The Role of Acidic Phospholipids in Glucagon Action on Rat Liver Adenylate Cyclase

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SUMMARY

We have examined the effects of phospholipase C from Bacillus cereus (Bc) and from Clostridium perfringens (Cp) on various parameters involved in the activity and response of adenylate cyclase to glucagon in rat liver plasma membranes. A crude preparation of Cp-phospholipase C hydrolyzes "neutral" phospholipids (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin) in these membranes. In contrast, highly purified Bc-phospholipase C hydrolyzes acidic phospholipids (phosphatidylserine, phosphatidylinositol) but not sphingomyelin. Treatment of the membranes with either type of phospholipase does not alter basal adenylate cyclase activity or the stimulatory effects of fluoride ion on the enzyme system. Bc-phospholipase C abolishes the effects of glucagon on adenylate cyclase whereas Cp-phospholipase C causes only partial loss of glucagon response even after hydrolysis of 60% of the membrane phospholipids. These findings provide evidence that acidic phospholipids are more specifically involved in glucagon activation of adenylate cyclase.

Acidic phospholipids are not directly involved in the binding of glucagon to its receptor. Treatment with Bc-phospholipase C results in a 10-fold reduction in the affinity but not the quantity of specific binding sites for glucagon. Binding of Des-His-glucagon, a competitive, inactive analogue of glucagon is unaffected by Bc-phospholipase C treatment and displays the same apparent affinity as does glucagon for the binding sites in phospholipase-treated membranes. These findings suggest that acidic phospholipids are involved in the liganding of the histidine residue of glucagon to a regulatory site responsible for glucagon action. GTP, which is required for glucagon action on adenylate cyclase and which increases the rate of dissociation of glucagon from its receptor (15) suggesting that the nucleotide regulatory site is somehow coupled to the sites of glucagon bind-

Several studies have suggested that lipids participate in the actions of hormones on adenylate cyclase, a membrane-bound enzyme system (1-9). A notable example of the role of specific phospholipids has been reported recently by Levey (10, 11) who found that addition of phosphatidylserine and phosphatidyl-inositol to "solubilized" nonresponsive preparations of cat heart adenylate cyclase restored the response of the enzyme to glucagon and epinephrine, respectively. In other studies (7), treatment of hepatic plasma membranes containing a glucagon-sensitive adenylate cyclase with either purified phospholipase A2 or with neutral detergents resulted in concomitant losses of both glucagon action and of binding of the hormone to specific sites having the characteristics of the receptor for glucagon (12). Partial restoration of binding and action of glucagon was observed upon addition of membrane lipids or purified phospholipids, the most effective being phosphatidylserine (7). Extraction of liver membranes with organic solvents also leads to reduction in glucagon action; phosphatidylinositol, but not phosphatidylserine, was reported to partially restore hormone action (9). Although these studies indicate that phospholipids, probably of the acidic type, are required for hormone action, the precise role of these lipids remains unknown.

Adenylate cyclase systems are complex regulatory enzymes (13). The glucagon-sensitive system in rat liver plasma membranes consists of a specific receptor for glucagon which binds the hormone specifically and reversibly (12) and a component which catalyzes the conversion of ATP to cyclic AMP. The system also contains a regulatory site which binds specific nucleotides (GTP, GDP, ATP, and ADP) and which is involved in glucagon action (14). These nucleotides increase the rate of dissociation of glucagon from the receptor (15) suggesting that the nucleotide regulatory site is somehow coupled to the sites of glucagon binding on the receptor. Thus, the loss of glucagon binding and ac-

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tion that occurs upon treatment of the membranes with surfactants or phospholipase A may result from alterations in the receptor, the catalytic component, the nucleotide regulatory site, or the "coupling" process, referred to as the "transducer" (13), between the receptor and catalytic component.

We report here the effects of phospholipase C (EC 3.1.4.3) from Bacillus cereus (Bc) and from Clostridium perfringens (Cp) on various parameters involved in the activity and hormonal regulation of the hepatic adenylate cyclase system. The crude preparation of C. perfringens phospholipase C used in this study contains a sphingomyelinase (16) and a phospholipase C that hydrolyzes phosphatidylcholine and phosphatidylethanolamine, but not acidic phospholipids such as phosphatidylserine and phosphatidylinositol (17-19). In contrast, B. cereus phospholipase C hydrolyzes acidic phospholipids but not sphingomyelin (20-23). The relative specificities of these preparations of phospholipase C provides a means of distinguishing between the various classes of phospholipids that may be involved in glucagon action. An additional advantage of their use is that the products of phospholipase C action (diglyceride and phospho-residue) are not potent surfactants or phospholipase A action (lyso-phospholipids and fatty acids).

**EXPERIMENTAL PROCEDURE**

**Materials**

Phospholipase C from C. perfringens, purchased from Worthington, was used without further purification. Phospholipase C from B. cereus was kindly supplied in partial and highly purified forms by Dr. J. Shiloach of the Department of Biological Chemistry, Hebrew University (Jerusalem). Dr. P. R. Wagelos (Department of Biological Chemistry, Washington University, St. Louis) also generously provided a highly purified preparation of Bc-phospholipase C that is free of proteolytic enzyme (24) (for purification procedures see Ref. 22). Oxoid filters were obtained from Fischer Scientific Co., Inc. Bovine serum albumin was obtained from Pentex. Glucagon was supplied by Lilly. Des-His-glucagon (DH-glucagon) was a gift from Dr. Finn Sundby (Novo Research Institute, Copenhagen, Denmark), and was contaminated with 5 to 10% glucagon as estimated from adenylate cyclase assays. The sources of all other materials have been specified previously (12).

**Methods**

Partially purified plasma membranes from rat liver were prepared as described previously (25).

**Determination of Phospholipase C Activity**—The activities of the various preparations of phospholipase C were determined by a previously described titrimetric procedure (26) using egg yolk lipoprotein phospholipids (27) as substrate. Incubations were carried out at room temperature in 1 ml of medium containing 154 mM NaCl and 4 mM CaCl₂. The pH was maintained at 7.35. One unit of activity is defined as the amount of enzyme that causes the liberation of 1 pmole of H⁺ per min.

**Treatment of Membranes with Phospholipase C**—Plasma membranes containing 1.9 to 2.7 mg of protein were incubated at 15° for 5 min (with gentle agitation) in 3.0 ml of medium consisting of 10 mM Tris-HCl, pH 7.5, 1 mM KHCO₃, and 1 mM CaCl₂ in the absence or presence of Bc-phospholipase C or Cp-phospholipase C. The concentrations of added phospholipases were adjusted according to the units of activity determined as described above in order to give essentially identical rates of hydrolysis of membrane phospholipids. The amount of phospholipase C used in this study was 0.01 to 0.7 unit. The results are supplied in the figures and table as the percentage of membrane phospholipid hydrolyzed in 5 min of incubation.

After incubation, the samples were diluted to 10 ml with ice-cold buffer (10 mM Tris-HCl, 1 mM KHCO₃, pH 7.5) and centrifuged for 10 min at 4° at 10,000 x g in a Sorvall centrifuge. The supernatant fluid was discarded and the pellet was suspended in 10 ml of cold buffer and centrifuged as above. This washing procedure was repeated once more and the pellet was suspended in 0.5 ml of the buffer. Various assays, described below, were carried out on aliquots of these preparations.

**Adenylate Cyclase Activity**—Assay conditions were the same as described previously (25). The incubation medium contained, in 0.1 ml, 5.0 mM MgCl₂, 1 mM EDTA, 3.2 mM [γ-³²P]ATP, 10 mM theophylline, creatine kinase (1 mg per ml), 25 mM phosphocreatine, 25 mM Tris-HCl, pH 7.6, and 50 to 60 μg of membrane protein. Solutions of creatine kinase and phosphocreatine were prepared freshly for each experiment since it was found that diluted preparations stored even in the frozen state lost considerable ATP-regenerating capacity and did not maintain constant levels of ATP during incubation. Response of adenylate cyclase to glucagon was measured, unless stated otherwise, in the presence of 10⁻⁴ M glucagon; fluoride response was to 20 mM NaF. Incubations were carried out for 10 min at 30°. The method of Krishna et al. (28) was employed for measuring the amount of cyclic AMP formed in the reaction.

**Binding of ³¹¹I-Glucagon or ³²P-DH-Glucagon—³²P-Glucagon (specific activity 200,000 to 600,000 cpn per pmole) and ³²P-DH-gluca- gon (specific activity 250,000 cpn per pmole) were prepared as described previously (6). Binding of the labeled peptides to the membrane preparations was carried out as follows. Liver membranes (40 to 80 μg of protein) were incubated in 100 μl of medium containing 20 mM Tris-HCl, pH 7.6, 1% bovine serum albumin, and the labeled peptides for 15 min (unless specified otherwise) at 25°. The samples were diluted rapidly with 1 ml of ice-cold solution of 1% albumin in 20 mM Tris-HCl, pH 7.6, and immediately filtered on oxoid filters. The oxoid filters were soaked in 10% albumin for 30 min prior to use and then washed with 1 ml of 1% albumin in 10 mM Tris-HCl, pH 7.6; this procedure reduced adsorption of the labeled peptides to the filter. As a control for nonspecific adsorption of the peptides to the liver plasma membranes, incubation of membranes in the presence of 4 X 10⁻⁴ M unlabelled glucagon was carried out concurrently with membranes incubated with labeled peptide alone. The difference in bound radioactivity was used to determine the amount of glucagon bound to its specific binding sites (12) in liver membranes. Radioactivity was determined in a well-type scintillation counter.

**Phospholipid Extraction and Analyses**—Lipids were extracted from washed membranes by the method of Ways and Hanahan (29). Phospholipid phosphorus was determined by the method of Bartlett (30). The types of phospholipids present in the lipid extracts were evaluated by thin layer chromatography (Adesibols-5 standard Prekotes, Applied Sciences Laboratories) using a developing solvent system containing (v/v) chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10). The lipid-containing spots were first located under ultraviolet light after spraying the plates with 0.1% 1-aminonaphthalene-8-sulfonate (magnesium salt) in water (31). After marking the lipid spots, the chromatograms were sprayed with 0.3% ninhydrin in a solution of 2% acetic acid in ethanol (32); ninhydrin-positive lipids were visualized after heating the plates for 5 min at 120°.
PHOSPHATIDYL ETHANOLAMINE
PHOSPHATIDYL CHOLINE
SPHINGOMYELIN
PHOSPHOLIPIDS LIVER PLASMA MEMBRANES (LPM)
PHOSPHOLIPIDES LPM TREATED WITH B. CEREUS
PHOSPHATIDYLSEERINE
PHOSPHOLIPIDS LPM TREATED WITH C1. PERFRINGENS
PHOSPHOLIPASE C
NINHYDRIN POSITIVE

Fig. 1. Thin layer chromatography of phospholipids extracted from liver plasma membranes (LPM) before and after treatment with phospholipase C from Bacillus cereus and Clostridium perfringens. Plasma membranes (2.5 mg of protein) were incubated in the absence or presence of 0.1 unit of either type of phospholipase. Other incubation conditions and the procedures for extraction, chromatography, and phospholipid detection are described under "Experimental Procedure."

RESULTS

In all experiments described in this study, incubations of hepatic plasma membranes were kept to 5 min at 15°C in the absence and presence of Bc-phospholipase C or Cp-phospholipase C in order to minimize changes in the stability of adenylate cyclase that occurred with longer times and higher temperatures of incubation. The concentrations of the enzymes were adjusted, based on their capacity to hydrolyze egg yolk phospholipids (see "Methods"), to give approximately equal rates of hydrolysis of membrane-bound phospholipids.

Fig 1 shows the types of membrane phospholipids hydrolyzed by Bc-phospholipase C and Cp-phospholipase C under standard incubation conditions with sufficient concentrations of the enzymes to cause, respectively, 57 and 58% hydrolysis. Four major phospholipid spots, representing phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and phosphatidylserine, were detected in chromatograms of lipids extracted from untreated membranes. Phosphatidylinositol was not separated from phosphatidylserine with the solvent system used. Be-phospholipase C hydrolyzed phosphatidylserine (phosphatidylinositol), phosphatidylethanolamine, and phosphatidylcholine, but not sphingomyelin. In contrast, Cp-phospholipase C did not hydrolyze phosphatidylserine (phosphatidylinositol). This qualitative pattern of hydrolysis by the two phospholipases was confirmed by Dr. William Weglicki who analyzed the phosphorus content of the individual phospholipids chromatographed with a solvent system that separated completely all of the phospholipids present in hepatic membranes (34-37). The specificities of the two types of phospholipases confirm the observations of others (16-23, 38) using either purified phospholipids or membrane-bound phospholipids as substrates.

Fig. 2 illustrates the effects of Bc-phospholipase C and Cp-phospholipase C on the response of adenylate cyclase to glucagon and fluoride ion; the effects are expressed relative to the percentage of phospholipids hydrolyzed with increasing concentrations of the phospholipases. Fluoride ion stimulates adenylate cyclase by a different process from hormonal activation and is thought to act directly on the catalytic component (14). The effects of fluoride ion are useful, therefore, for distinguishing the characteristics of the catalytic component from those of the components through which hormonal stimulation is expressed. It can be seen that the stimulatory effects of fluoride ion were not affected after hydrolysis of 55% of the phospholipids by either of the phospholipases. Basal activity (not shown) was also not altered by the phospholipases. In contrast, both phospholipases, at concentrations sufficient to hydrolyze more than 20% of the membrane phospholipids, caused increasing losses of glucagon response. Be-phospholipase C abolished the hormone response completely when 49 to 50% of the membrane phospholipids were hydrolyzed. However, in twelve experiments of this type, Cp-phospholipase C did not cause more than 50% loss of hormone

PHOSPHOLIPASE C
NINHYDRIN POSITIVE

Fig. 2. Effects of Bc-phospholipase C (□) and Cp-phospholipase C(●) on response of adenylate cyclase to glucagon (10−4 M) and fluoride ion (20 mM). Incubation conditions and procedures for assaying adenylate cyclase activity are described under "Experimental Procedure."

Protein—Protein was measured by the procedure of Lowry et al. (33) using bovine serum albumin as standard.

We are indebted to Dr. William B. Weglicki (Peter Bent Brigham Hospital, Boston, Mass.) for analyzing the percentage of phosphatidylserine, phosphatidylinositol, and sphingomyelin hydrolyzed by the two phospholipases under considerations described in Fig. 1. Be-phospholipase C hydrolyzed, respectively, 72 and 50% of the phosphatidylserine and phosphatidylinositol; sphingomyelin was not hydrolyzed. Cp-phospholipase C hydrolyzed 52% of the sphingomyelin and about 11% phosphatidylserine; phosphatidylinositol was not hydrolyzed. Both enzymes hydrolyzed approximately equal percentages of phosphatidylcholine and phosphatidylethanolamine.
response even when 60% of the phospholipids were hydrolyzed. Since the major differences in phospholipid specificity between Bc-phospholipase C and Cp-phospholipase C are their ability, respectively, to hydrolyze acidic phospholipids and sphingomyelin, we attribute the quantitative difference in their effects on hormone response to the hydrolysis of the acidic phospholipids by Bc-phospholipase C. The basis of the relatively small effects of Cp-phospholipase C on hormone response is unknown and requires further experimentation with purified preparations of the phospholipases in C. perfringens. We have observed that omission of calcium ion from the incubation medium reduces correspondingly both hydrolysis of membrane phospholipids and the effects of Cp-phospholipase C on hormone response, in keeping with the stimulatory effects of calcium ion on Cp-phospholipase C activity (38). The effects of Bc-phospholipase C, a zinc-requiring enzyme (21), were unchanged by the omission of calcium ion; the three preparations (partially purified and highly purified) of Bc-phospholipase C used in this study gave identical effects on hormone response when equivalent units of activity were added.

We have shown previously (5, 7) that phospholipase A2 causes both loss of glucagon action and binding of the hormone to specific sites having the characteristic of the receptor for glucagon. As shown in Fig. 3, Bc-phospholipase C and Cp-phospholipase C also caused decreases in the binding of $^{125}$I-glucagon to these sites. However, although the loss of binding bore some relationship to the effectiveness of the two phospholipases on glucagon action, glucagon binding was not diminished to the same extent as was glucagon action. In these experiments the concentration of glucagon used for binding was $10^{-8}$ M whereas that for action was $10^{-4}$ M. The same differences were obtained when equimolar ($10^{-4}$ M) concentrations of the hormone were employed.

In subsequent experiments we found that inclusion of 1 mM β-mercaptoethanol in the media used for incubating the membranes with phospholipase resulted in a significant decrease in loss of glucagon response due to Cp-phospholipase C treatment. In the presence of the sulfhydryl agent but under otherwise identical conditions (see "Experimental Procedure"), Cp-phospholipase C-treated membranes displayed (in three experiments) 52 ± 2% phospholipid hydrolyzed and 20 ± 2% loss of glucagon response rather than the 40 to 50% loss noted in Figs. 2 and 3. Inclusion of the sulfhydryl reagent during treatment with Bc-phospholipase C did not alter the extent of phospholipid hydrolysis or the loss of glucagon response.

Since Bc-phospholipase C did not diminish the specific binding sites for glucagon, it was apparent that a major change introduced by phospholipid hydrolysis is a reduction in the affinity of the binding sites for glucagon. As illustrated in Fig. 4, Bc-phospholipase C, at concentrations sufficient to catalyze 60% hydrolysis of membrane phospholipids, caused only a slight (10%) decrease in glucagon binding when the concentration of $^{125}$I-glucagon was increased to $10^{-7}$ M, indicating that the sites available for glucagon binding are not diminished significantly by hydrolysis of acidic phospholipids. Shown in Fig. 4 and consistently observed, is increased binding of glucagon (at concentrations higher than $10^{-8}$ M) after treatment with low concentrations of Bc-phospholipase C. The basis of the increased binding is not understood but may reflect either unmasking of cryptic binding sites by removal of phospholipids or by the introduction of "pores" in "inside-out" membrane vesicles (39) through which glucagon may penetrate the membrane and bind to glucagon binding sites on the interior face of the membrane. The latter possibility is raised since the receptor for glucagon is thought to be located exclusively on the outer surface of the plasma membrane (40).

![Diagram](https://example.com/diagram1.png) **Fig. 3.** Effects of Bc-phospholipase C and Cp-phospholipase C on the loss of glucagon response and on the binding of $^{125}$I-glucagon ($10^{-9}$ M) to liver plasma membranes. Incubation conditions and procedures for assaying adenylate cyclase activity and binding of labeled glucagon are described under "Experimental Procedure."
affinity of the binding sites for glucagon was reduced from $5 \times 10^{-8} \text{M}$ in untreated membranes to about $5 \times 10^{-9} \text{M}$ in Bc-phospholipase C-treated membranes. In these experiments, we also compared the binding of $^{125}\text{I}-\text{DH-glucagon}$, a biologically inactive, competitive inhibitor of glucagon binding and action (41), to that of native glucagon in treated and untreated membranes. The apparent affinity of DH-glucagon for the glucagon binding sites was unaltered by Bc-phospholipase C treatment and was similar (about $6 \times 10^{-8} \text{M}$) to that observed for native glucagon binding to the treated membranes.

We have shown in earlier studies (42) that binding of glucagon to its receptor is not sufficient per se to activate adenylate cyclase in hepatic membranes. GTP and other purine nucleotides (GDP, ATP, ADP) bind to regulatory sites required for the actions of glucagon on adenylate cyclase (42) and increase the rate of dissociation of bound glucagon from its receptor (15). It was of interest, therefore, to determine whether the hydrolysis of acidic phospholipids by Bc-phospholipase C would alter the effects of GTP on the rate of dissociation of bound $^{125}\text{I}$-glucagon. As shown in Table I, GTP did not alter the rates of dissociation of labeled glucagon or Bc-phospholipase C-treated plasma membranes in the absence or presence of excess ($10^{-4} \text{M}$) unlabeled hormone. In contrast, GTP did not alter the rates of dissociation of labeled glucagon in Bc-phospholipase C-treated membranes.

Since the affinity of the binding sites for glucagon in treated membranes is reduced to that of DH-glucagon, it seemed possible that the loss of the nucleotide effect on binding of glucagon to treated membranes is related to the reduction in affinity and that the histidine residue of the hormone is required for the GTP effect to be expressed. Accordingly, we examined the effects of the binding data yield, in the experiments described in Fig. 5, only relative changes in apparent affinities of the binding sites for glucagon and DH-glucagon. The apparent increase in binding sites for DH-glucagon relative to glucagon in treated and untreated membranes (note the intercepts on the ordinate) may be explained in part by the lower rates of inactivation of DH-glucagon relative to glucagon (8). Lower rates of inactivation might yield, in a kinetic sense, an apparent increase in the binding sites for DH-glucagon since the concentration of DH-glucagon would not change as rapidly as that of glucagon.

#### Table I

| Treatment of membranes | $^{125}\text{I}$-Glucagon bound | $^{125}\text{I}$-DH-glucagon bound |
|------------------------|----------------------------------|----------------------------------|
| Control                |                                  |                                  |
| GTP                    |                                  |                                  |
| Untreated              | 4.1                              | 1.0*                             |
| Treated                | 1.4                              | 0.4*                             |
| GTP                    | 1.2                              | 0.6*                             |
| Untreated              | 1.2                              | 0.6*                             |
| GTP                    | 0.6                              | 0.5*                             |

* These values have been corrected for the contamination of native glucagon (5 to 10%) in the DH-glucagon preparation.
corticotropic hormone-sensitive adrenal adenylate cyclase systems have been reported subsequently (43-46) and over the same range of concentrations, stimulate the rate of dissociation of bound DH-glucagon which binds with affinity binding sites for glucagon. Most importantly, the affinity of DH-glucagon compared to native glucagon observed (41); (b) phospholipids transmit or modulate the physicochemical functionally in hormone regulation if, for example, a metal ion, which acidic phospholipids form strong ligands (48, 49), were a component, it should be noted that histidine forms complexes with metal ions, particularly those of the transition series (50). With regard to a metal ion as a component, it is suggested that the nucleotides bind to a regulatory site which controls the state of activity of adenylate cyclase as well as the rate of dissociation of the hormone from its receptor. The results suggest that acidic phospholipids are involved in the action of glucagon (i.e. the histidine residue) and the nucleotides at the regulatory site.

The nature of the regulatory site remains unknown. It is not possible, therefore, to assign a strictly structural or functional role to phospholipids in hormone action. The negatively charged polar group of the acidic phospholipids might participate functionally in hormone regulation if, for example, a metal ion, with which acidic phospholipids form strong ligands (48, 49), were a component of the regulatory site. With regard to a metal ion as a component, it should be noted that histidine forms complexes with metal ions, particularly those of the transition series (50). As for a structural role, phospholipids may serve to maintain the quaternary structure of the receptor-enzyme complex such that the regulatory site is oriented in a configuration necessary for interaction with glucagon and nucleotides.

In conclusion, acidic phospholipids are required for the interrelated effects of glucagon and nucleotides on the hepatic adenylate cyclase system. A relationship is clearly indicated between these lipids and the liganding of the histidine residue of glucagon and of nucleotides to a site that controls the activity of this complex regulatory enzyme system.

REFERENCES

1. Sutherland, E. W., Rall, T. W., and Menon, T. (1962) J. Biol. Chem. 237, 1220-1227

2. Pastan, I., Pricer, W., and Blanchette-Mackie, J. (1970) Metabolism 19, 809-817

3. Wolff, J., and Jones, A. B. (1970) Proc. Nat. Acad. Sci. U. S. A. 65, 454-459

4. Perkins, J. P., and Moore, M. M. (1971) J. Biol. Chem. 246, 69-68

5. Birnbaumer, L., Pohl, S. L., and Rodbell, M. (1971) J. Biol. Chem. 246, 1857-1890

6. Wolff, J., and Jones, A. B. (1971) J. Biol. Chem. 246, 3090-3097

7. Pohl, S. L., Krans, H. M. J., Kozyleff, V., Birnbaumer, L., and Rodbell, M. (1971) J. Biol. Chem. 246, 4447-4454

8. Pohl, S. L., Krans, H. M. J., Birnbaumer, L., and Rodbell, M. (1972) J. Biol. Chem. 247, 2295-2301

9. Kretsinger, A., Tomasi, V., and Trestiani, A. (1971) Arch. Biochem. Biophys. 147, 36-40

10. Levvy, G. S. (1971) Biochem. Biophys. Res. Commun. 43, 108-113

11. Levvy, G. S. (1971) J. Biol. Chem. 246, 7405-7407

12. Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1861-1871

13. Rodbell, M., Birnbaumer, L., and Pohl, S. L. (1971) in The Role of Adenyl Cyclase and Cyclic 3',5'-AMP in Biological Systems (Rall, T. W., Rodbell, M., and Condliffe, P., eds) pp. 59-92, Fogerty International Center, Bethesda, Maryland

14. Rodbell, M. (1972) in Glucagon: molecular Physiology, Clinical and Therapeutic Implications (Leff, E. S., and Unger, R. H., eds) pp. 61-75, Pergamon Press, New York

The precise nature of the acidic phospholipids (or phospholipids) involved in glucagon action on the hepatic system has not been established. The studies of Levey (10) suggest that phosphatidylserine, which is required for glucagon action on a "solubilized" preparation of myocardial adenylate cyclase, may be specifically involved. Studies of the restoration of glucagon action by addition of phospholipids to membranes treated with Be-phospholipase C may help to resolve this question.

Our principle objectives are to establish at what stage of hormonal regulation phospholipids may participate and to assign a specific role for lipids in this process. The following possibilities are considered. (a) The phospholipids form a complex with the receptor and participate in the binding of glucagon, possibly with the intensely hydrophobic COOH-terminal region of the hormone (41); (b) phospholipids transmit or modulate the physiochemical signals that may be produced by hormone-receptor interaction; (c) phospholipids play a strictly structural role possibly by maintaining the receptor-enzyme complex in its hormonally responsive state.

The possibility that phospholipids participate directly in the binding of glucagon to its receptor can be ruled out. Hydrolysis of acidic phospholipids by Be-phospholipase C did not diminish the number of specific binding sites for glucagon. The major change observed was a 10-fold decrease in the affinity of the binding sites for glucagon. Most importantly, the affinity of glucagon for the binding sites in the treated membranes became the same as that for DH-glucagon which binds with affinity identical with the receptors in either untreated or phospholipase C-treated membranes.

The NH2-terminal histidine residue of glucagon plays a unique role in both the binding and action of glucagon on adenylate cyclase systems (41). Histidine is required for biological action and contributes substantially to the forces involved in the binding of glucagon to its receptor. The 10-fold difference in the binding affinity of DH-glucagon compared to native glucagon observed here and in a previous report from this laboratory (8) calculates to be (at 30°) about 1 Cal per mole. This difference in binding energy is consistent with the histidine residue binding through hydrogen bonds to a special site involved in the regulation of adenylate cyclase activity by glucagon. Since DH-glucagon specifically competes with glucagon at the receptor (41), stereospecificity of glucagon binding resides in the region encompassing the 2 to 29 amino acid residues of the hormone. Removal of the acidic phospholipids did not change the stereospecificity or the affinity of the 2 to 20 region for the receptor binding site (cf. Fig. 5). Thus, phospholipids play an important role in the binding of the histidine residue to a regulatory site involved in hormone action.

Another function lost upon removal of the acidic phospholipids is the ability of nucleotides such as GDP to enhance the rate of dissociation of glucagon from its receptor. We have shown previously (14) that purine nucleotides (GTP, GDP, ATP, and ADP) are required for glucagon action on adenylate cyclase and, over the same range of concentrations, stimulate the rate of dissociation of bound hormone. Similar requirements for purine nucleotides in the actions of several hormone-sensitive adenylate cyclase systems have been reported subsequently (45-46) and have been extended recently in this laboratory to the adrenocorticotropin hormone-sensitive adrenal adenylate cyclase system and the multireceptor system in rat fat cells (47). It has been suggested that the nucleotides bind to a regulatory site involved in the binding and action of glucagon as one means of accounting for the dual effects of the nucleotides (14).

The present study showed that GTP does not alter the rate of dissociation of bound DH-glucagon from either phospholipase C-treated or untreated membranes and affects the dissociation rate of glucagon only when the hormone is bound to untreated membranes (Table 1). Thus, GTP only affects the rate of glucagon dissociation from the receptor when the histidine residue can bind to the regulatory site involved in glucagon action. This finding can be taken as strong evidence that GTP (or other nucleotide modifiers) and the histidine residue of glucagon bind to a common regulatory site which controls the state of activity of adenylate cyclase as well as the rate of dissociation of the hormone from its receptor. The results suggest that acidic phospholipids are involved in the action of glucagon (i.e. the histidine residue) and the nucleotides at the regulatory site.

In conclusion, acidic phospholipids are required for the interrelated effects of glucagon and nucleotides on the hepatic adenylate cyclase system. A relationship is clearly indicated between these lipids and the liganding of the histidine residue of glucagon and of nucleotides to a site that controls the activity of this complex regulatory enzyme system.
15. Rodbell, M., Krantz, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1872–1876
16. Pastan, I., Macchi, V., and Katzen, R. (1968) J. Biol. Chem. 243, 3759–3765
17. Duttnera, S. M., Byrne, W. L., and Ganoza, M. C. (1968) J. Biol. Chem. 243, 2216–2228
18. Coleman, R., Finean, J. B., Knutton, S., and Limbrick, A. R. (1970) Biochim. Biophys. Acta 219, 81–92
19. Kahlerding, A., and Banjo, B. (1972) J. Biol. Chem. 247, 1150–1160
20. Sleij, M. W., and Logan, G. F., Jr. (1965) J. Bacteriol. 90, 69–81
21. Ottolenghy, A. C. (1965) Biochim. Biophys. Acta 106, 510–518
22. Zwaal, R. F. A., Roelofsen, B., Compurius, P., and Van Deenen, L. L. M. (1971) Biochim. Biophys. Acta 233, 474–479
23. Roelofsen, B., Zwaal, R. F. A., Compurius, P., Woodward, C. B., and Van Deenen, L. L. M. (1971) Biochim. Biophys. Acta 241, 925–929
24. Mavis, R. D., Bell, R. M., and Vagelos, P. R. (1972) J. Biol. Chem. 247, 2535–2541
25. Pohl, S. L., Birnbaumer, L., and Rodbell, M. (1971) J. Biol. Chem. 246, 1849–1856
26. Rodbell, M. (1966) J. Biol. Chem. 241, 130–139
27. Schmidt, G., Bessman, M. J., Hickey, M. D., and Thannhauser, S. J. (1968) J. Biol. Chem. 243, 1027–1031
28. Krishna, G., Weiss, B., and Brodie, B. B. (1968) J. Pharmacol. Exp. Ther. 163, 379–385
29. Ways, P., and Hanahan, J. (1994) J. Lipid Res. 5, 318–323
30. Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
31. Rubalcava, B. (1970) Ph.D. Thesis, p. 17, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, México
32. Farhy, A. R., Niederwieser, A., Pataki, G., and Brenner, M. (1961) Helv. Chim. Acta 44, 2022–2026
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
34. Skipski, V. P., Barclay, M., Archibald, F. M., Kekish, O. T., Reichman, E. S., and Good, J. J. (1965) Life Sci. 4, 1673–1680
35. Doo, B. J., and Gray, G. M. (1968) Biochim. Biophys. Acta 160, 397–404
36. Stahl, W. L., and Trams, E. G. (1968) Biochim. Biophys. Acta 168, 459–471
37. Ray, T. K., Skipski, V. P., Barclay, M., Essnee, E., and Archibald, F. M. (1969) J. Biol. Chem. 244, 5528–5536
38. Bangham, A. D., and Dawson, R. M. C. (1962) Biochim. Biophys. Acta 59, 103–115
39. Weck, T. L., Weinstein, R. S., Straus, I. H., and Wallach, D. F. H. (1970) Science 168, 255–257
40. Rodbell, M., Birnbaumer, L., and Pohl, S. L. (1970) J. Biol. Chem. 245, 715–722
41. Rodbell, M., Birnbaumer, L., Pohl, S. L., and Sundby, F. (1970) Proc. Nat. Acad. Sci. U. S. A. 56, 900–913
42. Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krantz, H. M. J. (1971) J. Biol. Chem. 246, 1877–1882
43. Goldfine, I. D., Roth, J., and Birnbaumer, L. (1972) J. Biol. Chem. 247, 1211–1218
44. Krishna, G., Harwood, J. P., Barber, A. J., and Jamieson, G. A. (1972) J. Biol. Chem. 247, 2253–2254
45. Rockaert, J., Roy, C., and Jard, S. (1972) J. Biol. Chem. 247, 7073–7081
46. Lefay, F., Chamodot, A. M., and Ilanoune, J. (1972) Biochem. Biophys. Res. Commun. 48, 1385–1391
47. Birnbaumer, L., and Rodbell, M. (1969) J. Biol. Chem. 244, 3477–3482
48. Abramson, M. B., Katzman, R., and Grogan, H. P. (1964) J. Biol. Chem. 239, 70–76
49. Barton, P. G. (1968) J. Biol. Chem. 243, 3384–3390
50. Scherber, J. (1964) in Chemical Specificity in Biological Interactions (Guth, F. R. N., ed) pp. 114–163, Academic Press, New York
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