SOLUBILIZATION OF GLUCAGON AND EPINEPHRINE SENSITIVE ADENYLATE CYCLASE FROM RAT LIVER PLASMA MEMBRANES

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SUMMARY

Hormonally sensitive adenylate cyclase has been solubilized from rat liver plasma membranes using Triton X-305 in Tris buffers containing mercaptoethanol and $MgCl_2$. The solubilized enzyme was stimulated 5 fold by NaF, 7 fold by glucagon and 20 fold by epinephrine. Criteria for solubilization included lack of sedimentation at 100,000 x g for one hour, the absence of particulate material in the 100,000 x g supernatant when examined by electron microscopy, and inclusion of hormonally sensitive adenylate cyclase activity in Sephadex G 200 gels. The molecular weight of the solubilized, hormonally sensitive enzyme was approximately 200,000 in the presence of Triton X-305.

INTRODUCTION

Although there has been considerable interest in the mechanism for hormonal stimulation of mammalian adenylate cyclase activities, progress toward the solution of this problem has been severely hindered by one major technical barrier. The enzyme has not been solubilized and purified in a hormonally sensitive form from any mammalian source. However, the bacterial enzyme from Brevibacterium lique-

faciens which is not stimulated by hormones has been purified to homogenity and crystallized (1). In addition to the problems often encountered when purifying membrane associated enzymes there are additional complications associated with the adenylate cyclase system since it is possible that it is a multisubunit system consisting of a catalytic subunit and hormone receptors (2). There are two general approaches which could be used to solve this problem. In the first case, hormone receptors and catalytic activity could be independently solubilized and purified with the intention of ultimately reconstituting the system in a hormonally sensitive form. Considerable progress has been made in solubilizing individual hormone binding activities and adenylate cyclase catalytic activities (3-7) although no successful reconstitution has been reported for solubilized preparations. Alternatively, an attempt can be made to solubilize the enzyme in a hormonally sensitive form in one step. If indeed the adenylate cyclase system is a multisubunit system, the second approach may be difficult since this may require breaking lipid-protein interactions while still maintaining protein-protein interactions between the associated subunits. A systematic approach for screening nonionic detergents has been successfully used to solubilize adenylate cyclase in a hormonally sensitive form (8). In this study we report the solubilization of rat liver plasma membrane adenylate cyclase sensitive to both glucagon and epinephrine.

MATERIALS AND METHODS

[^{3}H] ATP was purchased from ICN and [^{14}C] 3',5' c-AMP was purchased from New England Nuclear. Creatine phosphokinase, creatine phosphate, 3',5' c-AMP, Na_2ATP and Triton X-305 were obtained from Sigma.

Partially purified rat liver plasma membranes were prepared by the Neville procedure (9) as modified by Rodbell (10) with one further modification. The dounce homogenizer used had a drop time of 15 seconds through water. Adenylate cyclase was assayed by the Krishna technique (11) using [^{3}H] ATP as a substrate and [^{14}C] c-AMP in the recovery medium. The final assay mixture was 50 µl in volume containing 5 mM $MgCl_2$, 1 mM EDTA, 5 mM theophylline, 2 mM c-AMP, 1 mM [^{3}H] ATP (2.5 µC), 20 mM creatine phosphate, 1 mg/ml creatine kinase, 20 mM Tris/HCl, pH 7.6, 1 mM mercaptoethanol and 0.1% bovine serum albumin. Reactions were initiated by addition of 20 µl of membrane or solubilized extract containing 40 to 60 µl of protein. All experimental points were done in triplicate.

Adenylate cyclase was solubilized in a hormone sensitive form by treating membranes with 0.1% Triton X-305 in .01 M Tris buffer containing 1 mM mercaptoethanol and 1 mM $MgCl_2$ at a ratio of 1 ml of detergent solution per 300 µg of membrane protein. Membranes were treated with detergent solution for 0.5 hour at 4° with periodic vortexing. The mixture was then centrifuged for 1 hour at 100,000 x g. After careful removal of the supernatant the sample was rapidly frozen and lyophilized to dryness. Lyophilized samples were stored at -10°C and dissolved in water for the adenylate cyclase assay. Samples of the 100,000 x g supernatant were fixed with phosphotungstic acid for negative staining and electron microscopy.

RESULTS

Glucagon and epinephrine sensitive adenylate cyclase activity solubilized from rat liver plasma membranes by 0.1% Triton X 305

in .01 M Tris buffer containing 1 mM mercaptoethanol and $MgCl_2$ was non-sedimentable at 100,000 x g for extended periods of time. This solubilization procedure was repeated seven times with three different membrane preparations and solubilized hormonally sensitive adenylate cyclase was obtained in all cases. The solubilized enzyme was stimulated 7 fold by glucagon and 20 fold by ephinephrine whereas the membrane associated activity was only stimulated 4.4 fold and 3.4 fold by glucagon and epinephrine (Table 1). The increase in hormone

Table I. Adenylate Cyclase Activity
(N Moles c-AMP/mg protein/10 min.)

	Membranes	Stimulation Over Basal	Solubilized*	Stimulation Over Basal
Basal	0.45	---	0.11	---
NaF	4.58	10.3x	0.59	5.4x
Epinephrine	1.52	3.4x	2.21	20x
Glucagon	1.97	4.4x	0.774	7x

* Solubilized adenylate cyclase activity remaining in the supernatant following treatment with 0.1% Triton X 305 and centrifugation for one hour at 100,000 x g.

stimulation obtained with the solubilized preparation could be due to a number of different effects including exposure of hormone binding sites or loss of specific inhibitors. Although NaF stimulated the solubilized adenylate cyclase activity, the degree of stimulation was diminished relative to the membranes. The latter is consistent with the finding that NaF stimulation of adenylate cyclase activity can be lost or lowered in the presence of certain detergents (12). The stability

of the solubilized enzyme was not systematically studied, however hormonally sensitive adenylate cyclase activity was retained after lyophilization, concentration by ultrafiltration and Sephadex G-200 column chromatography at 4^{o} C.

The minimum criteria for solubilization of a membrane enzyme is failure to sediment at centrifugal forces $\geqslant$ 100,000 x g for extended periods of time. Several other criteria for solubilization were applied to the 100,000 x g supernatant. Electron microscopy of this sample demonstrated that the 100,000 x g supernatant contained little or no particulate material. Over 90% of the fields examined were totally free of particles. The maximum number of particles seen in any field at 10,000 fold magnification is shown in figure 1. No membrane particles were observable and particulate material, when seen, was sparsely distributed. In addition, when the supernatant was concentrated and run on Sephadex G-200 in the presence of triton X 305, hormonally sensitive adenylate cyclase activity and epinephrine binding activity were included in the gel (8). The molecular weight of epinephrine sensitive adenylate cyclase in the presence of 0.1% triton X 305 was approximately 200,000. Greater than 95% of the total protein solubilized by triton X 305 was included in the Sephadex G-200 column, indicating that the protein non-sedimentable at 100,000 x g was indeed solubilized and not associated with dispersed membrane fragments. Thus, it can be concluded, at least on the basis of these criteria, that adenylate cyclase was solubilized in a hormonally sensitive form. If there are separate subunits for hormone receptors and catalysis, they apparently remain associated in triton X 305 solubilized extracts.

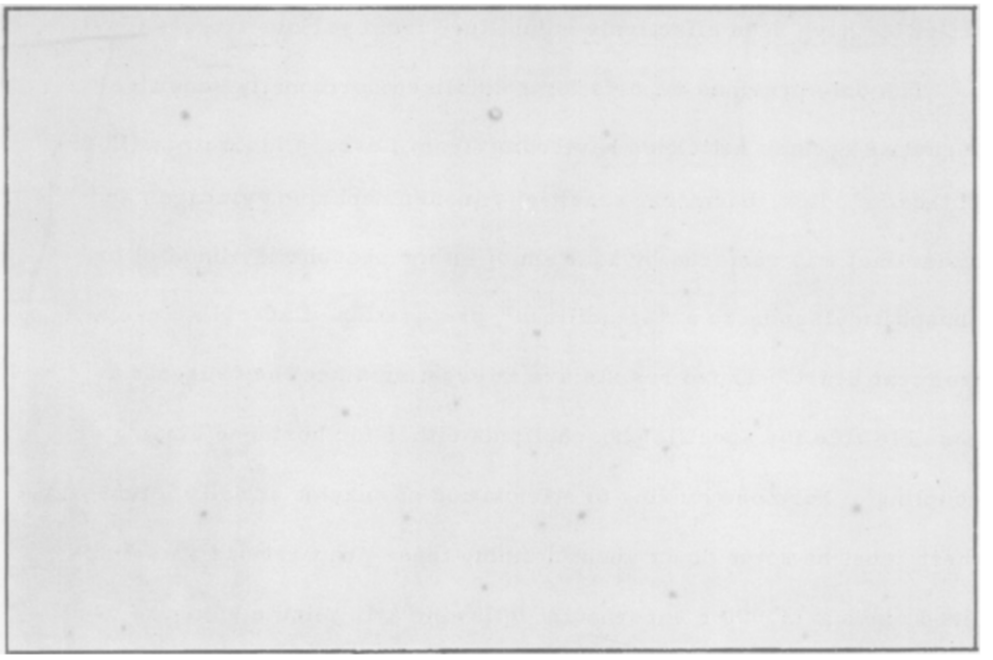

Figure 1. Electron Microscopy of 100,000 x g supernatant.

Rat liver plasma membranes were treated with 0.1% triton X 305 in .01 M Tris buffer at pH 7.5 containing 1 mM $MgCl_2$ and 1 mM mercaptoethanol for 30 minutes at 4°C. After centrifugation at 100,000 x g for one hour, the supernatant was concent rated and fixed with phosphotungstic acid. Magnification was 10,000 x.

DISCUSSION

There are a number of reports in the literature where components of the adenylate cyclase system have been solubilized by nonionic detergents. For example, adenylate cyclase activity has been solubilized from rat brain by Swislocki and Tierney (4) using Lubrol PX. This detergent was first used successfully by Levey (3) to solubilize myocardial adenylate cyclase. In both cases, catalytic activity was nonsedimentable at centrifugal forces $\geqslant$ 100,000 g for extended periods of time. Similarly, glucagon, epinephrine and norepinephrine binding

activities have been effectively solubilized from various tissues (5-7).

The only previous reports for solubilized hormonally sensitive adenylate cyclase acticities have come from Levey's laboratory (13-15). In those studies, hormonal sensitivity (nonepinephrine, glucagon and histamine) was restored by addition of either phosphatidylinositol or phosphatidylserine to a "solubilized" preparation of adenylate cyclase from cat heart. These results are interesting since they suggest a possible role for specific phospholipids either for hormone binding or coupling of hormone binding to stimulation of enzyme activity. However, there must be some doubt about defining these preparations as solubilized since a 12,000 g supernatant following a 10 minute spin was used for solubilized adenylate cyclase activity. The 12,000 g supernatant, which was rich in particulate material (13), was freed from detergent by treatment on a DEAE-cellulose column. Hormonal sensitivity was restored by adding specific phospholipids to a fraction from the DEAE-cellulose column. In order to convincingly establish that this material was indeed solubilized, it would be necessary to demonstrate that the hormonally sensitive adenylate cyclase activities are nonsedimentable at centrifugal forces $\geq$ 100,000 g.

Within the limitations discussed above, it can be concluded that the solubilization procedure described in this report is the first successful attempt to solubilize adenylate cyclase in a hormonally sensitive form from a mammalian tissue.

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