Acidic phospholipid species inhibit adenylate cyclase activity in rat liver plasma membranes

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Incubation of rat liver plasma membranes with liposomes of dioleoyl phosphatidic acid (dioleoyl-PA) led to an inhibition of adenylate cyclase activity which was more pronounced when fluoride-stimulated activity was followed than when glucagon-stimulated activity was followed. If Mn^{2+} (5 mM) replaced low (5 mM) $[Mg^{2+}]$ in adenylate cyclase assays, or if high (20 mM) $[Mg^{2+}]$ were employed, then the perceived inhibitory effect of phosphatidic acid was markedly reduced when the fluoride-stimulated activity was followed but was enhanced for the glucagon-stimulated activity. The inhibition of adenylate cyclase activity observed correlated with the association of dioleoyl-PA with the plasma membranes. Adenylate cyclase activity in dioleoyl-PA-treated membranes, however, responded differently to changes in $[Mg^{2+}]$ than did the enzyme in native liver plasma membranes. Benzyl alcohol, which increases membrane fluidity, had similar stimulatory effects on the fluoride- and glucagon-stimulated adenylate cyclase activities in both native and dioleoyl-PA-treated membranes. Incubation of the plasma membranes with phosphatidylserine also led to similar inhibitory effects on adenylate cyclase and responses to Mg^{2+} . Arrhenius plots of both glucagon- and fluoride-stimulated adenylate cyclase activity were different in dioleoyl-PA-treated plasma membranes, compared with native membranes, with a new 'break' occurring at around 16 °C, indicating that dioleoyl-PA had become incorporated into the bilayer. E.s.r. analysis of dioleoyl-PA-treated plasma membranes with a nitroxide-labelled fatty acid spin probe identified a new lipid phase separation occurring at around 16 °C with also a lipid phase separation occurring at around 28 °C as in native liver plasma membranes. It is suggested that acidic phospholipids inhibit adenylate cyclase by virtue of a direct headgroup specific interaction and that this perturbation may be centred at the level of regulation of this enzyme by the stimulatory guanine nucleotide regulatory protein N_s .

INTRODUCTION

Adenylate cyclase is a key regulatory enzyme whose activity is modulated by a variety of hormones and neurotransmitters (see, e.g., Ross & Gilman, 1980; Limbird, 1981). These ligands bind to specific cell surface receptors which interact with, and subsequently activate, a distinct guanine nucleotide regulatory protein called N_s . The activated N_s moiety then activates the catalytic unit of adenylate cyclase (Gilman, 1984; Northup, 1985). These components are asymmetrically orientated in the plasma membrane with the binding site of the receptor facing the cell surface and the substrate sites for N_s and adenylate cyclase facing the cell cytosol (Gilman, 1984; Houslay *et al.*, 1980).

The lipid bilayer of rat liver plasma membranes is structurally asymmetric with the acidic phospholipids found almost exclusively in the cytosol half of the bilayer (Higgins & Evans, 1978). It also exhibits a lipid phase separation occurring at around 28 °C (see Houslay & Gordon, 1983, for review) being due to the lipids of the external half of the bilayer only (Whetton *et al.*, 1982; Houslay & Gordon, 1983). The physical properties of the two halves of the bilayer can be perturbed selectively by charged local anaesthetics (Houslay *et al.*, 1981), by Ca^{2+} (Gordon *et al.*, 1983) and by cholesterol manipulation

(Whetton *et al.*, 1983a,b; Needham *et al.*, 1985). We have shown in rat and hamster liver plasma membranes (see Houslay & Gordon, 1983) and in rat brain plasma membranes (Needham & Houslay, 1982) that the activity of adenylate cyclase when stimulated through N_s , by either NaF or guanosine 5'-[β] γ limido-triphosphate (p[NH]ppG), is influenced only by the lipid environment of the inner half of the bilayer (see Houslay & Gordon, 1983; Houslay *et al.*, 1981; Whetton *et al.*, 1982). This is presumably because the functional, globular domains of both N_s and the catalytic unit of adenylate cyclase reside there (see Houslay & Gordon, 1983, for discussion). However, the presence of glucagon causes its receptor to couple structurally and functionally to N_s and adenylate cyclase (Houslay *et al.*, 1977, 1980; Martin *et al.*, 1979) forming a transmembrane complex whose activity is regulated by the lipid environment of both halves of the bilayer (Houslay & Gordon, 1983; Houslay *et al.*, 1976a,b; Whetton *et al.*, 1982).

Various attempts have been made in order to ascertain whether the activity of adenylate cyclase is dependent on or modulated by specific headgroup species of phospholipids (see Houslay & Gordon, 1983, for review). Many of these have been based upon studies using phospholipases with specificity towards preferred phospholipids. However, results obtained with certain of these enzymes

Abbreviations used: PA, phosphatidic acid; I(12,3), *N*-oxyl-4',4'-dimethyloxazolidine 5-oxostearate.

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have been equivocal due to the effect of products of their action upon adenylate cyclase activity (see Houslay & Palmer, 1979). More recently, medium supplements have been used to alter plasma membrane composition in cultured cells (Engelhard *et al.*, 1978) and reconstitution experiments have been performed using purified N_s and partially purified preparations of adenylate cyclase (Hebdon *et al.*, 1979, 1981; Ross, 1982). These studies have implied a role for phosphatidylcholine in supporting adenylate cyclase activity. In the present study we use a liposome fusion method *in vitro* to increase the content of the acidic, minor, phospholipid, phosphatidic acid, in rat liver plasma membranes and show that it inhibits ligand-stimulated adenylate cyclase activity.

MATERIALS AND METHODS

Liver plasma membranes were isolated from male Sprague-Dawley rats weighing 200–300 g as described previously by us (Houslay *et al.*, 1976a; Marchmont *et al.*, 1981). Fluoride- and glucagon-stimulated adenylate cyclase activities (EC 4.6.1.1) were assayed over linear timecourses at constant pH, under all conditions, as previously described by us (Houslay *et al.*, 1976a). Assays were normally performed with 5 mM- Mg^{2+} present except when stated otherwise, where either Mn^{2+} (5 mM) or high Mg^{2+} (20 mM) were employed. All assays were performed at 30 °C except for Arrhenius plot studies where the temperature was varied over approximately 1 °C intervals. The determination of breakpoints in Arrhenius plots was performed using a least-squares fitting procedure as described previously by us (Houslay & Palmer, 1978). Protein was determined with a modified biuret method (see Houslay & Palmer, 1978).

E.s.r. procedures were performed as described by us elsewhere (Houslay *et al.*, 1976a; Whetton *et al.*, 1982, 1984). Briefly, the *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-oxostearic acid [I(12,3)] was used at an experimentally determined 'low probe' concentration (9 µg/mg of membrane protein). The outer and inner hyperfine splittings, $2T_{11}$ and $2T_1$ were used to calculate the polarity-corrected order parameter S . Arrhenius plots were constructed using S , which has been found to be a useful parameter for identifying lipid phase separations occurring in these membranes (Houslay *et al.*, 1976a; Whetton *et al.*, 1984; Needham *et al.*, 1985). A Varian E9 e.p.r. spectrometer was used on a 2.0 G modulation amplitude, 0.30 G time constant, 5×10^3 receiver gain, 10 mW microwave power, 9.24 GHz microwave frequency, all set on a scan time of 4 min.

Fusion of dioleoyl-PA liposomes, dioleoyl phosphatidylcholine liposomes, dioleoyl-PA/cholesterol liposomes and phosphatidylserine liposomes was performed by a modification of procedures described previously by us (Houslay *et al.*, 1976b). Briefly, a sonicated phospholipid suspension was made first. This involved taking 12.5 mg of solid phospholipid, in some instances together with the appropriate molar amount of cholesterol, dissolving it in 0.5 ml of chloroform/methanol (2:1, v/v) in a glass scintillation vial, evaporating off the solvent with a stream of N_2 and then removing residual solvent in a dessicator under vacuum for about 3 h. After this time 1 ml of 50 mM-triethanolamine/HCl buffer (N_2 -saturated), neutralized to pH 7.4 with KOH, was added and the vial sealed with Parafilm and capped. It was then shaken for 10 min and sonicated to optical clarity. This was done

either in a bath type sonicator, kept below 20 °C for 2 h, or by using a probe type sonicator for 1–2 min. After this the suspension was centrifuged at 15000 g_{av} for 1 min. This fresh suspension was used to incubate with the plasma membranes. A 10 min preincubation at 8 °C was first performed after mixing 1.0 ml of plasma membranes (15 mg of protein/ml) in 1 mM- $KHCO_3$, pH 7.2, with 2.0 ml of 50 mM-triethanolamine/HCl, pH 7.4, and 1 ml of water. To this preincubated mixture was added 1.0 ml of the appropriate liposome suspension (12.5 mg of lipid/ml) and incubation was performed with constant gentle stirring and using N_2 -saturated buffers. After appropriate periods of time, 0.5 ml aliquots were taken from the incubation and immediately diluted with 0.8 ml of 1 mM- $KHCO_3$, pH 7.2, at 8 °C. Samples were centrifuged at 15000 g_{av} for 5 min, the supernatant was removed and the sample was resuspended in 0.6 ml of 1 mM- $KHCO_3$, pH 7.2, for two further 'centrifugation washes'. The pellet was finally resuspended in 0.5 ml of 1 mM- $KHCO_3$, pH 7.2, and kept on ice prior to taking 20 µl for assay of adenylate cyclase activity. When dioleoyl-PA membranes were prepared for Arrhenius plot studies then they were prepared after a 30 min incubation of native membranes with either dioleoyl-PA or dioleoyl-PA/cholesterol liposomes as described above.

Incorporation of phosphatidic acid into the membranes was performed using 1,2-di[1- ^{14}C]oleoyl-PA (kindly given by Dr. G. Smith, Department of Biochemistry, University of Cambridge) in the incubations and taking samples from the above procedure for scintillation counting.

Creatine kinase, ATP, cyclic AMP and triethanolamine/HCl were from Boehringer (U.K.), Lewes, East Sussex, U.K. Phosphocreatine, dioleoyl-PA, phosphatidylserine (bovine brain), cholesterol and benzyl alcohol were from Sigma, Poole, Dorset, U.K. Glucagon was kindly given by Dr. W. W. Bromer, Eli Lilly, Indianapolis, IN, U.S.A. [3H]Cyclic AMP was from Amersham International, Amersham, Bucks., U.K. The spin probe I(12,3) was obtained from Syva, Palo Alto, CA, U.S.A.

RESULTS

Incubation of liver plasma membranes with dioleoyl-PA caused an inhibition of glucagon-stimulated adenylate cyclase activity (Fig. 1a). A similar degree of inhibition was elicited if the membranes were incubated with a cholesterol/dioleoyl-PA mixture having a cholesterol:phospholipid molar ratio (0.7) identical with that of the native rat liver plasma membranes (Whetton *et al.*, 1983a,b). If glucagon-stimulated adenylate cyclase activity was measured either in the presence of 'high' (20 mM) Mg^{2+} concentrations or with Mn^{2+} (5 mM), rather than 'low' (5 mM) Mg^{2+} concentrations, then the perceived inhibitory action of dioleoyl-PA was enhanced (Fig. 1a). Over the time courses studied there was little change in glucagon-stimulated adenylate cyclase activity of control incubations whether assays were performed in the presence of high Mg^{2+} , low Mg^{2+} or Mn^{2+} .

When assayed using NaF to stimulate adenylate cyclase activity then, after incubation of the plasma membranes with either dioleoyl-PA or cholesterol/dioleoyl-PA (molar ratio 0.7) liposomes, a large decrease in enzyme activity ensued (Fig. 1b). In marked contrast with our observations with glucagon-stimulated activity, when either Mn^{2+} or high Mg^{2+} concentrations were employed in the adenylate cyclase assays then the

Fig. 1. Inhibition of adenylate cyclase activity by dioleoyl-PA

(a) Glucagon-stimulated adenylate cyclase activity. This shows a time course of changes in activity upon incubation with either dioleoyl-PA (Δ , $\circ$, $\bullet$) or cholesterol/dioleoyl-PA (ratio = 0.7) ($\square$, $\blacksquare$) liposomes, or for control incubations with no liposomes ($\blacktriangle$). Assays were performed in the presence of low Mg^{2+} (5 mM) ($\circ$, $\square$), high Mg^{2+} (20 mM) ($\bullet$, $\blacksquare$) and Mn^{2+} (5 mM) (Δ). (b) Fluoride-stimulated adenylate cyclase: symbols are as in (a). Representative experiments of ones performed at least five times are shown. Activity is shown as a percentage of that displayed by the membranes prior to start of the experiment, when assayed with the appropriate metal ion.

perceived inhibitory effect of dioleoyl-PA was much reduced (Fig. 1b).

In native rat liver plasma membranes then, for the fluoride- and glucagon-stimulated responses respectively, adenylate cyclase activities assayed at high (20 mM) Mg^{2+} concentrations were some $47\% \pm 6\%$ and $74\% \pm 8\%$ of the activities observed (100%, 100%) employing low (5 mM) Mg^{2+} . In marked contrast to this, using dioleoyl-PA-treated membranes (40 min incubation with liposomes) these values were $214\% \pm 15\%$ and $73\% \pm 10\%$ for the fluoride- and glucagon-stimulated responses respectively. Using phos-

Fig. 2. Association of dioleoyl-PA with liver plasma membranes

Using ^{14}C -labelled PA the association of this lipid with the plasma membrane was followed. A typical plot of an experiment performed three times is shown.

phatidylserine-treated membranes (40 min incubation with liposomes), activities at high (20 mM) Mg^{2+} concentrations were some $136\% \pm 8\%$ and $44\% \pm 8\%$ of that seen at low (5 mM) Mg^{2+} (100%, 100%) for the fluoride- and glucagon-stimulated responses respectively.

The time course of association of dioleoyl-PA with the liver plasma membranes is shown in Fig. 2.

Both glucagon- and fluoride-stimulated adenylate cyclase activities were activated similarly by the local anaesthetic benzyl alcohol in native and dioleoyl-PA-treated plasma membranes (Fig. 3).

Arrhenius plots of glucagon-stimulated adenylate cyclase activity in dioleoyl-PA-treated membranes exhibited two distinct break points (Fig. 4) at $29.0 \pm 1.5^\circ C$ and $15.7 \pm 2.0^\circ C$. These had activation energies of $12.5 \pm 2.9 \text{ kJ} \cdot \text{mol}^{-1}$ above the upper break point, $48.8 \pm 4.8 \text{ kJ} \cdot \text{mol}^{-1}$ above the lower break point and $105.8 \pm 12.9 \text{ kJ} \cdot \text{mol}^{-1}$ below the lower break point. Arrhenius plots of the fluoride-stimulated adenylate cyclase activity showed a single break at $15.5 \pm 1.8^\circ C$ with activation energies of $53.7 \pm 3.7 \text{ kJ} \cdot \text{mol}^{-1}$ above the break point and $90.0 \pm 17.2 \text{ kJ} \cdot \text{mol}^{-1}$ below the break point (Fig. 4).

Arrhenius-type plots of the order parameter S for the I(12,3) spin probe incorporated into dioleoyl-PA-treated plasma membranes showed three breaks (Fig. 5). These occurred at $28.2 \pm 1.6^\circ C$, $15.2 \pm 1.0^\circ C$ and $7.5 \pm 1.1^\circ C$. The break occurring at $7.5^\circ C$ in the e.s.r. studies, but not observed in the adenylate cyclase assays (Fig. 4), presumably reflects the association, rather than integration, of a fraction of pure dioleoyl-PA which itself undergoes a lipid phase transition at around $8^\circ C$ (Lee, 1977). The break occurring at around $15^\circ C$ represents a new lipid phase separation occurring in the dioleoyl-PA-treated membranes which is also experienced by adenylate cyclase activities (Fig. 4). Native rat liver plasma membranes showed a lipid phase separation occurring at around $28^\circ C$ (see Houslay *et al.*, 1976a; Gordon *et al.*, 1983; Whetton *et al.*, 1982, 1984).

Phosphatidylserine also inhibited adenylate cyclase activity (Fig. 6). However, the inhibitory effect exerted on

Fig. 3. Activation of adenylate cyclase by benzyl alcohol

(a) Glucagon-stimulated adenylate cyclase. The presence of increasing concentrations of benzyl alcohol on adenylate cyclase activity was followed in both native (○) and dioleoyl-PA-treated (●) membranes. (b) Fluoride-stimulated activity: symbols are as in (a). Activity is shown as a percentage change with respect to control values in the absence of benzyl alcohol.

fluoride-stimulated activity was entirely abolished if assays were performed at high (20 mM) Mg^{2+} concentrations. Furthermore, the inhibitory effect of this lipid on glucagon-stimulated activity was enhanced markedly if assays were done at such high Mg^{2+} concentrations (Fig. 6).

DISCUSSION

The acidic phospholipids, phosphatidic acid (PA) and phosphatidylserine, are minor constituents of rat liver plasma membranes, each representing some 5–6% of the total phospholipid pool (see Van Hoesen & Emmelot, 1972). It is possible therefore that they might play a role in influencing the magnitude of the expression of adenylate cyclase activity. Indeed the concentrations of these acidic phospholipids have been shown to increase in tumour (hepatoma) states (see Van Hoesen & Emmelot, 1972; Bergelson *et al.*, 1970) where decreases in the magnitude of adenylate cyclase activity have also

Fig. 4. Arrhenius plots of adenylate cyclase activity in dioleoyl-PA-treated membranes

Dioleoyl-PA-treated rat liver plasma membranes were assayed in the presence of either glucagon (●) or fluoride (▲). Activity is shown as pmol/min per mg of protein. Typical plots of ones performed three times are shown. Similar results were obtained using membranes which had been incubated with dioleoyl-PA/cholesterol liposomes.

Fig. 5. E.s.r. studies in dioleoyl-PA-treated membranes

This shows an Arrhenius type plot of the order parameter S for the I(12,3) fatty acid spin probe inserted into dioleoyl-PA membranes. A representative plot is shown.

Fig. 6. Inhibition of adenylate cyclase activity by phosphatidylserine

Liver plasma membranes were incubated with phosphatidylserine liposomes for various periods of time prior to harvesting, washing and assay for adenylate cyclase activity. Assays were performed in the presence of either glucagon (○, ●) or fluoride (□, ■) at either low (5 mM) Mg^{2+} (○, □) or high (20 mM) Mg^{2+} (●, ■). Activity is shown as a percentage change compared with membranes assayed at the start of the experiment in the absence of liposomes. A typical experiment of one performed at least three times is shown.

been observed (Granner *et al.*, 1968; Makman, 1971; Friedman, 1976).

In the present study we have shown that incubation of rat liver plasma membranes with PA liposomes caused an inhibition of both glucagon- and fluoride-stimulated activity (Fig. 1). The time course of this inhibition (Fig. 2) followed the association of PA with the membranes.

Others have shown that liposomes can fuse with, and be incorporated into, biological membranes (see Poste *et al.*, 1976, for review; Houslay *et al.*, 1976b). Our conclusion here, that dioleoyl-PA has been incorporated into the liver plasma membrane, is supported by both the changes in the form of the Arrhenius plots of ligand-stimulated adenylate cyclase activity (Fig. 4) and by the e.s.r. studies (Fig. 5). We have shown previously that the native lipid phase separation occurring in rat liver plasma membranes (Houslay *et al.*, 1976b) and those induced or altered by a variety of very different drugs (see Houslay & Gordon, 1983, for review) are reflected in corresponding breaks in the Arrhenius plots of ligand-stimulated adenylate cyclase activity. In this regard the fluoride-stimulated activity, reflecting N_s -controlled adenylate cyclase, is affected by lipid phase separations occurring in the inner half of the bilayer, whereas the transmembrane complex formed between the occupied glucagon receptor and both N_s and the catalytic unit of adenylate cyclase is affected by lipid phase separations occurring in both halves of the bilayer (see Houslay & Gordon, 1983). Arrhenius plots of fluoride-stimulated adenylate cyclase activity in native rat liver plasma membranes are linear, whereas those for the glucagon-stimulated activity are biphasic with a well-defined break

occurring at around 28 °C, reflecting a lipid phase separation occurring at this temperature in the external half of the bilayer. Here we see that, in dioleoyl-PA-treated membranes, Arrhenius plots of the fluoride-stimulated adenylate cyclase activity exhibited a single break at around 16 °C and those of the glucagon-stimulated activity two breaks, one at around 28 °C and one at around 16 °C. On the basis of considerable work done with a variety of agents (see Houslay & Gordon, 1983; Whetton *et al.*, 1982), which suggests that fluoride-stimulated adenylate cyclase activity monitors the inner half of the bilayer, we would like to suggest that a new lipid phase separation occurring at around 16 °C is induced in the inner half of the bilayer by the incorporation of dioleoyl-PA into rat liver plasma membranes. This is supported by our e.s.r. studies, which identified a novel lipid phase separation occurring at around 15 °C as well as detecting the one occurring at around 28 °C (Fig. 5). This apparently selective modulation of the inner half of the bilayer is of interest in that acidic phospholipids have been found almost exclusively to reside in this half of the bilayer of native liver plasma membranes (Higgins & Evans, 1978).

The inhibitory effect of dioleoyl-PA incorporation on adenylate cyclase activity is unlikely to be due to any change in the 'fluidity' of the bilayer, as dioleoyl-PA exhibits a lipid phase transition at 8 °C (Lee, 1977) which is not only well below the assay temperature employed (30 °C) but it is also well below the lipid phase separation occurring at 28 °C in native membranes. If anything then, one might expect dioleoyl-PA to increase membrane 'fluidity', an alteration which is well known to enhance and not to decrease adenylate cyclase activity (Houslay & Gordon, 1983). We have shown that the degree of activation of adenylate cyclase elicited by the membrane fluidizing agent benzyl alcohol at 30 °C can be enhanced by decreasing the initial level of fluidity of the membranes (Whetton *et al.*, 1983b). Thus, our observation showing that benzyl alcohol elicited a similar activation of adenylate cyclase in both native and dioleoyl-PA-treated membranes (Fig. 3) suggests that the fluidity of the microenvironment of adenylate cyclase is unlikely to have been reduced in these treated membranes.

Inhibitory effects were also observed using dioleoyl-PA/cholesterol liposomes, which ensured that no marked depletion of cholesterol occurred during the incubations. Indeed, cholesterol depletion could not be responsible for the changes in adenylate cyclase activity noted here. This is because, firstly, cholesterol depletion (Whetton *et al.*, 1983b) leads to a more pronounced inhibitory effect on glucagon-stimulated adenylate cyclase activity than for the fluoride-stimulated activity, rather than the opposite which is seen here (Figs. 1 and 5). Secondly, in cholesterol-depleted membranes, Arrhenius plots of fluoride- and glucagon-stimulated activity exhibit low temperature breaks at a different temperature where, importantly, the activation energies are greater above this break than below it (Whetton *et al.*, 1983b): the opposite of this is seen here (Fig. 4). Thirdly, if true equilibrium of the dioleoyl-PA associated with the membranes had occurred then the lipid phase separation occurring at 28/29 °C would have been depressed some 6–7 °C (Whetton *et al.*, 1983b) and no break occurring at around 8 °C, due to pure dioleoyl-PA, would have been evident. Fourthly, after 10 min incubation with dioleoyl-PA liposomes, true equilibrium would have resulted in a

reduction of the cholesterol:phospholipid molar ratio from 0.7 to 0.64, effecting a 15–20% reduction in both the fluoride and glucagon-stimulated adenylate cyclase activities, rather than the 40% decrease in glucagon-stimulated activity and 55% decrease in fluoride-stimulated activity seen here (Whetton *et al.*, 1983*b*; Figs. 1 and 2). It is likely then that only a small fraction of the total phosphatidic acid associating with the membranes is actually integrated into the bilayer of the liver plasma membrane. This would be consistent with our observation that, under the conditions employed, dioleoyl-PA exerted little effect (Fig. 4) on the 28 °C lipid phase separation attributed to the lipids of the external half of the bilayer. Furthermore, the conditions at which the liposome incubations were performed here, i.e. short time, low concentration and low temperature, would not allow for cholesterol loss from the membranes (Whetton *et al.*, 1983*b*). For these reasons, and that a similar inhibitory effect (Fig. 5) was observed with phosphatidylserine liposomes derived from beef brain lipids, which exhibit a wide spectrum of associated fatty acyl chains, and not after incubation with dioleoyl phosphatidylcholine liposomes under the conditions used in this study (results not shown), we believe that acidic phospholipids inhibit adenylate cyclase activity by a direct headgroup interaction.

Interestingly, if either Mn^{2+} or high concentrations of Mg^{2+} were present in the adenylate cyclase assays then the perceived inhibitory effect of either dioleoyl-PA (Fig. 1) or phosphatidylserine (Fig. 5) was enhanced as regards glucagon-stimulated activity and reduced as regards fluoride-stimulated adenylate cyclase activity. As Mg^{2+} is relatively ineffective at binding to phospholipids (Hoekstra, 1982) and Arrhenius plots of glucagon-stimulated adenylate cyclase activity in dioleoyl-PA-treated plasma membranes were unchanged in the presence of high $[Mg^{2+}]$, we consider it unlikely that high Mg^{2+} exerts its effect by inducing lipid segregation. Both N_s and adenylate cyclase, however, exhibit Mg^{2+}/Mn^{2+} -binding sites (Birnbaumer *et al.*, 1985) where stimulatory receptors effect a decrease in the K_a for Mg^{2+} expressed by N_s . Thus either 'high' concentrations of Mg^{2+} or the presence of Mn^{2+} , which exhibits a high affinity for N_s , can effect conformational and functional changes in N_s . Indeed, we have shown (Houslay *et al.*, 1983) that Mn^{2+} selectively augments the fluoride-stimulated and not the glucagon-stimulated activity of adenylate cyclase. Given then the surprisingly different responses of glucagon- and fluoride-stimulated adenylate cyclase to acidic phospholipids, when assayed with Mn^{2+} rather than low Mg^{2+} concentrations, it is tempting to suggest that acidic phospholipids might exert their inhibitory action at the level of N_s .

Our conclusions drawn here are in direct contrast to those of Rubaclava & Rodbell (1973) who suggested that acidic phospholipids were required for glucagon-stimulated adenylate cyclase activity. Their conclusion was based upon observations that a bacterial phospholipase C preparation, suggested to exhibit a preference for degrading acid phospholipids, caused a selective inhibition of the glucagon- rather than fluoride-stimulated activity. However, in excess of 20% of the total membrane phospholipids were degraded by the phospholipase in their study before inhibition ensued, with total loss of glucagon-stimulated adenylate cyclase activity ensuing after the degradation of 50% of the total phospholipids.

The ability actually to digest such a high percentage of plasma membrane phospholipids, where the acidic species make up only 10–15% of the total, clearly leads one to question the specificity of the phospholipase used in their study. Also, as the glucagon receptor interacts with adenylate cyclase components through productive collisions within the bilayer (see Houslay *et al.*, 1977), it is likely that loss of stimulatory response ensued because of the breakdown of the bilayer itself.

We would like to suggest that acidic phospholipids inhibit ligand-stimulated adenylate cyclase activity by virtue of a specific headgroup interaction. This inhibitory effect is probably localized at the level of N_s coupling to adenylate cyclase and such acidic lipid species may be involved in influencing the Mg^{2+} -sensitivity of ligand-stimulated adenylate cyclase in various biological membranes.

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REFERENCES

- Bergelson, L. D., Dyatlovitskaya, E. V., Torkhovskaya, T. I., Sorokina, I. B. & Gorkova, N. P. (1970) *Biochim. Biophys. Acta* **210**, 287–298
- Birnbaumer, L., Codina, J., Mattera, R., Cerione, R. A., Hildebrandt, J. D., Sunya, T., Rojas, F. J., Caron, M. J., Lefkowitz, R. J. & Iyengar, R. (1985) in *Molecular Aspects of Cellular Regulation* (Cohen, P. & Houslay, M. D., eds.), vol. 4, pp. 131–182, Elsevier Biomedical Press, Amsterdam
- Engelhard, V. H., Glaser, M. & Storm, D. R. (1978) *Biochemistry* **17**, 3191–3200
- Friedman, D. L. (1976) *Physiol. Rev.* **56**, 652–708
- Gilman, A. G. (1984) *Cell* **36**, 577–579
- Gordon, L. M., Whetton, A. D., Rawal, S., Esgate, J. A. & Houslay, M. D. (1983) *Biochim. Biophys. Acta* **729**, 104–114
- Granner, D. K., Chase, L., Aurbach, G. & Tomkins, G. M. (1968) *Science* **162**, 1018–1020
- Hebdon, G. M., Le Vine, H., III, Minard, R. B., Sahyoun, N. E., Schmitges, C. J. & Cuatrecasas, P. (1979) *J. Biol. Chem.* **254**, 10459–10465
- Hebdon, G. M., Le Vine, H., III, Sahyoun, N. E., Schmitges, C. J. & Cuatrecasas, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 120–123
- Higgins, J. A. & Evans, W. H. (1978) *Biochem. J.* **174**, 563–567
- Hoekstra, D. (1982) *Biochemistry* **21**, 1055–1061
- Houslay, M. D. & Gordon, L. M. (1983) *Curr. Topics Membr. Transport* **188**, 179–231
- Houslay, M. D. & Palmer, R. W. (1978) *Biochem. J.* **174**, 909–919
- Houslay, M. D. & Palmer, R. W. (1979) *Biochem. J.* **178**, 217–221
- Houslay, M. D., Metcalfe, J. C., Warren, G. B., Hesketh, T. R. & Smith, G. A. (1976*a*) *Biochim. Biophys. Acta* **436**, 489–494
- Houslay, M. D., Hesketh, T. R., Smith, G. A., Warren, G. B. & Metcalfe, J. C. (1976*b*) *Biochim. Biophys. Acta* **436**, 495–504
- Houslay, M. D., Ellory, J. C., Smith, G. A., Hesketh, T. R., Stein, J. M., Warren, G. B. & Metcalfe, J. C. (1977) *Biochim. Biophys. Acta* **467**, 208–219
- Houslay, M. D., Dipple, I. & Elliott, K. R. F. (1980) *Biochem. J.* **186**, 649–658
- Houslay, M. D., Dipple, I. & Gordon, L. M. (1981) *Biochem. J.* **197**, 675–681
- Houslay, M. D., Heyworth, C. M. & Whetton, A. D. (1983) *FEBS Lett.* **155**, 311–316
- Lee, A. G. (1977) *Biochim. Biophys. Acta* **472**, 237–281
- Limbird, L. L. (1981) *Biochem. J.* **195**, 1–13

- Makman, M. H. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2127–2130
- Marchmont, R. J., Ayad, S. R. & Houslay, M. D. (1981) *Biochem. J.* **195**, 645–652
- Martin, B. R., Stein, J. M., Kennedy, E. L., Doberska, C. A. & Metcalfe, J. C. (1979) *Biochem. J.* **184**, 253–260
- Needham, L. & Houslay, M. D. (1982) *Biochem. J.* **206**, 89–95
- Needham, L., Finnegan, I. & Houslay, M. D. (1985) *FEBS Lett.* **183**, 81–86
- Northup, J. K. (1985) in *Molecular Aspects of Cellular Regulation* (Cohen, P. & Houslay, M. D., eds.) vol. 4, pp. 91–116, Elsevier Biomedical Press, Amsterdam
- Poste, G., Papahadjopoulos, D. & Vail, W. J. (1976) *Methods Cell Biol.* **14**, 34–68
- Ross, E. M. (1982) *J. Biol. Chem.* **257**, 10751–10758
- Ross, E. M. & Gilman, A. G. (1980) *Annu. Rev. Biochem.* **49**, 533–564
- Rubacava, B. & Rodbell, M. (1973) *J. Biol. Chem.* **248**, 3831–3837
- Van Hoeven, R. P. & Emmelot, P. (1972) *J. Membr. Biol.* **9**, 105–126
- Whetton, A. D., Johannsson, A., Wilson, S. R., Wallace, A. V. & Houslay, M. D. (1982) *FEBS Lett.* **143**, 147–152
- Whetton, A. D., Gordon, L. M. & Houslay, M. D. (1983a) *Biochem. J.* **210**, 437–449
- Whetton, A. D., Gordon, L. M. & Houslay, M. D. (1983b) *Biochem. J.* **212**, 331–338
- Whetton, A. D., Needham, L. M., Margison, G. P., Dodd, N. J. F. & Houslay, M. D. (1984) *Biochim. Biophys. Acta* **773**, 106–112

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