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Localization of Adenylate Cyclase in Adrenal Medulla

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SUMMARY

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The localization of adenylate cyclase in bovine adrenal medulla was explored using a continuous sucrose density gradient to separate the various subcellular components of this tissue. The results indicate that adenylate cyclase is not a component of chromaffin vesicle membranes as reported earlier by Zinder *et al.* [(1976) *J. Biol. Chem.*, 251, 2179-2181]. Adenylate cyclase activity observed in chromaffin vesicle membrane fractions is most likely due to contamination by plasma membranes.

Recent communications have reported the presence of an adenylate cyclase activity in chromaffin vesicle membranes of the adrenal medulla (1-3) and have suggested a function for adenylate cyclase in the secretory process. Since earlier work in our laboratory had indicated that adenylate cyclase was a plasma membrane constituent (4), we undertook an analysis of the localization of adenylate cyclase by continuous sucrose density gradient centrifugation.

Adrenal glands were obtained from freshly slaughtered cattle and transported to the laboratory on ice, and the medullae were separated from the cortices. Ten grams of tissue were forced through an Arbor tissue press and homogenized in a final volume of 100 ml of buffer consisting of 0.3 M sucrose, 10 mM Tris-HCl (pH 7.4, at 4°), and 1 mM dithiothreitol, with a Kontes Duall tissue grinder. The homogenate was centrifuged at 800 × *g* for 10 min, and the resulting pellet was resuspended and washed once in the homogenization buffer. The two 800 × *g* supernatant fluids were combined and centrifuged at 26,000 × *g* for 20 min. The pellet obtained was resuspended in 0.5 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, and 300 units/ml of catalase.

Five milliliters of the resuspended 26,000 × *g* pellet, corresponding to 2 g of original tissue weight, were applied to each of three linear sucrose gradients formed from equal volumes of 2.25 M and 1.0 M sucrose, containing 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, and 300 units/ml of catalase. The gradients were centrifuged for 5 hr at 90,000 × *g* in a Beckman SW 27 rotor (26,000 rpm) at 3°. Fractions were collected from the bottom of the gradient, and individual fractions from the three gradients were pooled. The fractions were diluted and washed four times by centrifugation in 10 mM Tris-HCl (pH 7.4) and 1 mM dithiothreitol to lyse vesicles and remove soluble components. The first two washes were pooled and retained for catecholamine and soluble dopamine β-hydroxylase analysis.

Enzyme activities were assayed by the

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following methods: acetylcholinesterase (EC 3.1.1.7), Ellman *et al.* (5); dopamine β-hydroxylase (EC 1.14.17.1), Nagatsu and Udenfriend (6); monoamine oxidase (EC 1.4.3.4), Wurtman and Axelrod (7); and adenylate cyclase (EC 4.6.1.1), Salomon *et al.* (8). The last assay was modified to include 25 mM Tris-HCl (pH 7.5, at 37°), 10 mM $MgCl_2$, 10 mM creatine phosphate, 50 units/ml of creatine phosphokinase, 1 mM cyclic 3',5'-AMP, 1 mM [α-^{32}P]ATP, 1 mg/ml of bovine serum albumin, 1 mM dithiothreitol, and membranes in a final volume of 0.1 ml. The reaction was allowed to proceed for 10 min at 37°. All enzymatic assays were carried out under conditions in which the reactions were linear with respect to time and protein concentration.

Protein was determined by the method of Lowry *et al.* (9). Membranes were first solubilized in 1 N NaOH. Catecholamines (epinephrine and norepinephrine) were determined by the trihydroxyindole method of von Euler and Lishajko (10). Sucrose concentration was determined by refractive index.

To determine whether adenylate cyclase is a chromaffin vesicle membrane component, a crude granule fraction from adrenal medulla was subjected to density gradient centrifugation. Figure 1 shows the distribution of markers for various subcellular components on this gradient. Intact chromaffin vesicles sedimented into the

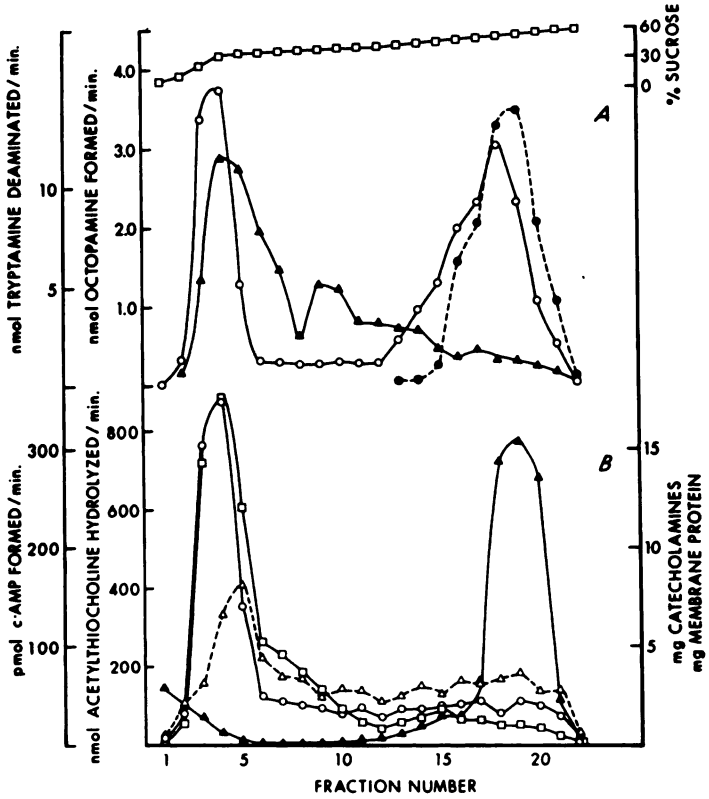

FIG. 1. *Distribution of marker enzymes in a typical density gradient centrifugation of crude chromaffin vesicle fraction*

All enzyme assays were linear with respect to time and amount of protein used. Recoveries were 69% for membrane-bound dopamine β-hydroxylase, 79% for acetylcholinesterase, 96% for monoamine oxidase, 75% for adenylate cyclase, 72% for membrane-bound protein, and 98% for catecholamines. A. Distribution of monoamine oxidase activity (▲——▲) and dopamine β-hydroxylase activity, both membrane-bound (○——○) and soluble (●---●). The sucrose concentration (□——□) in each fraction is given at the top of the panel. B. Distribution of acetylcholinesterase activity (○——○), adenylate cyclase activity (□——□), membrane protein (△---△), and total catecholamines (▲——▲). c-AMP, cyclic 3',5'-AMP.

more dense part of the gradient, as indicated by the distribution of catecholamines (epinephrine and norepinephrine) and dopamine β-hydroxylase, both soluble and membrane-bound. The peak of membrane-bound dopamine β-hydroxylase near the top of the gradient represents chromaffin vesicles that were lysed in the preparation procedure (the amount varied from preparation to preparation). Monoamine oxidase activity, a marker for other mitochondrial membranes and intact mitochondria, showed a biomodal distribution, as previously reported (11). Both peaks of activity moved farther into the gradient than the lysed chromaffin vesicle membranes, and the activity trailed off through the more dense fractions of the gradient. Acetylcholinesterase activity, a plasma membrane marker (4, 12), fractionated in a sharp peak near the top of the gradient in parallel with dopamine β-hydroxylase, but a constant amount of activity trailed through the gradient.

Adenylate cyclase fractionated in a pattern similar to that of acetylcholinesterase. The bulk of the activity was present in fractions near the top of the gradient, which contained membranes from a variety of subcellular structures. Both adenylate cyclase and acetylcholinesterase were also present in much smaller amounts and in a similar distribution throughout the more dense portions of the gradient. However, no peaks of adenylate cyclase activity paralleling membrane-bound dopamine β-hydroxylase in the fractions containing chromaffin vesicles (fractions 13–22) were observed. If adenylate cyclase were a component of the chromaffin vesicle membrane, one would expect its activity to parallel that of dopamine β-hydroxylase, a known component of chromaffin vesicle membranes. The average specific activity of adenylate cyclase in fractions 13–22 was 7.2 pmoles/min/mg of membrane protein—large enough to account for the activity seen in purified chromaffin vesicle membranes by other investigators (1, 2). In two identical experiments the same patterns of marker enzymes were observed, and specific activities of adenylate cyclase were 2.7 and 9.0 pmoles/min/mg of membrane protein in the chromaffin vesicle region of the gradient.

Adenylate cyclase activity was also measured in the presence of sodium fluoride and 5′-guanylylimidodiphosphate, as well as with substitution of manganese for magnesium in the assay. All these agents stimulated the activity but did not affect the distribution of adenylate cyclase in the gradient. To test the possibility that adenylate cyclase activity in the chromaffin vesicle fractions was not measured under conditions of linearity, membrane fractions from the upper, middle, and lower portions of the gradient were re-evaluated for linearity with respect to time and protein concentration. These studies confirmed that the data in Fig. 1 were obtained under appropriate conditions. We concluded from these studies that adenylate cyclase is not a component of the chromaffin vesicle membrane, and that activity found in these fractions is most likely due to contamination by plasma membranes.

In previous reports (1, 2), chromaffin vesicles were isolated by means of a discontinuous sucrose density gradient and were collected as a pellet from that gradient. This gradient was followed by lysis of the vesicles and further fractionation of the membranes on discontinuous sucrose density gradients. We have also employed this procedure in attempts to separate chromaffin vesicle membranes from plasma membranes, using acetylcholinesterase as a marker for the plasma membrane (4, 12), but have not been able to achieve the extent of purification reported by Zinder *et al.* (1), who used other membrane markers. We and others (4, 12) have found that losses of $(Na^+ + K^+)$-ATPase activity occurred during membrane fractionation and that this enzyme activity is a poor marker for plasma membranes of the adrenal medulla. We have also found (4) that 5′-nucleotidase appears not to be a reliable plasma membrane marker. In all our preparations the adenylate cyclase activity closely paralleled the activity of acetylcholinesterase, and in no instance was there any indication that the adenylate cyclase was associated with membranes

that did not also contain acetylcholinesterase.

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