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A NEW METHOD FOR REMOVING NONIONIC DETERGENT THAT ALLOWS RECONSTITUTION OF DOPAMINE-SENSITIVE ADENYLATE CYCLASE

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Summary: The macromolecular components of dopamine-sensitive adenylate cyclase are soluble in solutions of the nonionic detergents Brij 56 or digitonin, however response of the adenylate cyclase to dopamine is lost. Removal of the nonionic detergent by replacement with cholate and phospholipid, followed by removal of the cholate, restored the dopamine sensitivity of adenylate cyclase. By this method, the functional complex was reassembled from two separate solutions of components, one deficient in adenylate cyclase activity, and the other unresponsive to dopamine.

Recently a procedure was reported for the solubilization of bovine caudate nucleus adenylate cyclase in the ionic detergent cholate. Successful reconstitution of the dopamine-sensitive adenylate cyclase was dependent upon the removal of cholate (1). Reconstitution with resolved, purified components is useful for determining the minimal number of components needed for the biological function. The components of several other adenylate cyclases have been resolved and partially purified in the presence of nonionic detergents, but none of these preparations exhibited sensitivity to hormones (2-6). Successful reconstitution of hormone sensitivity requires 1) stability of all the critical macromolecules in the detergent, 2) thorough removal of the detergent, and 3) reassembly of the functional complex from dissociated components.

The data in the present report show that dilution of the nonionic detergent extracts into a solution of cholate and phospholipid, followed by ammonium sulfate precipitation of the protein, removes nonionic detergent and allows reconstitution of the dopamine-sensitive adenylate. This method of removing

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetic acid; GMPPNP, guanyl-5'yl imidodiphosphate; NEM, N-ethylmaleimide.

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nonionic detergent from membrane proteins with retention of biological function has proven useful on other membrane proteins, e.g. the calcium and sodium transporters of bovine heart mitochondria (W. Dubinsky, M.A. Kandrach, and E. Racker, manuscript in preparation), and the carbamylcholine dependent sodium flux of Torpedo californica (F.M. Hoffmann and R.L. Huganir, unpublished observations).

Methods: Dopamine-sensitive adenylate cyclase (39-49% fraction) was obtained by fractionation of a cholate extract of bovine caudate nucleus as previously described (1). Aliquots of this material were diluted into 20-40 volumes of 25% v/v glycerol, 20 mM Tris/maleate, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA and the indicated type and concentration of nonionic detergent. After 30 min, the mixture was centrifuged for a minimum of 45 min at 150,000 x g. The supernatant was removed and either assayed directly or treated to remove nonionic detergent prior to reconstitution of dopamine-sensitive adenylate cyclase.

Removal of nonionic detergent was attained by dilution of the supernatant into a cholate/phospholipid buffer to yield final concentrations of 0.1-1.0 mg/ml protein and less than 0.05 mg/ml nonionic detergent. The cholate/phospholipid buffer contained 7.5 mM crude soybean phospholipid, 14 mM sodium cholate, 75 mM Tris/maleate, 2 mM EGTA, 15 mM $MgSO_4$, 1 mM ascorbic acid, 2 mM dopamine-HCl, 1 mM dithiothreitol, and 25% saturated ammonium sulfate (pH 7.5, 4°). After stirring for 30 min, the ammonium sulfate concentration was raised to 62% of saturation by adding 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) to yield a solution of 20 to 40 mg of protein per ml. This material was added to the cholate/phospholipid buffer at 1 mg protein per ml, stirred, precipitated and centrifuged as above. After a third cycle of suspension in cholate/phospholipid buffer, precipitation, and centrifugation, the resuspended pellet (20 to 40 mg protein per ml) was dialyzed against 100 to 200 volumes of vigorously stirred 10 mM Tris/maleate, 0.2 mM EGTA (pH 7.2) for 3-6h at 4°. Aliquots were then assayed for adenylate cyclase activity and protein as previously described (1). Aliquots of the detergent solutions were assayed for adenylate cyclase in the absence of phospholipid whereas aliquots of the reconstituted adenylate cyclase were assayed in the presence of phospholipid as described (1).

Brij 56 and Triton X-100 were obtained from Sigma. Octylglucoside was purchased from Calbiochem and [^{3}H] Triton was a gift from Dr. R.D. O'Brien. All other materials and procedures were identical to those previously described (1)

Results: As shown in Table I, the adenylate cyclase is active in the presence of several nonionic detergents. Digitonin or Brij 56 yielded optimal solubilization. After high speed centrifugation greater than 80% of the activity remained in the supernatant. Triton X-100 and octylglucoside were less effective while in the absence of added detergent, only 25% of the activity remained in the supernatant.

Table I

Solubility of Adenylate Cyclase Activity

Detergent	Units of Adenylate Cyclase pmoles cAMP x min^{-1} Before Centrifugation	After Centrifugation Supernatant
1) None	701	173
2) Digitonin 10 mg/ml	1148	957
3) Brij 56 10 mg/ml	1349	1254
4) Triton X-100 10 mg/ml	1482	585
5) Octylglucoside 10 mg/ml	1190	505

Aliquots (100 µl) of the dopamine-sensitive adenylate cyclase were diluted into 2 ml of 25% v/v glycerol, 20 mM Tris/maleate, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA containing none or the indicated type of detergent. After 30 min, one half of each mixture was centrifuged for 45 min at 150,000 x g. Aliquots of the supernatants, and of the mixtures not centrifuged were assayed for adenylate cyclase in the presence of 20 µM GMPPNP.

Table II

Reconstitution of Dopamine-Sensitive Adenylate Cyclase From Nonionic Detergent Solubilized Components

Solubilization Conditions	Adenylate Cyclase Activity After Reconstitution pmoles cAMP x min^{-1} x mg^{-1} Basal	Dopamine
Digitonin 2 mg/ml	309	1136
Brij 56 2 mg/ml	177	230
Brij 56 2 mg/ml + 2 mM Dopamine	260	730
Triton X-100 2 mg/ml	140	150
Triton X-100 2 mg/ml + 2 mM Dopamine	140	270

Aliquots of dopamine-sensitive adenylate cyclase (75 µl) were added to 3 ml of 20 mM Tris/maleate, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA, 25% v/v glycerol and the compounds indicated in the Table. After 20 min, the mixtures were centrifuged for 60 min at 200,000 x g. The supernatants were removed and used in the reconstitution of adenylate cyclase as described under "Methods."

Digitonin and Brij 56 have also been used successfully for solubilization, resolution and partial purification of adenylate cyclase from other tissue sources (2,6). As observed in those reports and the present work, the solubilized

adenylate cyclase is not responsive to hormones. However the dopamine-sensitive adenylate cyclase was reconstituted from material obtained by DEAE cellulose chromatography in the presence of digitonin (1). During reconstitution with digitonin, Brij 56 or Triton X-100-solubilized material, two new characteristics were observed. First, stability of the components differed in the various detergents. Dopamine was required during solubilization with Brij 56 or Triton X-100 but not during solubilization with digitonin (Table II). Second, as shown in Table III, the reconstitution procedure removed nonionic detergent from the protein. Three cycles of both resuspension in cholate/phospholipid buffer and subsequent ammonium sulfate precipitation were required to obtain a dopamine-sensitive adenylate cyclase from components solubilized in Brij 56 or Triton X-100, whereas only one cycle had been necessary with digitonin solubilized components (1).

Removal of nonionic detergents by ion exchange chromatography has been used to partially restore the hormone response of adenylate cyclase (10, 11). The advantage of replacing the nonionic detergent with cholate and phospholipid is that the protein is maintained in a dispersed state rather than forming an aggregate. The cholate can then be removed under conditions that permit optimal reassembly and reconstitution of the protein. For two multicomponent complexes, dopamine-sensitive adenylate cyclase or mitochondrial ATPase, optimal reconstitution was achieved when cholate was removed gradually by dialysis rather than rapidly by chromatography (12,13).

As discussed above, exposure to Brij 56 in the absence of dopamine resulted in a reconstituted adenylate cyclase with poor response to dopamine. Exposure to N-ethylmaleimide destroyed the catalytic activity of adenylate cyclase in this system and in others (8, 9). Mixing these two partially inactivated preparations resulted in restoration of the dopamine sensitivity after reconstitution (Table IV). The fact that the active components remaining in each preparation reassemble with each other to form a functional complex suggests that, under these conditions, the components are freely exchangeable. The procedure may therefore permit the reconstitution of dopamine-sensitive adenylate cyclase from resolved, purified components.

Table III

Removal of [^{3}H]-Triton and Reconstitution of Dopamine-Sensitive Adenylate Cyclase by Precipitation from Cholate/Phospholipid Buffer

Sample	^{3}H-Triton mg/ml	^{3}H-Triton total mg	Adenylate Cyclase Activity after Reconstitution pmoles cAMP x min^{-1} x mg^{-1}	
			Basal	+50 µM Dopamine
High speed supernatant of Triton solubilized components	2	4	not assayed	not assayed
1st Ammonium Sulfate pellet	1.5	0.15	65	65
3rd Ammonium Sulfate pellet	0.03 - 0.07	0.003 - 0.007	120	450

A 50 µl aliquot of the 39-49% fraction was diluted into 2 ml of 25% glycerol, 20 mM Tris/maleate, 2 mM dopamine, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EDTA and 2 mg/ml of ^{3}H-Triton (288,000 cpm). After stirring for 20 min at 4°, the solution was centrifuged for 50 min at 200,000 x g. The supernatant was diluted into 6 ml of cholate/phospholipid buffer. After 20 min, 8 ml of saturated ammonium sulfate solution was added, the solution was stirred for 10 min, and then centrifuged for 10 min at 150,000 x g. The pellet was designated the 1st Ammonium Sulfate pellet and an aliquot of it was counted by liquid scintillation to quantitate the Triton. Another aliquot was dialyzed and assayed, and the remainder was diluted into 2 ml of cholate/phospholipid buffer. To the latter solution, 2 ml of saturated ammonium sulfate were added, and the pellet obtained from centrifugation of this mixture was resuspended in 2 ml of cholate-phospholipid buffer. Addition of 2 ml of saturated ammonium sulfate solution and centrifugation as above produced the 3rd Ammonium Sulfate Pellet. An aliquot was dialyzed for 3 hrs and assayed, and another aliquot was counted by liquid scintillation to quantitate Triton.

Table IV

Restoration of Dopamine Sensitivity by N-Ethylmaleimide-Treated Adenylate Cyclase

Treatment	Specific Activity of Reconstituted Dopamine-sensitive Adenylate Cyclase pmoles cAMP x min^{-1} x mg^{-1}		Fold Stimulation
	Basal	+50 μM Dopamine	
A) 2 mg/ml Brij 56 (minus dopamine)	120	160	1.3
B) 2 mg/ml Digitonin - 5 mM NEM	5	15	3.1
A+B)	140	370	2.6
C) Control: 2 mg/ml Brij 56 (plus dopamine)	150	500	3.3

A 100 μl aliquot of dopamine-sensitive adenylate cyclase was diluted into 4 ml of 25% glycerol, 20 mM Tris/maleate, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA and 2 mg/ml Brij 56 or 2 mg/ml digitonin. After stirring for 30 min, the solutions were centrifuged for 50 min at 200,000 xg. The supernatant of the digitonin solublilized components was treated with 5 mM NEM for 20 min at 4°. This reaction was stopped by the addition of DTT to 8 mM. The solubilized components were added to cholate/phospholipid buffer and precipitated by the addition of saturated ammonium sulfate solution. The Brij-solubilized components were put through three cycles of suspension in cholate/phospholipid buffer and precipitation. The mixing of A with B was done immediately before the last precipitation from cholate/phospholipid buffer. The resuspended pellets were dialyzed and assayed.

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