Lysophosphatidylcholines Can Modulate the Activity of the Glucagon-Stimulated Adenylate Cyclase from Rat Liver Plasma Membranes

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1. Synthetic lysophosphatidylcholines inhibit the glucagon-stimulated adenylate cyclase activity of rat liver plasma membranes at concentrations two to five times lower than those needed to inhibit the fluoride-stimulated activity. 2. Specific ^{125}I -labelled glucagon binding to hormone receptors is inhibited at concentrations similar to those inhibiting the fluoride-stimulated activity. 3. At concentrations of lysophosphatidylcholines immediately below those causing inhibition, an activation of adenylate cyclase activity or hormone binding was observed. 4. These effects are essentially reversible. 5. We conclude that the increased sensitivity of glucagon-stimulated adenylate cyclase to inhibition may be due to the lysophosphatidylcholines interfering with the physical coupling between the hormone receptor and catalytic unit of adenylate cyclase. 6. We suggest that, *in vivo*, it is possible that lysophosphatidylcholines may modulate the activity of adenylate cyclase only when it is in the hormone-stimulated state.

Lysophosphatidylcholines have been demonstrated to inhibit the fluoride-stimulated adenylate cyclase activity of 3T3 mouse fibroblasts (Shier *et al.*, 1976) and the basal adenylate cyclase activity of neuroblastoma cells (Zwiller *et al.*, 1976) at concentrations where they activate the guanylate cyclase activity of these cell preparations. This observation led those authors to suggest that lysophosphatidylcholines may participate in the regulation of cellular cyclic nucleotide concentrations.

Lysophosphatidylcholines are present in rat liver in significant concentrations (see Ray *et al.*, 1969) and have been demonstrated to modulate the activity of a number of membrane-bound enzymes in this tissue (Mookerjee & Yung, 1974; Shier & Trotter, 1976; Houslay, 1978). We have demonstrated that the properties of the lipid environment of glucagon-stimulated adenylate cyclase can have a marked effect on its activity (Houslay *et al.*, 1976b; Dipple & Houslay, 1978; Houslay & Palmer, 1978). The present study demonstrates the effect of lysophosphatidylcholines on the adenylate cyclase activity of rat liver plasma membranes when stimulated directly by the ligand fluoride or through the hormone receptor by glucagon.

Methods

Rat liver plasma membranes from male Sprague-Dawley rats (250–300 g body wt.) were prepared by the method of Pilakis *et al.* (1974) modified as described previously (Houslay *et al.*, 1976a). The assay of

ligand-stimulated adenylate cyclase activity (EC 4.6.1.1) and the determination of cyclic AMP was carried out as described in full previously (Houslay *et al.*, 1976a), except that the creatine kinase (EC 2.7.3.2) concentration was 0.5 mg/ml and bovine serum albumin was added to the assay mixture to a concentration of 0.5 mg/ml. Before use the bovine serum albumin was dialysed against 10 mM-EDTA and then exhaustively dialysed against deionized water with subsequent freeze-drying. All assays were carried out at 30°C at a final membrane concentration of either 0.2 mg/ml or 0.4 mg/ml; results were similar and linear with respect to time.

By treating our membrane fraction with buffers of various ionic strengths, with short periods (5–15 s) of sonication, with freeze-drying, with homogenization and with repeated centrifugation and resuspension (Houslay *et al.*, 1976a), we were unable to identify any latent ligand-stimulated adenylate cyclase activity. Furthermore, all catalytic units have been shown to be able to couple structurally to the glucagon receptor on addition of hormone (Houslay *et al.*, 1977). We consider our fraction to consist of membrane sheets, as evidenced by electron microscopy, and this would be in accord with the results of other workers (see Pilakis *et al.*, 1974).

Protein determination was by the method of Goa (1953) as modified by Houslay & Palmer (1978). The 1-acyl-*sn*-glycero-3-phosphocholines (lysoacylphosphatidylcholines) were either prepared by treatment of their respective 1,2-diacyl-*sn*-glycero-3-phosphocholines with phospholipase A_2 (EC 3.1.1.4)

as described by Houslay (1978), or were prepared identically but given as a gift by Dr. G. A. Smith, Department of Biochemistry, University of Cambridge. The 1,2-diacyl-*sn*-glycero-3-phosphocholines were synthesized as described by Robles & van den Berg (1969). ^{125}I -labelled glucagon was prepared as described by Giorgio *et al.* (1974), and specific binding to glucagon receptors assessed as described by Houslay *et al.* (1977), except that the concentration of bovine serum albumin in the binding assay was 2mg/ml and the membrane concentration was 0.1mg/ml.

Reversibility of the effects of lysophosphatidylcholines was assessed routinely by diluting the assays 10-fold with 1mM- KHCO_3 , pH 7.2, containing 10mg of bovine serum albumin/ml and centrifuged for 6min at 14000g on a Microfuge. In some cases the suspension was centrifuged for 60min at 100000g on an MSE 65 ultracentrifuge. ATP, creatine kinase and triethanolamine were from Boehringer Corp., Lewes, East Sussex, U.K. Egg lysophosphatidyl-

ethanolamine and venom phospholipase A_2 were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of A.R. grade from BDH Chemicals Ltd., Poole, Dorset, U.K. Glucagon was kindly given by Dr. W. W. Bromer, Lilly Research Laboratories, Indianapolis, IN, U.S.A.

Results

All three lysophosphatidylcholines had a marked effect on the activity of both fluoride- and glucagon-stimulated adenylate cyclase activity (Fig. 1). The effects observed were apparently instantaneous (<1min), as time courses of activity at 30°C were linear over a 10min period. All the lysophosphatidylcholines augmented the fluoride-stimulated activity at the lower concentrations tested and then caused inhibition at concentrations greater than about 0.2mM (Fig. 1). The activation of the fluoride-

Fig. 1. Effect of lysophosphatidylcholines on the adenylate cyclase activity of rat liver plasma membranes. All data points are given with s.d. (a) Effect of lysomyristoylphosphatidylcholine on the glucagon- (□) and fluoride- (○) stimulated adenylate cyclase activity. Four different membrane preparations were tested in duplicate ($n = 8$). (b) Effect of lysomyristoylphosphatidylcholine on the specific ^{125}I -labelled-glucagon binding to the hormone receptor (▲). Non-specific binding was unchanged by additions. Two different membrane preparations were tested in duplicate ($n = 4$). (c) Effect of lyso-oleoylphosphatidylcholine on the glucagon- (□) and fluoride- (○) stimulated adenylate cyclase activity. Two membrane preparations were tested in duplicate ($n = 4$). (d) Effect of lysopalmitoylphosphatidylcholine on the glucagon- (□) and fluoride- (○) stimulated activity. Three membrane preparations were tested in duplicate ($n = 6$).

stimulated activity caused by the lysophosphatidylcholines was as much as 30% and in the case of oleoyl- and palmitoyl-lysophosphatidylcholine was most pronounced at the concentrations just below those at which inhibition set in (Figs. 1c and 1d). The effects of lysophosphatidylcholines on the fluoride-stimulated activity were fully reversible (>90%), although at concentrations greater than 0.3 mM, centrifugation at the higher speed (see the Methods section) together with a further washing was necessary to pellet the material and demonstrate reversibility over the range of concentrations tested.

The specific binding of ^{125}I -labelled glucagon to hormone receptors showed a similar sensitivity to lysomyristoylphosphatidylcholine as did the fluoride-stimulated activity (Fig. 1b).

The response of the glucagon-stimulated activity to the lysophosphatidylcholines was markedly different from that exhibited by both the fluoride-stimulated activity and specific ^{125}I -labelled glucagon binding to hormone receptors (Fig. 1). Very little, if any, activation occurred over the entire range, but its sensitivity to inhibition was far greater, occurring at between 2- and 5-fold lower concentrations. Reversibility of these effects could be demonstrated up to concentrations of around 0.4 mM-lysophosphatidylcholine, but above this, irreversible loss of glucagon-stimulated adenylate cyclase activity occurred.

The magnitude of these effects and the exact concentrations at which they occurred differed between the various-chain-length lysophosphatidylcholines (Fig. 1), but in all cases the glucagon-stimulated activity was inhibited at concentrations much lower than those needed to inhibit the fluoride-stimulated activity.

The action of egg lysophosphatidylethanolamine was investigated over an identical concentration range. The fluoride-stimulated activity was unaffected by the compound, and the glucagon-stimulated activity only began to be inhibited (reversibly) at concentrations greater than 0.2 mM, attaining approx. 20% inhibition at 2 mM.

Discussion

Lysophosphatidylcholines have the ability to modulate the activity of the adenylate cyclase activity from rat liver plasma membranes when it is in both the uncoupled state (activated by fluoride) and the coupled state (activated by glucagon through its hormone receptor). There appear to be slight differences in the effects observed when using the different lysophosphatidylcholine species (Fig. 1). Lysophosphatidylethanolamine had no effect on the fluoride-stimulated adenylate cyclase activity at the concentrations tested, and only achieved a moderate inhibition of the glucagon-stimulated activity. In

mouse 3T3 fibroblasts, the highest concentration of egg lysophosphatidylethanolamine tested by us achieved a 50% inhibition (Shier *et al.*, 1976) of the fluoride-stimulated activity and about a 25% inhibition of the basal adenylate cyclase activity of neuroblastoma cells (Zwiller *et al.*, 1976). Thus there seem to be differences in sensitivity to lysophosphatidylethanolamine inhibition when using adenylate cyclase from different sources.

The different lysophosphatidylcholine species used in the present study all achieved similar effects, namely, the ability to activate the fluoride-stimulated activity at low concentrations and then progressively inhibit it as the concentration increased. The effect on the glucagon-stimulated adenylate cyclase activity, which requires both the catalytic unit of adenylate cyclase and the glucagon receptor, appeared to be purely inhibitory, with a marked inhibition occurring at much lower concentrations than those required to inhibit the fluoride-stimulated activity. This increased sensitivity could have been due to inactivation of hormone binding to the receptor, but specific binding of ^{125}I -labelled glucagon to hormone receptors was only decreased at the higher lysomyristoylphosphatidylcholine concentrations needed to inhibit the activity of the uncoupled (fluoride) response. Thus the increased sensitivity of the glucagon-stimulated adenylate cyclase to inhibition by lysophosphatidylcholine is likely to be due either to interference with the physical interaction between the receptor and catalytic unit of the enzyme (coupling) that leads to activation of the catalytic unit, or to a changed sensitivity exhibited to lysophosphatidylcholines by the complex of receptor and catalytic unit. Lysophosphatidylcholines have surfactant properties, and detergents have been demonstrated to inhibit the glucagon-stimulated activity at lower concentrations than those required to inhibit the activity of the uncoupled catalytic unit (Dipple & Houslay, 1978). Such inhibition may be achieved by interacting directly with the protein species exposed to the aqueous environment or by partitioning into the bilayer and displacing phospholipid species that may be essential for coupling from around the proteins.

We consider it highly unlikely that the effects we observed were due to the presence of sealed vesicles in our sample. As discussed in the Methods section, we could obtain no evidence for latent activity. Neither sealed inside-out nor right-side-out vesicles would exhibit glucagon-stimulated activity, and we obtained no evidence for lysophosphatidylcholines removing a permeability barrier and increasing glucagon-stimulated activity. Only sealed right-side-out vesicles would show latent fluoride-stimulated activity, and that should increase in parallel with glucagon-stimulated activity, whereas specific glucagon-

gon-binding would be unaffected. This does not occur.

An interesting phenomenon common to the action of the lysophosphatidylcholines on all the activities monitored was that, at concentrations immediately below those effecting a rapid loss of activity/binding, there was a point at which activity/binding appeared to be enhanced. This again has analogies with the action of the detergent Lubrol PX on this system (Dipple & Houslay, 1978), and it is tempting to suggest that, at such concentrations, lysophosphatidylcholine increases the fluidity of the bilayer around the protein, relieving a physical constraint which activates the enzyme. Increasing concentrations may then displace annular lipid essential for activity. The elevated ^{125}I -labelled glucagon binding to hormone receptors at this point may then be due to either a change in K_A for binding or the exposure of cryptic binding sites as the membrane is disrupted.

The sensitivity of adenylate cyclase to both activation and inhibition by lysophosphatidylcholines shows that caution must be exercised in studies attempting to demonstrate the specific phospholipid-dependence of the enzyme by treating plasma membranes with phospholipase A_2 (see Pohl *et al.*, 1971; Birnbaumer *et al.*, 1971). It has been demonstrated (Birnbaumer *et al.*, 1971) that incubation of plasma membranes with increasing concentrations of phospholipase A_2 caused an initial activation of fluoride-stimulated adenylate cyclase followed by a progressive inhibition. The glucagon-stimulated activity showed a very slight activation at low concentration of phospholipase A_2 , followed by a very rapid inhibitory response as the concentration was raised. We would suggest that the lysophosphatidylcholines produced by the action of the phospholipase A_2 on the membrane lipids were in part responsible for the changes in activity.

It is interesting to consider whether the changes in adenylate cyclase activity on exposure to lysophosphatidylcholines has any relevance *in vivo*. Calculations have been carried out (Mookerjee & Yung, 1974) purporting to demonstrate that if all the lysophosphatidylcholine in rat liver cells were uniformly distributed, it would attain a concentration of 1.5 mg/ml (approx. 2.9 mM). Clearly this is extremely unlikely to reflect the free lysophosphatidylcholine concentration in the cell, as one would expect considerable compartmentation of this species and binding to cellular components (Reitz *et al.*, 1969). Indeed such concentrations would be expected to disrupt cell membranes, and irreversibly inhibit adenylate cyclase. Other information that may give an insight as to the concentration of lysophosphatidylcholine expected, is from kinetic data on the lysophosphatidylcholine acyltransferase enzymes (EC 2.3.1.23) responsible for converting lysophos-

phatidylcholine into diacylphosphatidylcholine and located in the plasma membrane, endoplasmic reticulum and mitochondrial outer membrane (see van den Bosch, 1974). Under conditions where acyl-CoA is saturating, these enzymes appear to exhibit K_m values for lysophosphatidylcholine in the range 0.02–0.2 mM (see Okuyama *et al.*, 1972; Jezyk & Hughes, 1973; Wittels, 1973). If the intracellular concentrations of lysophosphatidylcholine were of this magnitude, it is unlikely they would have significant inhibitory effects on the activity of the uncoupled catalytic unit, although they might have reversible stimulatory effects dependent on the species of lysophosphatidylcholine. Such concentrations could, however, act to inhibit the response of adenylate cyclase to stimulation by glucagon by affecting the coupling interaction, and thus modulate cellular responses to this hormone. Such concentrations of lysophosphatidylcholine would only be achieved intracellularly, and it is not apparent from our experiments which side of the bilayer they need to act on to achieve their effect or indeed if they need to penetrate the bilayer. There is evidence that demonstrates that although in liposomes the transfer of lysophosphatidylcholine from one side of the bilayer to the other ('flip-flop') is negligible (de Kruijff *et al.*, 1977; van den Besselaar *et al.*, 1977), on incorporation of proteins that span the bilayer into liposomes, then significant 'flip-flop' rates ($t_{1/2}$ about 1 h) are achieved (van Zoelen *et al.*, 1978). Thus one should not discount the possibility that lysophosphatidylcholines produced intracellularly *in vivo* could serve to modulate the activity of glucagon-stimulated adenylate cyclase. However, we consider it unlikely that lysophosphatidylcholines would reach concentrations *in vivo* high enough to inhibit the uncoupled catalytic unit.

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References

- Birnbaumer, L., Pohl, S. L. & Rodbell, M. (1971) *J. Biol. Chem.* **246**, 1857–1860
- de Kruijff, B., van den Besselaar, A.-M. H. P., Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta* **465**, 443–453
- Dipple, I. & Houslay, M. D. (1978) *Biochem. J.* **174**, 179–190
- Giorgio, N. A., Johnson, C. B. & Blecher, M. (1974) *J. Biol. Chem.* **249**, 428–437
- Goa, J. (1953) *Scand. J. Clin. Invest.* **5**, 218–222
- Houslay, M. D. (1978) *Biochem. Pharmacol.* **27**, 1287–1288
- Houslay, M. D. & Palmer, R. W. (1978) *Biochem. J.* **174**, 909–919

- Houslay, M. D., Metcalfe, J. C., Warren, G. R., Hesketh, T. R. & Smith, G. A. (1976a) *Biochim. Biophys. Acta* **436**, 489–494
- Houslay, M. D., Hesketh, T. R., Smith, G. A., Warren, G. B. & Metcalfe, J. C. (1976b) *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
- Jezyk, P. F. & Hughes, H. N. (1973) *Biochim. Biophys. Acta* **296**, 24–33
- Mookerjee, S. & Yung, J. W. M. (1974) *Biochem. Biophys. Res. Commun.* **57**, 815–822
- Pilkis, S. J., Exton, J. H., Johnson, R. A. & Park, C. R. (1974) *Biochim. Biophys. Acta* **343**, 250–267
- Pohl, S. L., Krons, H. M. J., Kozyreff, V., Birnbaumer, L. & Rodbell, M. (1971) *J. Biol. Chem.* **246**, 447–454
- Okuyama, H., Lands, W. E. M., Gunstone, F. D. & Barve, J. A. (1972) *Biochemistry* **11**, 4392–4398
- Ray, T. K., Skipski, V. P., Barclay, M., Essner, E. & Archibald, F. M. (1969) *J. Biol. Chem.* **244**, 5528–5536
- Reitz, R. C., A-Sheikh, M., Lands, W. E. M., Ismail, J. A. & Gunstone, F. D. (1969) *Biochim. Biophys. Acta* **176**, 480–490
- Robles, E. C. & van den Berg, B. (1969) *Biochim. Biophys. Acta* **187**, 520–526
- Shier, W. T. & Trotter, J. T., III (1976) *FEBS Lett.* **62**, 165–168
- Shier, W. T., Baldwin, J. H., Nilsen-Hamilton, M., Hamilton R. T. & Thanassi, N. M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1586–1590
- van den Besselaar, A.-M. H. P., van den Bosch, D. & van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta* **465**, 545–465
- van den Bosch, H. (1974) *Am. Rev. Biochem.* **43**, 243–277
- van Zoelen, E. J. J., De Kruijff, B. & van Deenen, L. L. M. (1978) *Biochim. Biophys. Acta* **508**, 97–108
- Wittels, B. (1973) *J. Biol. Chem.* **248**, 2906–2911
- Zwiller, J., Ciesielski-Treska, J. & Mandel, P. (1976) *FEBS Lett.* **69**, 286–290