in these membranes is localized exlusively to the external half of the bilayer. Thus only enzymes which have functional globular domains in this half of the bilayer would experience this lipid-phase separation. Indeed, this would seem to be the case for a number of asymmetrically-disposed enzymes (see Whetton *et al.* 1982). A similar distribution was observed in hamster liver plasma membranes. In this instance hibernation, which allowed body temperature to fall from 39 to 4°, appeared to exert a selective effect on the lipid-phase separation occurring in the outer half of the bilayer (Houslay & Palmer, 1978).

The asymmetric nature of the liver plasma membrane is such that the acidic phospholipids are found preferentially at the inner half of the bilayer. We have exploited this using charged local-anaesthetic drugs (Fig. 1) to exert a selective perturbation of each half of the bilayer (Houslay *et al.* 1980*b*, 1981). These show that an anionic drug like phenobarbital can activate glucagon-stimulated adenylate cyclase selectively by increasing the fluidity of the external half of the bilayer. It does not exert any effect whatsoever on the fluoride-stimulated adenylate cyclase activity. In contrast, a cationic drug like prilocaine will exert a selective effect on the inner half of the bilayer. However, like many cationic species it exerts a direct inhibitory effect on the coupling between the various protein components.

Ca^{2+} also exerts a selective perturbation of the inner half of the bilayer and, like prilocaine, seems to induce a new lipid-phase separation there (Gordon *et al.* 1983).

Thus the inherrent asymmetry of cell plasma membranes means that perturbing agents may well exert selective effects on enzymes located there (Fig. 1). This has, indeed, been shown to be true for 5′-nucleotidase (*EC* 3.1.3.5) (Dipple *et al.* 1982) as well as for adenylate cyclase (Houslay *et al.* 1980*b*, 1981). Thus alterations in membrane lipid properties caused by drugs, diet and disease are likely to have complex and profound effects on the functioning of globular proteins inserted in the membrane.

Physiological changes in the lipid environment and functioning of adenylate cyclase do occur

We have noted two distinct states which lead to changes in the lipid membrane environment with corresponding changes in the functioning of adenylate cyclase in

liver plasma membranes. These are during both hibernation (Houslay & Palmer, 1978) and maturation (Dipple & Houslay, 1982).

During the hibernation of hamsters, in an environment at 6°, their body temperature fell to around 4°. After 2 weeks of acclimatization we noted that this led to a selective perturbation of the plasma membrane. This took the form of lowering the temperature at which the lipid-phase separation in the outer half of the bilayer occurred from around 13° to around 4°. The temperature of the lipid phase separation occurring in the internal (cytosol) half of the bilayer was unaffected. Thus effects were exerted selectively on the hormone-stimulated activity of the enzyme.

Arrhenius plots of the activity of both adenylate cyclase and 5'-nucleotidase exhibit a well-defined break-point at around 28° in both mature and weanling animals. However, for the activities of both enzymes, the activation energies were higher at temperatures above the break-point using membranes from the weanling animals; the converse was true for the adults. As both enzymes were affected and significant changes in membrane lipids occur at this time, then this would appear to reflect lipid-mediated perturbations of the functioning of these plasma membrane enzymes.

Changes in membrane lipid environment can thus exert profound effects on the activity of functioning membrane proteins. This has been demonstrated here with the key regulatory enzyme adenylate cyclase. As dietary changes can lead to alterations in membrane lipid composition then, of course, we can expect that diet will influence the reactions that are mediated by biological membranes. Certainly, our preliminary findings with adenylate cyclase would support this contention.

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