Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 120–123, January 1981
Biochemistry

Specific phospholipids are required to reconstitute adenylate cyclase solubilized from rat brain

(sodium deoxycholate/cholesterol esters)

G. MATTHEW HEBDON*, HARRY LEVINE III*, NAJI E. SAHYOUN*, CLAUS J. SCHMITGES*, AND PEDRO CUATRECASAS

The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

Communicated by Solomon H. Snyder, September 16, 1980

ABSTRACT **Adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] was solubilized from a rat brain homogenate with sodium deoxycholate. This solubilized preparation had no detectable enzymic activity with either Mg-ATP or Mn-ATP as substrate. The activity could be restored by addition of either nonionic detergent or certain specific phospholipids. Maximal restoration of enzyme activity was obtained with Triton X-100, L-α-phosphatidylcholine, L-α-lysophosphatidylcholine, phosphatidyl-*N*-monomethylethanolamine, or sphingomyelin. Activity was only partially restored by phosphatidylethanolamine (40–60%) or phosphatidyl-*N*,*N*-dimethylethanolamine (10–20%). Other phospholipids tested, including phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid, could not restore enzyme activity but, instead, could inhibit the stimulation of enzyme activity by phosphatidylcholine. The restoration of activity by L-α-phosphatidylcholine was inhibited by cholesterol at concentrations above 33 mol %, although this effect was not observed with three different esters of cholesterol. These studies suggest a possible specific role of phospholipids in modulating adenylate cyclase activity.**

Adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] is a membrane-bound enzyme (1, 2) that, through the generation of cyclic AMP in response to hormones, exerts profound effects on cellular metabolism (3, 4). Reports have appeared suggesting a role for lipids in modulating the activity of membrane-bound enzymes (5, 6). Examples exist where this modulation seems to reflect changes in membrane fluidity (7, 8) and others where there appears to be a requirement for certain specific phospholipids for enzyme activity (9–11). Such investigations have been extended to the study of adenylate cyclase principally by the direct addition of phospholipids (or cholesterol) to cells or membranes by lipid fusion or exchange or by supplemention of the medium of auxotrophic mutant cells with phospholipid precursors (12–15). Although these studies have yielded much new and interesting information, the added lipids may be exerting uncontrolled effects on the metabolism of the cells or they may not partition equally throughout the membrane.

We have reported the solubilization of adenylate cyclase from rat brain and its subsequent incorporation into liposomes of defined phospholipid composition (16). These studies showed that the enzyme activity in the liposomes was dependent on the particular phospholipids present. One drawback to this study is the use of nonionic detergents which, themselves, are capable of stimulating activity. In the present investigation we have used sodium deoxycholate to solubilize the enzyme; this preparation is totally dependent on addition of certain specific phospholipids or nonionic detergent for activity. The results further support the hypothesis that the activity of adenylate cyclase may be dependent on the presence of specific phospholipids.

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MATERIALS AND METHODS

Materials. [α-^{32}P]ATP and cyclic [2,8-^{3}H]AMP were from New England Nuclear; alumina, activity 1, was from ICN. Triton X-100 was obtained from Packard. Pyruvate kinase was purchased from Boehringer Mannheim. Phosphatidyl-*N*-methylethanolamine (no. 835-8125) and phosphatidylglycerol (no. 835-8126) were supplied by GIBCO. The following naturally derived lipids were from Sigma: phosphatidylcholine (P 5763), lysophosphatidylcholine (L 4129), sphingomyelin (S 7004), phosphatidylserine (P 6641), phosphatidic acid (P 9511), phosphatidylinositol (P 0639), phosphatidylethanolamine (P 4513), phosphatidyl-*N*,*N*-dimethylethanolamine (P 1634), cholesterol (CH-S), cholesterol acetate (CH-SA), cholesterol stearate (CH-SS), and cholesterol oleate (CH-SO). ATP, phospho*enol*pyruvate, and sodium deoxycholate were also obtained from Sigma. Triolein and 1,3-diolein were purchased from P-L Biochemicals.

Preparation of Solubilized Adenylate Cyclase. Male rats (Sprague–Dawley, 120–170 g) were decapitated and their brains were removed. Subsequent operations were performed at 0°C. Each brain was homogenized in 8 vol (vol/wt) of 3 mM $MgCl_2$/3 mM dithiothreitol/50 mM Tris·HCl, pH 8.2, and centrifuged for 10 min at 40,000 × g_{max}. This procedure was repeated once and the pellet was then homogenized in 8 vol of 1 mM $MgCl_2$/3 mM dithiothreitol/0.5% deoxycholate/50 mM Tris·HCl, pH 8.2. The homogenate was incubated at 0°C for 20 min and centrifuged for 40 min at 300,000 × g_{max}; further centrifugation of the supernatant for 45 min at 300,000 × g_{max} did not sediment any further enzyme activity. The supernatant, containing solubilized (but inactive) adenylate cyclase, was retained for further study.

Incubation Procedure. Phospholipid in organic solvent was dried under a stream of dry N_2 until the last trace of solvent was removed. The phospholipid was dispersed in the deoxycholate-solubilized adenylate cyclase preparation with a glass rod and a Vortex mixer to give the concentration noted in the text; this mixture was incubated at 0°C for 20 min prior to assay for adenylate cyclase activity.

Incorporation of Adenylate Cyclase into Liposomes. Phospholipid (10 mg/ml) was dispersed in the deoxycholate-solubilized adenylate cyclase (as described above). This mixture was diluted 20- to 40-fold with 50 mM Tris·HCl (pH 8.2) and centrifuged at 300,000 × g_{max} for 1 hr. The liposomal pellet was

* Authors' names arranged in alphabetical order.

resuspended in 50 mM Tris·HCl (pH 8.2) and assayed for adenylate cyclase activity.

Adenylate Cyclase Assay. Enzyme activity was measured for 20 min at 30°C as described (16). The results are the mean of duplicate determinations. The results presented here are representative of several (at least three) separate experiments because the absolute magnitude of the changes varies somewhat (±20%) with different preparations. Because the activity of the deoxycholate-solubilized enzyme was totally dependent on the nature of the added phospholipid, it did not seem appropriate to express it as specific activity, which may give a misleading appearance of purification.

Protein Determination. Protein concentration was estimated by the method of Lowry *et al.* (17) as modified by Torngvist and Belfrage (18).

RESULTS

Effect of Added Nonionic Detergent, Phosphatidylcholine, and Lysophosphatidylcholine. Adenylate cyclase solubilized from a rat brain homogenate with 0.5% deoxycholate had essentially no enzymic activity with either Mg^{2+} (see Table 1) or Mn^{2+} as the metal ion cofactor.

Fig. 1 shows the effect on enzyme activity of adding increasing concentrations of Triton X-100, phosphatidylcholine, and lysophosphatidylcholine. The maximal extent of activation with nonionic detergent, phosphatidylcholine, or lysolecithin was about the same as that achieved with sphingomyelin (not shown). The optimal concentration of lysophosphatidylcholine was about 5 mg/ml; that of the other phospholipids or detergent was about 10–20 mg/ml. Addition of nonionic detergent plus phosphatidylcholine gave an activity that was somewhat greater than either one alone. The activation by lysolecithin, in contrast, was inhibited by the presence of Triton X-100.

Table 1. Incorporation of adenylate cyclase into liposomes

Sample	Adenylate cyclase activity, pmol/min	Protein concentration, mg/ml
Original brain homogenate	17	13
Deoxycholate supernatant	0.5	5.1
Deoxycholate pellet	1.3	7.9
Deoxycholate supernatant + phosphatidylcholine	36.5	5.1
Liposomes	45.5	3.0

Rat brain was homogenized and treated with deoxycholate. Phosphatidylcholine (10 mg/ml) was dispersed in the detergent-solubilized enzyme preparation and the liposomes were collected by centrifugation (300,000 × g_{max} for 1 hr). All samples were resuspended to equivalent volumes, and aliquots were taken to measure enzyme activity.

Effects of Other Phospholipids. Phosphatidylserine, phosphatidic acid, phosphatidylglycerol, phosphatidylinositol, and di- or triglycerides, at 10 mg/ml, were unable to restore enzyme activity. Fig. 2 shows the activity obtained at constant lipid concentration for phosphatidylcholine in the presence of increasing proportions of phosphatidylserine or phosphatidic acid. Both of these phospholipids inhibited the reconstitution of activity.

Axelrod and his colleagues (19, 20) have proposed a mechanism for hormonal activation of adenylate cyclase involving the sequential methylation of phosphatidylethanolamine to phosphatidylcholine. We therefore decided to investigate the effects of phosphatidylethanolamine and the methylated intermediates

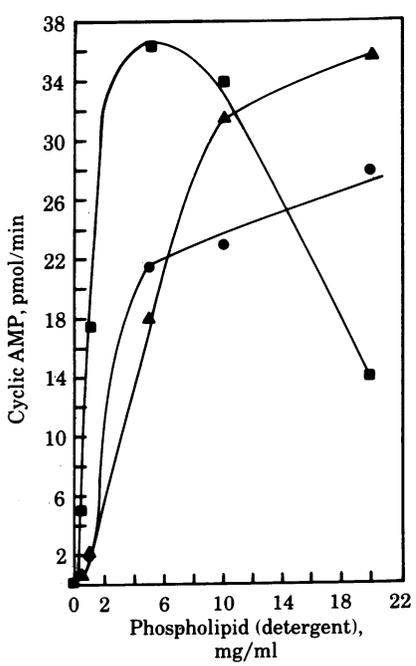

FIG. 1. Effect of nonionic detergent, phosphatidylcholine, and lysophosphatidylcholine on solubilized adenylate cyclase activity. Phospholipid was dried under a stream of nitrogen and resuspended at the concentration indicated in a solution of adenylate cyclase (5.1 mg of protein per ml) that had been solubilized in 0.5% deoxycholate. Triton X-100 was added directly to the solubilized enzyme preparation. The mixtures were incubated at 0°C for 20 min before 50-μl aliquots were assayed for enzyme activity. ●, Triton X-100; ▲, phosphatidylcholine; ■, lysophosphatidylcholine.

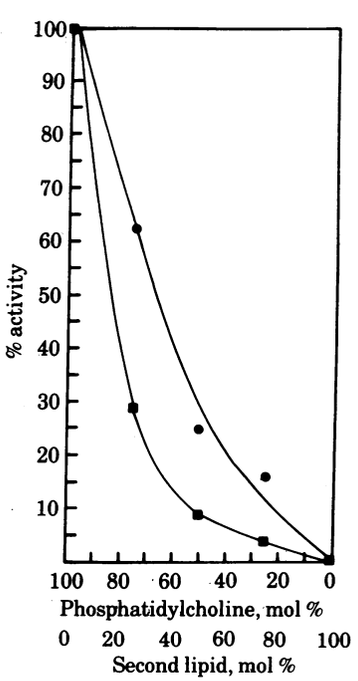

FIG. 2. Effect of phosphatidylserine and phosphatidic acid on solubilized adenylate cyclase. Phosphatidylserine and phosphatidic acid were mixed with phosphatidylcholine in the proportions indicated and the mixture was dried under a stream of nitrogen. The phospholipid mixture was resuspended in deoxycholate-solubilized adenylate cyclase (5.1 mg of protein per ml) to give a final concentration of 10 mg of phospholipid per ml. The suspension was incubated at 0°C for 20 min and then assayed for enzyme activity. ●, Phosphatidylserine plus phosphatidylcholine; ■, phosphatidic acid plus phosphatidylcholine.

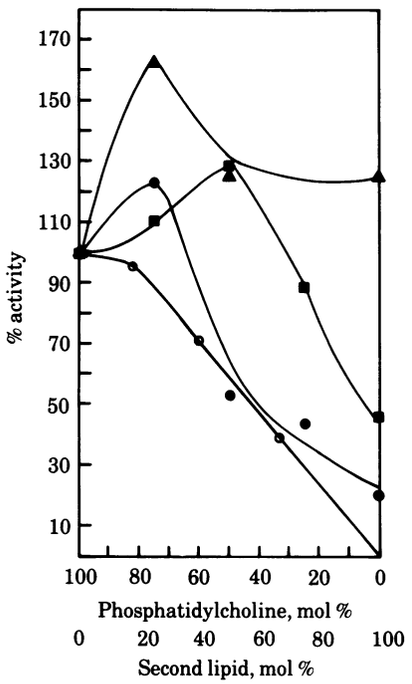

FIG. 3. Effect of phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine, and phosphatidyl-*N,N*-dimethylethanolamine on solubilized adenylate cyclase (5.1 mg of protein per ml). Mixed lipids were prepared, dried, resuspended, and measured as described in the legend to Fig. 2. ■, Phosphatidylethanolamine plus phosphatidylcholine; ▲, phosphatidyl-*N*-monomethylethanolamine plus phosphatidylcholine; ●, phosphatidyl-*N,N*-dimethylethanolamine plus phosphatidylcholine; ○, phosphatidylcholine.

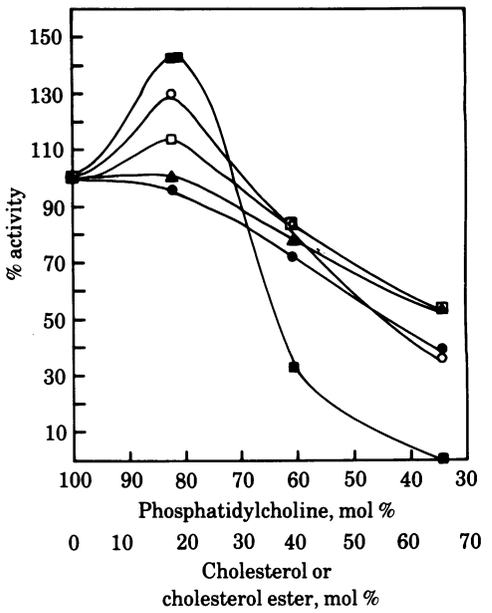

FIG. 4. Effect of cholesterol and cholesterol esters on solubilized adenylate cyclase. Mixed lipid films containing phosphatidylcholine and cholesterol or cholesterol ester in the proportions indicated were prepared as described in the legend to Fig. 2. The mixed lipid was resuspended in deoxycholate-solubilized adenylate cyclase (5.1 mg of protein per ml) to give a concentration of 10 mg of lipid per ml. The suspension was incubated at 0°C for 20 min and assayed for adenylate cyclase activity. ■, Cholesterol plus phosphatidylcholine; ○, cholesteryl acetate plus phosphatidylcholine; □, cholesteryl oleate plus phosphatidylcholine; ▲, cholesteryl stearate plus phosphatidylcholine; ●, phosphatidylcholine.

on the solubilized enzyme preparation (Fig. 3). In the absence of added phosphatidylcholine, phosphatidyl-*N*-monomethylethanolamine was capable of reconstituting full enzymic activity. Phosphatidylethanolamine could reconstitute about 40–60% and phosphatidyl-*N,N*-dimethylethanolamine about 10–20% of the activity found with phosphatidylcholine. When these phospholipids were mixed with phosphatidylcholine, there appeared to be a potentiation of the activity; the reason for this is obscure, but may reflect some physical property of the mixed lipid liposomes. For comparison, the effect of simply decreasing the phosphatidylcholine concentration is also shown.

Activity of Adenylate Cyclase in Liposomes. When the inactive deoxycholate-solubilized adenylate cyclase was mixed with phosphatidylcholine and diluted with large volumes (20- to 40-fold) of buffer, the enzyme activity was recovered in the liposomal pellet after centrifugation. With the phospholipids studied (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidyl-*N*-monomethylethanolamine) the activity recovered in the pellet was comparable with that detected in the mixture before dilution and centrifugation (see Table 1). No enzyme activity was detectable in the supernatant after centrifugation. Similar results were found after prolonged dialysis of the phospholipid/deoxycholate mixture against 50 mM Tris·HCl (pH 8.2) (data not shown).

Effect of Cholesterol and Cholesterol Esters. Fig. 4 shows the effect of cholesterol and cholesterol esters on the reconstitution of activity by phosphatidylcholine. For comparison, the effect of simply decreasing the concentration of phosphatidylcholine is also shown. At concentrations greater than about 33 mol %, cholesterol antagonized the stimulation by phosphatidylcholine. Esterification of the 3(OH) group (with acetate, stearate, or oleate) completely abolished this antagonism and the activity observed seemed to reflect only the amount of phosphatidylcholine present. At concentrations below about 30 mol %, cholesterol appeared to potentiate the phosphatidylcholine stimulation. This potentiation was reduced by esterification with acetate or oleate and was essentially abolished by esterification with stearate. When added alone, cholesterol or cholesterol esters did not reconstitute enzyme activity.

DISCUSSION

The results presented in this communication demonstrate that adenylate cyclase can be solubilized from rat brain by deoxycholate, albeit in an inactive form. Londos *et al.* (21) have suggested that adenylate cyclase can be solubilized from liver with deoxycholate in a form that can use only Mn-ATP as substrate. We have been unable to reproduce these results with liver membranes (unpublished data), and the solubilized brain enzyme has essentially no activity with either Mg-ATP or Mn-ATP. The activity of this solubilized brain preparation can be restored by the addition of nonionic detergent or specific phospholipids. At no time have we been able to demonstrate any selective loss or restoration of either Mg^{2+} or Mn^{2+} sensitivity.

The phospholipids most effective in restoring activity were phosphatidylcholine, sphingomyelin, phosphatidyl-*N*-monomethylethanolamine, and lysophosphatidylcholine. Partial restoration of activity was obtained with phosphatidylethanolamine (40–60%) and phosphatidyl-*N,N*-dimethylethanolamine (10–20%). The other lipids tested, including phosphatidic acid, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, diglycerides, triglycerides, cholesterol, and cholesterol ester, were ineffective.

There are several possible explanations for the lack of enzyme activity in the presence of deoxycholate and its subsequent re-

appearance on addition of nonionic detergent or phospholipid. Deoxycholate may directly and specifically inhibit the enzyme, and the phospholipids may be competing with (i.e., "displacing") deoxycholate. Alternatively, the inhibition by deoxycholate may be indirect, possibly due to removal of essential lipids. Because the activity of the enzyme following dialysis or in the liposomal pellet (i.e., when the detergent concentration has been reduced) reflects the activity in the mixture before dialysis or dilution and centrifugation, it seems unlikely that the phospholipids are acting merely to remove deoxycholate. Instead, it would appear more likely that the phospholipids are exerting a specific effect on the enzyme activity. The activation by nonionic detergent may represent increased availability and equilibration of stimulatory phospholipids solubilized from the brain along with the enzyme. The differences between phospholipids in their capacity to support adenylate cyclase activity may provide a mechanism by which the enzyme could be modulated *in vivo*. The enzyme could, for example, be activated by an exchange between an inhibitory and a stimulatory phospholipid.

The results with cholesterol (Fig. 4) are interesting. Cholesterol clearly has two very different effects depending on its concentration. Below about 30 mol % it potentiates phosphatidylcholine stimulation whereas above 33 mol % it progressively inhibits enzyme activity. Esterification of the 3(OH) group with any of the three substituents tested abolishes the inhibitory effects of high cholesterol concentration, possibly reflecting decreased incorporation of the ester into the lipid bilayer. Esterification also reduces the potentiating effect observed at lower cholesterol concentrations, but the extent of this reduction is dependent on the substituent. Cholesteryl acetate shows an approximately 30% reduction and cholesteryl oleate a 60% reduction in the potentiation by low cholesterol concentrations. There is no potentiation with cholesteryl stearate.

The results with cholesterol and its esters provide yet another potential method for modulating enzymic activity. Esterification of cholesterol might be predicted to reverse a possible inhibition induced by this moiety, leading to an increase in enzyme activity.

In conclusion, we believe that the variations in adenylate cyclase activity seen in the presence of different phospholipids, cholesterol, and cholesterol esters may reflect a phenomenon of biological relevance. We further suggest that this may be a means of modulating the enzyme activity *in vivo*.

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