Solubilization and Reconstitution of Dopamine-sensitive Adenylate Cyclase from Bovine Caudate Nucleus*

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SUMMARY

Dopamine-sensitive adenylate cyclase was reconstituted from the cholate-soluble components of caudate nucleus homogenate. Biological function was restored by precipitating the components from cholate and phospholipid with ammonium sulfate, dialyzing the resuspended precipitate, and activating the particulate complex with phospholipid in the assay.

The reconstituted adenylate cyclase was stimulated 3- to 4-fold by dopamine and 8- to 12-fold by guanyl-5'-yl imidodiphosphate. The catecholamine stimulation was specific for dopamine and required the addition of GTP. The cholate-soluble component(s) of the basal adenyl cyclase were separated from the component(s) that conferred dopamine sensitivity by gel filtration chromatography.

Dopamine-sensitive adenylate cyclase was also reconstituted from digitonin-soluble components. These were resolved into two fractions by DEAE-cellulose chromatography: one fraction contained adenyl cyclase, but both fractions were required for reconstitution of dopamine-sensitive adenyl cyclase.

The detergent-soluble adenyl cyclases do not exhibit physiological responses to hormones (1–4). Attempts to obtain a hormone-sensitive adenyl cyclase from soluble components have failed although reconstitution of β-adrenergic-sensitive adenyl cyclase was achieved by the addition of soluble adenyl cyclase to receptor-replete membranes (5).

In this report, biochemically resolvable, detergent-soluble components are reconstituted into a particulate complex that exhibits dopamine-sensitive adenyl cyclase.

EXPERIMENTAL PROCEDURES

Materials

Bovine brains were shipped on dry ice from Pel-Freez. Crude soybean phospholipid, from Sigma or Associated Concentrates, was acetone-washed (6). Cholic acid was purchased from Schwarz/Mann and recrystallized (7). Digitonin was obtained from Fisher. GMP-PNP1 and [32P]ATP from ICN, [3H]bolic acid and [3H]cAMP from New England Nuclear. All inorganic salts were reagent grade and all other biochemicals were purchased from Sigma Chemical Co.

Homogenization and Solubilization

Bovine brains were thawed slowly at 4°C. Caudate nuclei were dissected out and homogenized as described (8), except that 3 ml of 20 mM EGTA, 2 mM Tris/maleate (pH 7.4) per g wet weight were used instead of 50 ml of 9 mM EGTA. 2 mM Tris/maleate. Homogenates were not purified further by subcellular fractionation because such fractionation does not greatly increase the adenyl cyclase specific activity (8).

For solubilization, the following additions were made per ml of homogenate (20 mg of protein/ml): 38 μl of 2 mM Tris/maleate (pH 7.5), 50 μl of 0.3 mM MgSO4, 380 μg of solid dopamine/HCl, 38 μl of 0.5 M cholate (pH 7.5), and 0.5 ml of a saturated solution of ammonium sulfate (pH 7.5, 4°C). After stirring for 20 min at 4°C, the mixture was centrifuged at 100,000 x g for 20 min. The supernatant (cholate extract) was carefully removed from the floating lipid layer and the pellet (0 to 33% fraction). The cholate fraction was brought to 39% saturation by the addition of saturated ammonium sulfate solution (4°C). After stirring for 20 min, the solution was centrifuged as before. The precipitate (33 to 39% fraction) was resuspended in 100 mM Tris/maleate, 2 mM EGTA (pH 7.4) at a protein concentration of 20 to 40 mg/ml and stored at −70°C. The ammonium sulfate concentration of the 39% supernatant was raised to 49% saturation. After stirring for 15 min, the solution was centrifuged as before; the resulting precipitate (39 to 49% fraction) was resuspended and stored as above.

Reconstitution

Step 1—The appropriate fraction was suspended at 0.1 to 1.0 mg of protein/ml in a solution (pH 7.5, 4°C) containing 7.5 mM crude soybean phospholipid, 14 mM sodium cholate, 75 mM Tris/maleate, 2 mM EGTA, 15 mM MgSO4, 1 mM ascorbic acid, 2 mM dopamine/HCl, 1 mM dihydrothreitol, and 35% saturated ammonium sulfate. This solution will be referred to as the “cholate/phospholipid buffer.”

Step 2—After stirring for 10 min, the ammonium sulfate concentration was raised to 62% saturation by addition of an equal volume of saturated ammonium sulfate solution. The precipitate obtained by centrifugation (100,000 x g, 15 min) was resuspended in 100 mM Tris/maleate, 2 mM EGTA (pH 7.2) at 20 to 40 mg of protein/ml. At this high protein concentration, the resuspended pellets were determined to contain [3H]cholate (0.8 to 1.2%), phospholipid (15 to 25 μmol of phosphate/ml), and ammonium sulfate (10 to 20% of saturation).

Step 3—A aliquots (25 to 100 μl) of this concentrated solution were placed in dialysis tubing and dialyzed against 100 to 200 ml of vigorously stirred 10 mM Tris/maleate, 0.2 mM EGTA (pH 7.2) for 3 to 6 h at 4°C. The material in the dialysis tubing became turbid during the dialysis. The [3H]cholate concentration was reduced to approximately 0.1%. Aliquots were assayed for adenyl cyclase activity (in the presence of sonicated phospholipid) and protein.

Assays

Adenyl cyclase activity was assayed at pH 7.2 in 0.5 ml of 100 mM Tris/maleate, 100 mM KCl, 10 mM MgSO4, 0.5 mM papavereine, 0.5 mM EGTA, 8.0 mM dithiothreitol, 2 mg/ml of bovine serum albumin, 0.1 mM GTP (or 0.01 mM GMP-PNP), 2 mM phosphoenolpyruvate, 5.6 units of pyruvate kinase, 16 mM sonicated crude soybean phospholipid, and 10,000 cpm of [3H]cAMP. For maximal activation, the protein (30 to 100 μg/assay) was incubated with the assay mixture for 5 to 10 min at 30°C, then the reaction was initiated by the addition of [32P]ATP (2 x 104 cpm) to a final concentration of 0.5 mM. The enzymatic reaction was linear with time for at least 20 min. The reaction was terminated by boiling after addition of 0.1 ml of 6% sodium dodecyl sulfate, 3 mM CAPS, and 2 mM ATP. The [32P]cAMP

1 The abbreviations used are: GMP-PNP, guanyl 5’-y- imidodiphosphate; EGTA, ethylene glycol bis[b-aminoethyl ether]N,N’-tetraacetic acid.

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was isolated by the method of Salomon et al. (9). All values reported are the averages of duplicate or triplicate assays. The radioactive assay product was identified as cyclic \[^{32P}\text{AMP}\] by thin layer chromatography on (a) cellulose plates with a solvent system of chloroform:methanol:concentrated NH\(_4\)OH (2:2:1) and on (b) polyethyleneimine cellulose plates with a solvent system of 30 mM sodium acetate (pH 5.4) (10, 11).

Protein was determined by the method of Bensadoun and Weinstein (12) with bovine serum albumin as a standard.

Phospholipid was quantitated by the determination of phosphorus (13).

**RESULTS AND DISCUSSION**

**Fractionation of Dopamine-sensitive Adenylate Cyclase**—As shown in Table I, the crude homogenate was stimulated 2-fold by dopamine, the dialyzed 39 to 49% fraction 4-fold. For six separate preparations, the average basal and dopamine-stimulated adenylate cyclase specific activities of the 39 to 49% fraction were 240 and 980 pmol of cAMP/min/mg, respectively. It was essential that the samples were dialyzed at 20 to 40 mg of protein/ml prior to assay. Dialysis at lower protein concentration (e.g. the cholate extract) yielded preparations with low activity. This requirement for high protein concentration and the turbid appearance of the dialyzed 39 to 49% fraction indicate the formation of a particulate complex during dialysis.

The observation that an active preparation (30 to 40% fraction) was obtained from the cholate extract (a solution rich in endogenous phospholipid) by ammonium sulfate precipitation and dialysis suggested that composition of the ammonium sulfate precipitate (20 to 40 mg of protein/ml; 15 to 20 \(\mu\)mol of phospholipid/\(\mu\)l, 0.8 to 1.2% cholate, 10 to 20% saturation of ammonium sulfate) enabled the components to reassemble during removal of the detergent and salt by dialysis. This observation led to the reconstitution procedure described under “Experimental Procedures.” When the 39 to 49% fraction was taken through the reconstitution procedure, there was no change in the activities from those shown in Table I. Therefore, the reconstitution procedure successfully imitated the conditions used in obtaining the active preparation from the cholate extract. The 39 to 49% fraction was not routinely taken through the reconstitution procedure; however, any manipulation of the 39 to 49% fraction before dialysis required that the reconstitution procedure be used to obtain an active preparation.

**Phospholipid Requirement**—As shown in Fig. 1, the activity of the dialyzed 39 to 49% fraction was stimulated by addition of sonicated phospholipid to the assay mixture. This phospholipid in the assay was required whether or not the 39 to 49% fraction was taken through the reconstitution procedure.

**TABLE I**

**Fractionation of caudate nucleus homogenate with ammonium sulfate in the presence of cholate**

The fractionation procedure and the assays (containing phospholipid) were done as described under “Experimental Procedures.” Before the assay, all fractions were dialyzed for 8 h against 100 volumes of 10 mM Tris/maleate, 0.2 mM EGTA (pH 7.2) to remove cholate and ammonium sulfate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>50 (\mu)M Do-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>paminide (pmol cAMP \times \text{ min}^{-1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 (\mu)M Do-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>paminide (pmol cAMP \times \text{ min}^{-1})</td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>120</td>
<td>230</td>
</tr>
<tr>
<td>Cholate extract</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>0-33% Fraction</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>33-39% Fraction</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>39-49% Fraction</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>49% Supernatant</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**FIG. 1.** Activation of the dopamine- and GMP-PNP-sensitive adenylate cyclase. Aliquots of the dialyzed material (40 \(\mu\)g of protein) were assayed as described under “Experimental Procedures” except that the quantity of phospholipid in the assay was varied as indicated. The concentration of dopamine and GMP-PNP were 50 \(\mu\)M and 20 \(\mu\)M, respectively.

Maximal activity of the dopamine- or GMP-PNP-activated adenylate cyclase required that the amount of phospholipid be 100-fold greater than the amount of protein in the assay mixture. The mechanism of the phospholipid activation is not clear. Precipitation of membrane proteins from a cholate solution by ammonium sulfate generally reduces the amount of phospholipid associated with the protein (6). In the present study, the particulate material formed during dialysis of the 39 to 49% fraction had a lipid to protein ratio less than 1:1. There are several examples of restoring biological activity to preparations that were depleted of phospholipid (14-16). In all of these cases, and in the present work, excess phospholipid was simply mixed with the lipid-poor preparation. Therefore, as described under “Experimental Procedures,” all assays routinely contained a 100-fold excess of sonicated crude soybean phospholipid.

**Specificity of Activation**—As shown in Fig. 2, the reconstituted dopamine-sensitive adenylate cyclase exhibited a specificity similar to that of caudate nucleus homogenate (17). Half-maximal stimulation of the reconstituted adenylate cyclase occurred at 8 \(\mu\)M dopamine or 100 \(\mu\)M norepinephrine, and there was no response to isoproterenol. In agreement with the current hypothesis that the hormonal stimulation of the adenylate cyclase is mediated through the guanine nucleotide site (18), the dopamine stimulation of the reconstituted adenylate cyclase required the presence of GTP in the assay. This stimulation of dopamine was competitively inhibited by micromolar concentrations of fluphenazine, cloropromazine, or haloperidol. It is concluded that the stimulation of the reconstituted adenylate cyclase by dopamine occurs through a receptor and coupling apparatus that are similar to those that function physiologically.

**Separation of Two Essential Components**—Active preparations were obtained after further fractionation of the 39 to 49% fraction, provided that the fractionated material was taken through the reconstitution procedure before the assay. In the first procedure, the resuspended 39 to 49% fraction was applied to a column of Sepharose 6B equilibrated with the cholate/phospholipid buffer. Fractions were collected during elution with cholate/phospholipid buffer. Since the fractions were eluted under conditions identical to Step 1 of the reconstitution procedure, the components in each fraction were reconstituted simply by adding ammonium sulfate to 62% saturation and dialyzing at high protein concentration (20 to 40 mg). They were assayed in the presence of sonicated soybean phospholipid (16 mM). As can be seen from Fig. 3,
Reconstitution of Dopamine-sensitive Adenylate Cyclase

Add to 3 volumes of 1.33 times concentrated solutions of chololate/phospholipid buffer. Ammonium sulfate was added to 62% saturation and the resulting precipitate was taken up in 100 mM Tris/maleate, 0.2 mM EGTA to 20 to 40 mg/ml. After dialysis, the dopamine-sensitive adenylate cyclase was measured in the presence of 16 mM phospholipid. The basal adenylate cyclase activity and the responses to dopamine and GMP-PNP of the material reconstituted from the digitonin extract indicated that all of the essential components were solubilized by digitonin (Table II, Line 1). Evidently, the precipitation and the dialysis steps removed the detergents (digitonin and cholate, respectively) sufficiently to allow reassembly of the functional complex of dopamine-sensitive adenylate cyclase.

It can be seen from Table II that the 1 mM KCl fraction contained adenylate cyclase activity that was poorly responsive to dopamine and GMP-PNP. The low salt DEAE-fraction contained no adenylate cyclase activity. However, when both fractions were mixed prior to dialysis and assayed in the presence of phospholipid, a good response to dopamine and GMP-PNP was seen. The ability of the low salt fraction to restore dopamine sensitivity was destroyed by heating at 37°C for 20 min. This heat treatment did not, however, alter the ability of the fraction to enhance the GMP-PNP sensitivity of the adenylate cyclase (Table II, Experiment 2). It should be emphasized that, in order to observe dopamine sensitivity, the 1 mM KCl fraction and the heat-labile factor in the low salt fraction had to be reconstituted by the procedure under "Experimental Procedures" and activated by phospholipid in the assay mixture.

These experiments show that the 39 to 49% fraction can be resolved into at least two components that can be reassembled into a particulate complex exhibiting the biological function of dopamine-sensitive adenylate cyclase. The reconstitution procedure described here should be useful in assaying and characterizing the macromolecular components essential for the function of the dopamine-sensitive adenylate cyclase.

![Graph](http://www.jbc.org/)

**FIG. 3.** Elution of the components of dopamine-sensitive adenylate cyclase from a Sepharose 6B column. A Sepharose 6B column (2.6 × 90 cm) was equilibrated with 500 ml of cholate/phospholipid buffer. A 2-ml aliquot of resuspended 39 to 49% fraction was applied to the column and eluted with cholate/phospholipid buffer at 11 ml/h. Fractions of 3.7 ml were collected and ammonium sulfate was added to 62% saturation. The precipitated material was recovered by centrifugation, resuspended at a protein concentration of 20 to 40 mg/ml, and dialyzed as described under "Experimental Procedures." The emergence of thyroglobulin (600,000) from this column and ferritin (410,000) are indicated by arrows. The fractions were assayed as described under "Experimental Procedures" for basal (●), dopamine-stimulated (△, ○) or GMP-PNP-stimulated (■, □) adenylate cyclase activity. Open symbols indicate the fold increase in adenylate cyclase induced by dopamine (△) or GMP-PNP (□).

There was a partial separation of the basal activity from the GMP-PNP- and dopamine-stimulated activity. Judging from the markers shown in Fig. 3, the complex is less than 500,000 daltons.

In an alternative procedure, the 39 to 49% fraction was diluted into a solution of 2 mg/ml of digitonin. The material that was not soluble in digitonin was removed by high speed centrifugation. The supernatant contained adenylate cyclase activity that did not respond to dopamine when assayed directly. This supernatant was applied to a DEAE-cellulose column equilibrated with digitonin. Approximately half of the protein was not adsorbed to the column and was eluted as the "low salt fraction." The adenylate cyclase activity was adsorbed to the column and was eluted with a solution of 1 mM KCl in digitonin.

For reconstitution, 1 volume of each of the fractions (digitonin extract, low salt fraction, and 1 mM KCl fraction) was added to 3 volumes of 1.33 times concentrated solutions of cholate/phospholipid buffer. Ammonium sulfate was added to 62% saturation and the resulting precipitate was taken up in 100 mM Tris/maleate, 0.2 mM EGTA to 20 to 40 mg/ml. After dialysis, the dopamine-sensitive adenylate cyclase was measured in the presence of 16 mM phospholipid. The basal adenylate cyclase activity and the responses to dopamine and GMP-PNP of the material reconstituted from the digitonin extract indicated that all of the essential components were solubilized by digitonin (Table II, Line 1). Evidently, the precipitation and the dialysis steps removed the detergents (digitonin and cholate, respectively) sufficiently to allow reassembly of the functional complex of dopamine-sensitive adenylate cyclase.

### Table II

Reconstitution of dopamine-sensitive adenylate cyclase after fractionation of the components on DEAE-cellulose

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Adenylate cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td></td>
<td>pmol cAMP × min⁻¹ × mg⁻¹</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>150</td>
</tr>
<tr>
<td>Digitonin extract</td>
<td>0</td>
</tr>
<tr>
<td>Low salt fraction</td>
<td>150</td>
</tr>
<tr>
<td>1 mM KCl fraction</td>
<td>200</td>
</tr>
<tr>
<td>Low salt + 1 mM KCl fractions</td>
<td>120</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>80</td>
</tr>
<tr>
<td>Low salt (37°C, 20 min) + 1 mM KCl fraction</td>
<td>80</td>
</tr>
</tbody>
</table>

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F M Hoffmann


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