Evidence for a Dissociable Glucagon Binding Site in a Solubilized Preparation of Myocardial Adenylate Cyclase*

(Received for publication, April 16, 1973)

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SUMMARY

A solubilized preparation of myocardial adenylate cyclase was chromatographed on Sephadex G-100. Binding of 125I-glucagon and fluoride-stimulatable adenylate cyclase activity occurred in the elution fractions excluded from the gel suggesting a molecular weight greater than 100,000 for the enzyme-receptor site complex. Prior incubation of the binding peak with glucagon shifted its elution pattern on Sephadex G-100 to a smaller molecular weight peak of approximately 20,000 as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The findings are consistent with a dissociable receptor site for glucagon on myocardial adenylate cyclase and may provide a mechanism for activation-inactivation of the enzyme following hormone binding.

The mechanism by which hormones bind to membrane receptor sites and effect subsequent activation of adenylate cyclase is of great interest. Rodbell et al. (1) have proposed a three-component model of the hormone-responsive, membrane-bound adenylate cyclase. The regulatory site (receptor) located on the external surface of the cell membrane serves as the binding site for the hormone. The catalytic site, on the interior of the cell membrane, has access to ATP and generates adenosine 3':5'-monophosphate. An intermediate coupler serves to transmit a message initiated by the binding event to the catalytic site resulting in activation of adenylate cyclase. Although it is not clear how these subunits interact several investigations have demonstrated a clear separation of binding from activation of the enzyme (2, 3). We have been utilizing a solubilized preparation of cat myocardial adenylate cyclase in order to study the interrelationships of these various components (4-7). The data in this report provide evidence for a smaller molecular weight glucagon binding site which is dissociable both from a larger molecular weight component and catalytic adenylate cyclase activity.

Normal cats were anesthetized with pentobarbital, 25 to 35 mg per kg intraperitoneally, the heart was quickly excised, and the left ventricle was dissected free of endocardium and epicardium. About 300 mg of muscle were homogenized in 4.0 ml of a cold solution containing 0.25 m sucrose, 10 m Tris-HCl, pH 7.7, 20 m Lubrol PX, and 1 m EDTA magnesium chloride. The homogenate was centrifuged at 12,000 × g for 10 min at 4° and the supernatant was used for Sephadex chromatography described in the legend to Fig. 1.

Glucagon binding to the solubilized myocardial receptor was determined using 125I-labeled glucagon. Glucagon was iodinated by the method of Hunter and Greenwood (8). 125I-Glucagon was purified on a column of cellulose powder as described by Rodbell et al. (9). The 125I-glucagon was applied to a 2.5 cm cellulose column prepared in a Pasteur pipette, inside diameter 0.6 cm, washed with 3.0 ml of a solution of 1% albumin in 10 m sodium phosphate adjusted to pH 7.5, and then eluted with 0.6 ml of the same solution adjusted to pH 10.0 with concentrated ammonium hydroxide. The 125I-glucagon eluted in this manner was biologically active as determined by its ability to activate the particulate myocardial adenylate cyclase (10). The specific fractions of solubilized enzyme referred to in the text were incubated at 37° in a final volume of 100 μl containing 1.0% albumin in 10 m Tris-HCl, pH 7.7, and 125I-glucagon (0.370 μCi per pmole). After 90 min the incubation mixture was added to 2.5 cm cellulose columns in disposable Pasteur pipettes (inside diameter 0.6 cm) and washed with 1.4 ml of 1% albumin in 10 m Tris-HCl, pH 7.7. Bound 125I-glucagon did not adsorb to the column whereas free (unbound) 125I-glucagon did. The eluate was then counted in a Nuclear-Chicago Autogamma. This method removes more than 90% of the free (unbound) 125I-glucagon as determined by the number of counts found in the control samples of identical composition incubated simultaneously using 10 m Tris-HCl, pH 7.7, in place of the enzyme fraction (10). Similar control values are obtained when Lubrol-PX is included in the incubation mixture. Each value represents the mean of duplicate experiments.

Adenylate cyclase was assayed by the method of Krishna et al. (11) in a total volume of 60 μl containing 1.6 m [α-32P]ATP (2.5 to 3.5 × 106 cpm), 8 μm theophylline, 2 m MgCl2, 21 m Tris-HCl, pH 7.7, 0.8 mgl per ml of bovine serum albumin, 8 m sodium fluoride, and 1 to 11 μg of enzyme protein. The assay was initiated by adding the enzyme-fluoride mixture prepared at 1° to the other components which were at 25°. After 5 min at 37° the incubations were stopped and the cyclic 3':5'-monophosphate accumulated was determined as previously described (7). Protein was measured by the method of Lowry et al. (12), using bovine serum albumin as a standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed utilizing a modification of the method of Weber and Osborn (13, 14). Acrylamide concentration was 7.5% with a ratio of bisacrylamide to acrylamide of 1:37. The SDS concentration in the gels and buffer was 0.1%. Prior to electrophoresis the samples were incubated at 37° for 30 min in 0.01 m phosphate, pH 7.0, containing 0.1% SDS and 0.1% mercaptoethanol.

We have previously demonstrated that almost all of the solu-

The abbreviation used is: SDS, sodium dodecyl sulfate.
Hormones which exert specific effects upon certain cells and tissues are presumed to act by attaching to the cell surface receptors and in many cases to activate subsequently membrane-bound adenylate cyclase systems (15). Glucagon is thought to increase cardiac inotropy through such a sequence of events. Little is known about the nature of the interaction between the receptor and the adenylate cyclase that yields increased enzyme activity. It is not certain if the receptor is a discrete molecule or just a functional part of the catalytic unit. Current theory based upon the work of Rodbell et al. points to a three-component model comprising the hormone-responsive, membrane-bound adenylate cyclase system (1). In their model the receptor or discriminator located on the external cell surface binds certain hormones and initiates the process leading to activation. Evidence from a number of laboratories suggests that the process of binding and that of activation are separate events which can be dissociated by several experimental interventions (2, 3). Thus, while the activation of the solubilized adenylate cyclase by glucagon requires the presence of phospholipids, the binding of glucagon to a solubilized preparation of cat myocardium. Two hundred microliters of the 12,000 X g supernatant derived from the original Lubrol-PX homogenate of heart muscle were applied to a Sephadex G-100 column (6 X 96 mm), equilibrated with 10 mM Tris-HCl, pH 7.7, at room temperature. The column fractions were eluted with 10 mM Tris-HCl, pH 7.7; 0.25-ml fractions were collected doublewise and a 25-μl aliquot of each fraction was assayed for 125I-glucagon binding activity. The maximum binding activity was found in the eluate at 1.0 to 1.2 ml, with a peak at approximately 1.0 to 1.2 ml. The maximum binding fractions (0.9 to 1.8 ml) were pooled, a 200-μl aliquot was rechromatographed on the Sephadex G-100 column (H), and 0.25-ml fractions were collected doublewise. Twenty-five-microliter aliquots of each of these fractions were assayed for 125I-glucagon binding (see text). The bound and free 125I-glucagon were separated by cellulose chromatography as described in the text, and the total cellulose efficiency (1.0 ml) which contained bound 125I-glucagon was counted in an Autogamma. In a separate set of experiments (R) 20 μl of the original maximum binding fraction were incubated with 125I-glucagon (see text) and then chromatographed on cellulose. Next 200 μl of this eluate were applied to the identical Sephadex G-100 column and eluted with 10 mM Tris-HCl, pH 7.7, and the binding profile was obtained by determining the bound 125I-glucagon in each fraction eluted from the Sephadex column (H). In one experiment, glucagon, 1 X 10^-6 M, was added to the incubations prior to the addition of the 125I-glucagon in order to determine the specificity of the binding (C). Appropriate controls were utilized at each step in the procedure. Less than 10% of unbound (free) glucagon was counted in an Autogamma. In a separate set of experiments (B) 200 μl of the binding peak. The small amount of protein in the Sephadex fraction precluded visualization of bands with either Coomassie blue or periodic acid-Schiff stains. The crude heart muscle preparation showed a complex pattern of over 20 peptide bands.

It is also noteworthy that the peak of adenylate cyclase activity did not shift after incubation with glucagon (Fig. 1B), all of the activity appearing in the eluate between 0.9 and 1.3 ml. The minor peak of adenylate cyclase activity in the small molecular weight area (Fig. 1A) was not observed after glucagon preincubation (Fig. 1B). Furthermore, in experiments in which the solubilized preparation was freed of detergent by DEAE-cellulose chromatography (5) no adenylate cyclase activity was found in the area of the small molecular weight binding peak under any conditions.

The binding profile, including the shift upon prior incubation with glucagon, was identical whether the detergent was present or absent.

Hormones which exert specific effects upon certain cells and tissues are presumed to act by attaching to the cell surface receptors and in many cases to activate subsequently membrane-bound adenylate cyclase systems (15). Glucagon is thought to increase cardiac inotropy through such a sequence of events. Little is known about the nature of the interaction between the receptor and the adenylate cyclase that yields increased enzyme activity. It is not certain if the receptor is a discrete molecule or just a functional part of the catalytic unit. Current theory based upon the work of Rodbell et al. points to a three-component model comprising the hormone-responsive, membrane-bound adenylate cyclase system (1). In their model the receptor or discriminator located on the external cell surface binds certain hormones and initiates the process leading to activation. Evidence from a number of laboratories suggests that the process of binding and that of activation are separate events which can be dissociated by several experimental interventions (2, 3). Thus, while the activation of the solubilized adenylate cyclase by glucagon requires the presence of phospholipids, the binding of glucagon is more sharply defined and appears in the eluate between 0.9 and 1.3 ml. The minor peak of adenylate cyclase activity in the small molecular weight area (Fig. 1A) was not observed after glucagon preincubation (Fig. 1B). Furthermore, in experiments in which the solubilized preparation was freed of detergent by DEAE-cellulose chromatography (5) no adenylate cyclase activity was found in the area of the small molecular weight binding peak under any conditions.

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gon to this same preparation occurs in the absence of such lipid (10).

The present report describes the physical dissociation of a small molecule of approximate molecular weight 20,000 from a fraction of solubilized cat myocardium that contains adenylate cyclase. The dissociation occurs only under conditions where the larger molecular weight fraction is first incubated with glucagon at concentrations that are capable of stimulating the adenylate cyclase. In the absence of preincubation with glucagon, all of the glucagon binding activity is found in a fraction of the Sephadex eluate which is excluded from the column and which also contains almost all of the adenylate cyclase activity. The catalytic activity of the enzyme remains in the large molecular weight fraction even after dissociation of the glucagon binding peak. It is also of interest that SDS is capable of preventing the initial binding of glucagon when present in the incubation but does not dissociate bound 125I-glucagon. This finding is consistent with the observation of Krug et al. that glucagon bound to its specific receptor represents a strong bond (16) and may reflect hormone (ligand)-induced allosteric interaction as postulated by Koshland (17).

Results similar to the findings in this investigation have recently been reported in studies of the adenosine 3':5'-monophosphate dependent protein kinase from the laboratories of Garren et al. (18) and Tao (19), in which they demonstrated the dissociation of a smaller molecular weight adenosine 3':5'-monophosphate binding protein from the larger molecular weight catalytic unit. Their observations suggested that in the process of binding, the receptor, whose association with the enzyme was inhibitory to catalytic function, was dissociated from the protein kinase and the enzyme was rendered active. The data in this study are consistent with such a sequence of events occurring in the solubilized adenylate cyclase system. However, whether the smaller molecular weight glucagon binding peak described in these experiments is inhibitory to the adenylate cyclase or interacts directly with the catalytic moiety in some other manner cannot be determined from the present results. Further studies with a more purified preparation of solubilized adenylate cyclase are required to define this interaction more precisely.

Acknowledgments—We would like to thank Mrs. Eva Ruiz and Mrs. Antoinette Schenk for excellent technical assistance.

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J. Biol. Chem. 1973, 248:5552-5554.

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