The Glucagon-sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver

VI. EVIDENCE FOR A ROLE OF MEMBRANE LIPIDS*

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SUMMARY

Plasma membranes prepared from rat livers have been treated with digitonin or phospholipase A under conditions which result in substantial loss of glucagon-stimulated adenyl cyclase activity but no loss of fluoride-stimulated activity. These results are thought to reflect extensive modification of the structures responsible for hormone sensitivity without destruction of the catalytic component of the adenyl cyclase system in these membranes. Corresponding decreases in binding of 125I-glucagon to the membranes are observed following digitonin or phospholipase A treatment. Both glucagon sensitivity of adenyl cyclase and binding of 125I-glucagon can be partially restored by exposing treated membranes to aqueous suspensions of membrane lipids. The mechanism of the effects of these lipids has not been established. Pure phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine are all capable of partially restoring glucagon-stimulated adenyl cyclase activity and binding of 125I-glucagon to phospholipase A-treated membranes. Of these, phosphatidylserine produces the greatest effect. Chromatographic fractions of membrane lipids produce positive and negative effects on control and treated membranes which are difficult to interpret. However, it appears that there is some specificity in the effects of the lipid fractions. These results may have significant implications regarding the relationship of adenyl cyclase systems to membrane structure and attempts to purify the components of these systems.

* Some of the studies have been reported in preliminary form (1-3). The previous papers in this series are References 4 to 8.
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neous states unless the role of lipids is understood. For these reasons, we have examined further the lipid requirements of the liver membrane adenyl cyclase system. The studies described in the present report show that the hormone-response of the adenyl cyclase system and the ability of the membranes to bind glucagon can be partially restored following digitonin or phospholipase A treatment by exposing the treated membranes to aqueous dispersions of lipids.

**EXPERIMENTAL PROCEDURE**

**Materials**

Digitonin, from Mann Research Laboratories, was recrystallized twice from ethanol. Highly purified phospholipase A (EC 3.1.1.4), an type, 1400 units per mg (12), was a gift from Dr. Michael A. Wells (University of Arizona, Tucson). Chloroform and methanol were reagent grade products of the J. T. Baker Chemical Company, and were used without redistillation. Glucagon (crystalline) was a gift from Eli Lilly Company. Phosphatidylserine and phosphatidylcholine were obtained from Applied Science Labs and phosphatidylethanolamine from Supeco, Inc., Bellefonte, Pennsylvania. These lipids were found to yield one spot with the thin layer chromatographic system described below and were used as supplied. The sources of other materials have been specified previously (4, 6).

**Methods**

**Liver Membranes**—Plasma membranes were prepared from rat livers by a minor modification of the procedure of Neville (13) as described previously (4). All preparations used for this study were partially purified membranes (4).

**Extraction of Lipids**—An antioxidant, 2,6-di-tert-butyl-4-methylphenol (Aldrich Chemical Company), at 0.005%, was added to all solvents. One batch of liver membranes (80 to 120 mg of membrane protein) suspended in 1 ml KHCO₃ in a total volume of about 5 ml, was extracted with 90 ml of CHCl₃-CH₃OH, 2:1 (v/v), for 2 hours in an ice bath. The insoluble material was removed by filtration through Whatman No. 1 filter paper. The filtrate was extracted with 0.2 volume of 0.1 M CaCl₂. The upper phase was discarded and the lower phase was filtered to remove any remaining water droplets. The solvents were removed by evaporation under a stream of N₂ in a flash evaporator (Buchler Instruments, Inc., Fort Lee, New Jersey) at 40°C. Following evaporation, the lipid residue was dissolved immediately in 1 to 2 ml of CHCl₃.

**Column Chromatography of Lipids**—The lipids extracted from one batch of liver membranes were dissolved in 2 ml of CHCl₃. One ml of this solution was removed for comparison with the subsequent lipid fractions. The remainder was applied to a column (0.8 x 10 cm) of silicic acid (Unisol, Clarkson Chemical Company, Williamsport, Pennsylvania; 200 to 325 mesh) packed in CHCl₃. The lipids were washed into the column with 1 to 2 ml of CHCl₃, and the column was eluted at 22°C, with a flow rate of 0.5 ml per min, using 20 ml of the following eluents: Fraction I, CHCl₃; Fraction II, CHCl₃-CH₃OH, 9:1 (v/v); Fraction III, CHCl₃-CH₃OH, 4:1; Fraction IV, CHCl₃-CH₃OH, 1:4; Fraction V, CH₃OH. Solvents were removed from these fractions, which were collected in tared vials, by flash evaporation as described above, and the lipid residues were weighed and then dissolved immediately in 1 ml of CHCl₃.

**Dispersion of Lipids in Buffer**—Lipids dissolved in CHCl₃ were transferred to either glass test tubes (10 x 75 mm) or 13-ml conical glass centrifuge tubes depending on the volume required. The solvent was removed by evaporation with a stream of N₂, and the buffer, 25 mM Tris-Cl, pH 7.6, 1 mM EDTA, was added over the lipid film. In some experiments, the lipids were dissolved in diethyl ether which was layered on and evaporated from the surface of the buffer. The former procedure was found to be simpler. The lipids were then dispersed by sonication with a Biosonik III (Browell Scientific Company, Rochester, New York) at 35% intensity until continuous sonication failed to clarify the solution further (2 to 10 min). The vessel in which the sonication was performed was immersed in an ice bath and a stream of N₂ was directed over the surface of the buffer. The dispersed lipids were stored at 4°C and were used within 1 week of preparation.

The membrane lipids used in this study were not weighed routinely. Typically, one batch of membranes yielded 60 mg of lipid which was dispersed in 2.5 ml of buffer. The lipid suspensions were used at this concentration except as specified in "Results" when they were diluted in 25 mM Tris-Cl, 1 mM EDTA. In early experiments, a small amount of sedimentable material was removed from the milky whole lipid suspensions by centrifugation for 15 min at 25,000 x g. However, this was found not to affect the subsequent effects of the lipids and was not continued.

**Thin Layer Chromatography of Lipids**—Glass thin layer chromatography plates precoated with Silica Gel H (Analtech, Inc., Wilmington, Delaware), were activated by heating for 30 min at 110°C. Lipids were spotted in 2 to 10 µl of CHCl₃, and the plates were developed with CHCl₃-CH₃OH-CH₂COOH-H₂O, 10:62:18:24 (14). Detection of spots was accomplished by spraying the plates with 5% H₂SO₄ in ethanol and heating for 10 min at 220°C. Purified phospholipids described above were used as standards.

**Adenyl Cyclase Assay**—Adenyl cyclase activity was determined by following the conversion of ATP-γ-32P to cyclic AMP-32P using the method of Krishna, Weiss, and Brodie (15). Standard incubation media and conditions have been specified previously (4).

**[3H]Glucagon Binding Assay**—The methods for preparation of [3H]glucagon and measurement of binding have been described previously (6). The incubation medium contained 2.5% albumin, 20 mM Tris-Cl, pH 7.6, 0.2 to 1.0 mM EDTA, 0.5 to 2.0 x 10⁻⁷ M [3H]glucagon, and 0.2 to 0.4 mg per ml of membranes in a volume of 0.125 ml. Incubation was for 15 min at 30°C.

**Protein Determination**—Protein was measured by the method of Lowry et al. (16) using bovine serum albumin as standard.

**Expression of Results and Terminology**

In all figures and tables "Adenyl cyclase activity" refers to nanomoles of cyclic AMP formed in 10 min per mg of membrane protein and "Glucagon bound" refers to picomoles of [3H]glucagon per mg of membrane protein. Basal adenyl cyclase activity is the activity measured in the absence of glucagon or fluoride ion. Glucagon- and fluoride-stimulated activities are the activities measured in the presence of these compounds and from which basal activity has been subtracted.

1 The abbreviation used is: cyclic AMP, cyclic adenosine 3',5'-monophosphate.

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**References**

1. Krishna, Weiss, and Brodie (15).
2. Lowry et al. (16).
In the present studies we have used the fluoride-stimulated adenylyl cyclase activity only to indicate that treated membranes are capable of catalyzing the conversion of ATP to cyclic AMP. Conditions of treatment with digitonin or phospholipase A have been selected to produce substantial loss of glucagon-stimulated adenylyl cyclase activity and glucagon binding but little or no loss of the fluoride-stimulated activity. This approach is justified by our previous finding that glucagon and fluoride ion stimulate the same adenylyl cyclase activity in the liver membrane preparation but do so by different mechanisms (5).

Digitonin and phospholipase A, the agents which were found to produce a selective loss of the glucagon-stimulated adenylyl cyclase activity and of glucagon binding, would be expected to interact primarily with membrane lipids. Therefore, it was reasonable to suspect that lipids might be able to restore these functions to treated membranes. Accordingly, membrane lipids were extracted and dispersed in buffer and added to suspensions of treated membranes.

A representative experiment with digitonin is shown in Fig. 1. The glucagon-stimulated adenylyl cyclase activity has been reduced to 36% or 9% of the control by treatment of the membranes with 1 or 10 mg per ml of digitonin, respectively. These activities are increased to 65% or 22% of control by exposing the treated membranes to a suspension of membrane lipids. The basal activity (no glucagon or fluoride, no digitonin) in this experiment was 0.20 nmoles of cyclic AMP per 10 min per mg of protein and was unaffected by the addition of the lipid dispersion. Digitonin treatment decreased the basal activity and it remained unaffected by lipids. The control for this experiment is the glucagon-stimulated activity of the membranes which were carried through the entire treatment procedure but in media containing no digitonin. As is apparent from Fig. 1, this activity is unaffected by the addition of the lipid dispersion. Therefore, the lipid dispersion has partially restored the glucagon response of the adenylyl cyclase activity in the treated membranes. Similarly, the lipid dispersion partially restores the binding of $^{125}$I-glucagon to digitonin-treated membranes (Table I).

The fluoride-stimulated adenylyl cyclase activities are included in Fig. 1 to illustrate the fact that the mechanism through which fluoride acts on adenylyl cyclase can also be modified by agents which interact with membrane lipids. However, for reasons mentioned above and because the mechanism of action of fluoride on the system is unknown, the fluoride-stimulated adenylyl cyclase activity can only serve to demonstrate the integrity of the catalytic site and cannot serve as a control for interpreting effects on the glucagon-stimulated activity. In general, digitonin and phospholipase A tend to increase the fluoride-stimulated activity; however, relatively high concentrations of these agents decrease this activity. Addition of lipid dispersins mimics the effects of digitonin and phospholipase A and, as illustrated in Fig. 1, does not restore the fluoride-stimulated activity of treated membranes toward the value of the untreated control. Thus, lipids appear to be involved in the mechanism through which fluoride stimulates adenylyl cyclase but in a manner which is clearly different from their role in the mechanism through which glucagon acts.

Representative experiments with phospholipase A are shown in Figs. 2 and 3. These effects are attributed to hydrolysis of lipids by phospholipase A on two grounds. (a) the enzyme is physically homogeneous on polyacrylamide gel electrophoresis (12) and is, therefore, unlikely to have other enzymatic activity, and (b) the observed effects require the presence of calcium (5, 6), a characteristic requirement of the purified phospholipase A from snake venom (17). As reported previously (5), treatment of the membranes with a relatively low concentration of phospholipase A slightly but significantly increases the glucagon-stimulated adenylyl cyclase activity (Fig. 2). In addition, treatment with a lower concentration of phospholipase A increases the binding of

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**TABLE I**

Effect of membrane lipids on binding of glucagon to digitonin-treated membranes

Liver membranes were incubated with or without digitonin, 10 mg per ml, washed, and exposed to membrane lipids as described in the legend to Fig. 1. Binding of $^{125}$I-glucagon to the membranes and protein concentrations were determined as described under "Methods." Values are the mean ± half the range of duplicate determinations.

| Lipids | Glucagon bound |
|--------|----------------|
| Control | Digitonin treated |
| - | 0.74 ± 0.04 | 0.20 ± 0.02 |
| + | 0.74 ± 0.05 | 0.92 ± 0.02 |
FIG. 2. Effects of phospholipase A on adenylyl cyclase activity and reversal by lipids. Liver membranes, 3 mg of membrane protein per ml, were incubated in 0.38 ml of a medium containing 25 mM Tris-HCl, pH 7.6, and 1 mM CaCl₂, with phospholipase A at the indicated concentrations for 5 min at 30°. The reaction was stopped by adding 0.02 ml of 20 mM EDTA. Aliquots of the treated membrane suspension were incubated with an equal volume of either 25 mM Tris, 1 mM EDTA, or membrane lipid suspension, 1:8 (see "Methods") in 25 mM Tris, 1 mM EDTA for 5 min at 22°. Adenylyl cyclase activities in the absence or presence of 10 μg per ml of glucagon and protein concentrations were measured as described under "Methods." Values are the increase in adenylyl cyclase activity due to the addition of glucagon to the assay medium and are the mean ± half the range of duplicate determinations. U, units.

At higher concentrations, phospholipase A reduces both the glucagon-stimulated adenylyl cyclase activity and the binding of glucagon without reducing the fluoride-stimulated adenylyl cyclase activity (fluoride data not shown; see Reference 5). Exposing phospholipase A-treated membranes to membrane lipids partially restores the response of adenylyl cyclase activity to glucagon. In Fig. 2, this effect is most striking at the highest phospholipase A concentration; under this condition, no stimulation of adenylyl cyclase by glucagon was observed unless the membranes were exposed to lipids. The positive effect of the lipids on the control (no phospholipase A) membranes is probably due to the fact that diluted membrane lipids were used in this experiment (see below). In other experiments using 4- to 8-fold higher lipid concentrations, the glucagon-stimulated adenylyl cyclase activity of the control membranes was unaffected or reduced. In all experiments, basal adenylyl cyclase activity was either unaffected or reduced by either phospholipase A or lipids, or both. As with digitonin, either phospholipase A treatment or lipids slightly increased fluoride-stimulated adenylyl cyclase activity, but both together failed to produce an additional effect (data not shown).

Exposing phospholipase A-treated membranes to lipids also partially restores the ability of the membranes to bind glucagon. In Fig. 3, at the highest concentration of phospholipase A, lipids have restored glucagon binding from 20 to 89% of control. It should be noted that the membranes, phospholipase A, and treatment conditions were identical in the experiments shown in Figs. 2 and 3. As shown previously (6), the effects of varying concentrations of phospholipase A on glucagon-stimulated adenylyl cyclase and on glucagon binding do not correlate perfectly. The binding data presented in this paper must be considered to apply to the characteristics of a glucagon-specific binding site in the same membranes which contain the glucagon-stimulated adenylyl cyclase system and not necessarily to the characteristics of the site which actually mediates the stimulation of adenylyl cyclase activity by glucagon. On the other hand, the studies presented here do not prove that the observed binding sites are not those which mediate the stimulation of adenylyl cyclase activity. The problem of establishing or ruling out a cause-effect relationship between observed binding of glucagon and activation of adenylyl cyclase has been treated in a preliminary way elsewhere (3, 6) and is beyond the scope of this paper.

In general, the restoration effects of lipids on phospholipase A-treated membranes have been larger than the effects on digitonin-treated membranes. For this reason, phospholipase A treatment was selected for studies of the specificity of the effects of lipids. In Figs. 1 to 3, effects of membrane lipids on treated membranes were emphasized by selecting conditions in which lipids produced little or no effect on control membranes. Under these conditions, exposure to lipids restored adenylyl cyclase and binding properties of treated membranes toward the values of relatively unaffected controls. However, the control membranes may also be affected by exposure to lipids. For example, Fig. 4 shows both positive...
and negative effects of lipids on control and phospholipase A-treated membranes depending on lipid concentration.

It is clear, therefore, that addition of an aqueous dispersion of lipids can affect the system in more than one way. This fact greatly complicates the problem of establishing the specificity or non-specificity of the effects of these lipids since both kinds of effects may occur simultaneously and may either mimic or counteract each other. Phospholipids have surface-active properties which may be either strong or weak depending on the composition of the phospholipid and may, therefore, mimic the effects of detergents on the system. Furthermore, addition of a lipid dispersion to the system adds a new phase, lipid micelles, to a system which already contains two phases, an aqueous medium and membranes. The physical properties of the micelle phase and its interactions with the other two phases will depend on the composition of the micelle phase. Finally, although the membranes are free of significant contamination by other organelles (4), they are still a highly complicated material for biochemical investigation. The components of the hormone-sensitive adenyl cyclase system probably represent only a small fraction of the total protein and lipid in the membranes, and alterations in the properties of the membrane as a whole may be reflected secondarily by changes in the adenyl cyclase system. In view of these considerations, it is not surprising that the question of the specificity of the effects of adding lipids to the system cannot be answered easily.

Despite these problems, we have obtained some information regarding the effectiveness of different lipid classes. In a typical experiment, one batch of partially purified membranes, approximately 120 mg of membrane protein, was extracted as described in “Methods.” One-half of this extract was dried and weighed. The remainder was applied to the column and eluted as described in “Methods.” The amounts of lipid in these fractions, determined gravimetrically, were as follows: total applied, 30 mg; Fraction I, 6.3 mg; Fraction II, 3.6 mg; Fraction III, 2.6 mg; Fraction IV, 14.2 mg; Fraction V, 1.0 mg. By thin layer chromatography, neutral lipids were seen exclusively in Fraction I, and no spots corresponding to polar lipids were seen in this fraction. Phosphatidylycerine was seen primarily in Fraction II, phosphatidylserine in Fraction III, and phosphatidylcholine in Fraction IV. However, there was considerable overlap of lipids in adjacent fractions. These results agree well with analyses of rat liver plasma membrane lipids reported from other laboratories (14, 18).

The effects of the membrane lipid fractions on phospholipase A treated and control membranes are shown in Table II. For this experiment, the amount of lipid and buffer were adjusted so that the concentration of lipids in the suspension of each fraction would approximate the concentration of those lipids in the undiluted membrane lipid suspensions used in experiments above. These amounts could have been chosen so that the total lipid concentration would have been the same for all fractions. However, for reasons mentioned above, either choice is arbitrary and subject to the same criticisms. In order to compare the effects of fractions of membrane lipids to those of whole membrane lipids it was intuitively more appealing to use the former condition. The high concentrations of lipids probably accounts for the negative effects of the various fractions on the control membranes (see Fig. 4). Fraction I reduced the glucagon-stimulated adenyl cyclase activity of treated membranes in proportion to its effect on control membranes. Fractions II and III contained low and roughly equal concentrations of lipids and affected adenyl cyclase activity of the control membranes to about the same extent. Fraction III, however, produced a much larger positive effect on the glucagon-stimulated adenyl cyclase activity of the treated membranes than did Fraction II. Fraction IV had a substantial negative effect on both control and treated membranes but differed from the other phospholipid fractions in having a much larger total concentration of lipid. Fraction III had the largest positive effect on glucagon binding in treated membranes. However, in contrast to the adenyl cyclase findings, all of the fractions except Fraction I produced positive effects on binding in the treated membranes.

Because of the difficulties involved in purifying lipids and the apparent multiplicity of the effects of lipids, further fractionation of membrane lipids was not undertaken. Instead, three of the principal membrane phospholipids were obtained in purified form from other sources. The effects of these phospholipids on phospholipase A-treated and control membranes are illustrated in Table III. It is clear that all three lipids tested are capable of partially restoring glucagon-stimulated adenyl cyclase activity and binding of glucagon to treated membranes. Of the three, phosphatidylserine is clearly the most effective. Phosphatidylethanolamine differs from the others in consistently showing a marked positive effect on control membranes. Combinations of lipids do not produce additional effects; if anything, these combinations produce less effect than the individual lipids.

Although the specificity experiments are difficult to interpret, four conclusions seem warranted. (a) Different lipids may affect the system in different ways; (b) more than one phospholipid are capable of restoring glucagon-stimulated adenyl cyclase activity and glucagon binding of treated membranes toward the values of a relatively unaffected control; (c) certain lipids do not produce this restoration; (d) the effects of lipids on adenyl cyclase and on glucagon binding are not always quantitatively or qualitatively the same. The question of specificity of the role of lipids in hor-

![Fig. 4. Effect of varying lipid concentration on phospholipase A-treated and control membranes. Liver membranes were treated with (Phospholipase A Treated) or without (Control) phospholipase A, 3.5 units per ml, as described in the legend to Fig. 2. Membrane lipids diluted in 25 mM Tris, pH 7.6, 1 mM EDTA were added and incubated and glucagon binding was determined as described in the legend to Fig. 2. Each point is the mean of duplicate determinations.](http://www.jbc.org/)

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mammalian adenyl cyclase systems probably will not be answered until the system has been simplified and separated from the bulk of the extraneous membrane material.

**DISCUSSION**

The structures responsible for hormone-sensitivity of a mammalian adenyl cyclase system can be extensively modified by agents which modify membrane lipids without destroying the catalytic site of the system and these effects can be partially reversed by exposing the treated membranes to aqueous dispersions of lipids. These findings illustrate further the complexity of mammalian adenyl cyclase systems and are consistent with the hypothesis (19, 20) that these systems are multimolecular with separate hormone-sensitive and catalytic components.

While the present study is, to our knowledge, the first of its type reported for a hormone-sensitive adenyl cyclase system, effects of lipids on detergent and phospholipase-treated preparations have been reported for (Na+ + K+)-dependent ATPase (21, 22), mitochondria (23), and several microsomal enzymes (24–26). In no case has the mechanism of action of lipids been established. The most attractive hypothesis is that lipid suspensions act by replacing essential membrane lipids which have been either removed or destroyed by detergents or phospholipase treatments (20). However, Emmelot and Bos (27), in reference to deoxycholate-treated preparations of (Na+ + K+)-dependent ATPase, have suggested that lipids act by removing bound detergent. Zakim (24) has presented evidence that one effect of phospholipase A on microsomal glucose 6-phosphatase is to produce an unstable form of the microsomal enzyme and that lipids act by stabilizing this form. Any one or combination of these mechanisms could explain the effects of lipids reported here. The concentration-dependent positive and negative effects of lipid suspensions on control membranes may be due to detergent properties of phospholipids in these suspensions.

The molecular basis of the involvement of membrane lipids in adenyl cyclase systems is far from clear. Hormone-specific binding sites appear to contain lipoproteins since they are sensitive to both trypsin (28) and phospholipase A (6). To some extent,

### Table II
**Effect of membrane lipid fractions on phospholipase A-treated membranes**

Liver membranes were treated with (“Phospholipase A treated”) or without (“Control”), phospholipase A, 5 units per ml, as described in the legend to Fig. 2. Membrane lipids were fractionated by column chromatography on silicic acid, and 0.20 of each fraction was dispersed in 0.5 ml of buffer as described under “Methods.” Equal volumes of membrane and lipid suspensions or buffer were incubated for 5 min at 22°, and adenyl cyclase activities in the absence and presence of 10 μg per ml of glucagon and binding of 125I-gluca
gon to the membranes were determined as described under “Methods.” Each value is the mean ± half the range of duplicate determinations.

| Addition             | Adenyl cyclase activity increase due to glucagon | Glucagon bound |
|----------------------|-----------------------------------------------|----------------|
|                      | nmoles/10 min/mg protein                      | nmoles/mg protein |
| None                 | 3.14 ± 0.03                                  | 1.60 ± 0.01     |
| Whole lipids         | 2.30 ± 0.03                                  | 1.80 ± 0.01     |
| Fraction I           | 2.45 ± 0.02                                  | 2.14 ± 0.02     |
| Fraction II          | 2.56 ± 0.05                                  | 1.68 ± 0.06     |
| Fraction III         | 2.74 ± 0.10                                  | 2.21 ± 0.01     |
| Fraction IV          | 1.47 ± 0.02                                  | 0.97 ± 0.06     |
| Fraction V           | 3.09 ± 0.02                                  | 1.26 ± 0.03     |

### Table III
**Effect of exogenous lipids on phospholipase A-treated membranes**

Liver membranes were treated with (“Phospholipase A treated”) or without (“Control”) phospholipase A, 5 units per ml, as described in the legend to Fig. 2. Phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE), alone or in combination were dispersed in 25 mM Tris-HCl, pH 7.6, 1 mM EDTA at a concentration of 2 mg per ml for each lipid by sonication and incubated for 5 min at 22°. Adenyl cyclase activities in the absence and presence of 10 μg per ml of glucagon and binding of 125I-gluca
gon to the membranes were determined as described under “Methods.” Each value is the mean ± half the range of duplicate determinations.

| Addition | Adenyl cyclase activity increase due to glucagon | Glucagon bound |
|----------|-----------------------------------------------|----------------|
|          | nmoles/10 min/mg protein                      | nmoles/mg protein |
| None     | 3.45 ± 0.05                                  | 1.18 ± 0.06     |
| PS       | 3.09 ± 0.09                                  | 2.52 ± 0.10     |
| PC       | 3.41 ± 0.03                                  | 1.92 ± 0.01     |
| PE       | 4.38 ± 0.22                                  | 2.26 ± 0.10     |
| PS + PC  | 3.16 ± 0.15                                  | 1.71 ± 0.04     |
| PE + PC  | 3.13 ± 0.10                                  | 2.35 ± 0.10     |
| PS + PE  | 3.16 ± 0.03                                  | 2.17 ± 0.12     |

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changes in adenyl cyclase activity produced by manipulating the membrane lipids undoubtedly reflect changes in the state of the hormone-binding site. However, at least three observations suggest that lipids are involved at multiple sites in the system: the increase in fluoride-stimulated adenyl cyclase activity produced by relatively low concentrations of digitonin or phospholipase A (5), the complete loss of fluoride-stimulated activity produced by sufficiently high concentrations of these agents, and the lack of absolute correlation between the effects of digitonin, phospholipase A, and lipid suspensions on glucagon binding and activation of adenyl cyclase. The ability of more than one kind of phospholipid to reverse the effects of phospholipase A indicates that part of the effect of membrane lipids may be nonspecific, i.e., related only to maintaining a generally hydrophobic environment for the components of the adenyl cyclase system. However, the differences in the effects of various membrane lipid fractions on phospholipase A-treated membranes suggests that specific lipids may be required at specific sites in the system.

The apparent involvement of lipids at multiple sites in the liver membrane adenyl cyclase system is consistent with certain concepts of adenyl cyclase organization and membrane structure. Davoren and Sutherland (29) first proposed that the hormone binding component of the adenyl cyclase system might be located on or near the extracellular surface and the catalytic component on or near the intracellular surface of the plasma membrane. This hypothesis has been supported by the finding that trypsin treatment of isolated fat cells results in loss of sensitivity of these cells to the effects of the lipolytic hormone (28) without disrupting the integrity of the plasma membrane (30, 31) or destroying the fluoride-stimulated adenyl cyclase activity in "ghosts" prepared from these treated cells (28). However, trypsin treatment of ghosts, a hypotonic lysate of isolated fat cells, inactivates both the hormone- and the fluoride-stimulated adenyl cyclase activities (32). These results probably reflect a difference in accessibility of trypsin to the outer and inner surfaces of the plasma membrane in isolated fat cells and ghosts. Although numerous objections have been raised (33), it is generally accepted that the unit of membrane structure is the extended phospholipid bilayer (34). Even if the "unit membrane" theory is not correct in detail, there is evidence for extended, lipid-rich regions in cell membranes (35). Consequently, the hormone binding and catalytic components of mammalian adenyl cyclase systems may be separated by a lipid-rich layer.

Zakim (24) has observed increases in microsomal glucose-6-phosphatase activity due to treatment with phospholipase A and albumin and, on this basis, has suggested that membrane lipids "constrain" the maximum activity of the enzyme. The increase in glucagon- or fluoride-stimulated adenyl cyclase activities produced by certain conditions of treatment with digitonin, phospholipase A, and lipid suspensions suggests a similar hypothesis for the adenyl cyclase system. The interaction between glucagon and its binding site on the membrane might then activate adenyl cyclase by changing the state of the membrane lipids in such a way that a limitation on the activity of the catalytic component is relieved. If such a mechanism is operative, it should be possible to activate adenyl cyclase in the absence of glucagon by modifying membrane lipids. Such a phenomenon has not yet been observed with the liver membrane system. However, stimulation of adenyl cyclase by detergents in the absence of added hormone has been observed with homogenates of brain (36) and avian erythrocytes (37) and in fat cell ghosts (3).

The studies with lipids described here have important implications for future work with adenyl cyclase systems. In attempting to purify the components of these systems, it has generally been assumed that these structures might retain functional configurations after separation from membrane lipids. Such an expectation may be unreasonable since the factors which govern protein configurations in membranes are poorly understood. However, it may be possible to purify components of adenyl cyclase systems and retain or restore function by performing purification steps in media containing phospholipids or by adding phospholipids to membrane protein fractions. Pastan, Pricer, and Blanchette-Mackie (38) have made a step in this direction by devising a method for preparing a particulate but nonsedimentable adrenocorticotropin-sensitive adenyl cyclase activity from an adrenal tumor; the method requires the addition of phospholipid to media. Very recently Levey has shown (39) that addition of phosphatidylserine to a hormone-insensitive adenyl cyclase, which has been solubilized from rat heart with Lubrol-PX and then freed of detergent by ion exchange chromatography, restores the sensitivity of the enzyme to glucagon.

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