DIRECT EVIDENCE FOR CO-LOCALIZATION OF ADENYLATE CYCLASE, DOPAMINE-β-HYDROXYLASE AND CYTOCHROME b_{562} TO BOVINE CHROMAFFIN GRANULE MEMBRANES

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ABSTRACT: Adenylate cyclase, dopamine-β-hydroxylase and cytochrome b_{562} have been found to co-equilibrate on equilibrium sucrose gradients of lysed chromaffin granule membranes from bovine adrenal medulla. Peak activities for these enzymes, as well as maximum membrane protein concentration, were found to coincide at $d = 1.11$ gm cm^{-3}, and the ratios of adenylate cyclase to both dopamine-β-hydroxylase and cytochrome b_{562} were constant across the entire peak. Adenylate cyclase activity has been reported previously to co-purify with chromaffin granule membranes, and we conclude, on the basis of these new data, that adenylate cyclase is also an intrinsic granule membrane enzyme.

INTRODUCTION: Adenylate cyclase activity has been reported in preparations of isolated bovine chromaffin granules and purified granule membranes (1-3). Catecholamines suppress granule adenylate cyclase, and cyclase activity in intact granules can be activated only under conditions that induce catecholamine release, such as exposure to Mg^{2+}-ATP and chloride (1), or treatment with NH_4Cl (1,2). Granule membranes can be prepared by hypotonic lysis of granules, but also must be washed with NH_4Cl in order to detect full cyclase activity (3).

Wilson and Krishner (4) have recently reported their inability to detect adenylate cyclase activity in association with chromaffin granules or granule membranes. This was in apparent contrast to our own findings (1-3) and to recent results of others who were also able to detect adenylate cyclase activity in association with pituitary neurosecretory vesicle membranes (5). To resolve this issue unambiguously, we subjected lysed granule membranes to equilibrium

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centrifugation on sucrose gradients and analyzed fractions for conventional granule membrane marker enzymes such as dopamine-β-hydroxylase (DBH) and cytochrome b_{562} as well as adenylate cyclase. In this paper we report that adenylate cyclase activity co-equilibrates with both DBH and cytochrome b_{562} across the entire gradient, and that all three enzymes peak in the same fraction at d = 1.11 gm cm^{-3}. We conclude that adenylate cyclase is indeed a granule membrane enzyme as are DBH and cytochrome b_{562}.

MATERIALS AND METHODS:

Chromaffin Granules. Bovine adrenal glands were obtained at the slaughter house within 20 minutes of killing and transported to the laboratory on ice within two hours. The medullary tissue was dissected free of cortical contamination and highly purified, homogeneous chromaffin granules were prepared in osmotically intact form by differential centrifugation in 0.3 M sucrose as previously described (6,7).

Chromaffin Granule Membranes. Granules were lysed by exposure to 20 volumes of 0.1 M NH_4Cl containing 5 mM tris-HCl buffer, pH 7.4 and centrifuged at 100,000 xg for 30 minutes (2). The membrane pellet was resuspended in 0.3 M sucrose (30 mg protein in 5 ml) and layered on a continuous sucrose gradient (range: d = 1.04 - 1.17 gm cm^{-3}) having a volume of 30 ml. The gradient was then placed in an SW 27 rotor and centrifuged at 100,000 xg for two hours. One ml fractions were then collected by needle puncture from below and subjected to enzyme and protein analysis.

Chemical and Enzymatic Analysis. Protein was determined by the Bradford Coomassie Blue assay (8) using crystalline bovine serum albumin as the standard. Protein determined by this method is identical to that obtained using the Lowry method. Dopamine-β-hydroxylase (DBH; EC1.14.17.1) was assayed by the spectrophotometric method of Nagatsu and Udenfriend (9). Adenylate cyclase (EC 4.6.1) was assayed by the method of Salomon and Rodbell (10). Cytochrome b_{562} was determined from a reduced-minus-oxidized difference spectrum taken with the Aminco DW2 dual beam spectrophotometer using the absorbance difference between 416 and 429 nm. A reduced-minus-oxidized scan revealed that cytochrome b_{562} was the only detectable cytochrome in the granule membrane preparation, and a reduced, carbon monoxide minus reduced spectrum verified the absence of either hemoglobin or cytochrome P450. The density of various fractions was determined from the index of refraction using an Abbe refractometer.

RESULTS: Lysed chromaffin granule membranes were found to distribute on an equilibrium sucrose gradient with a protein concentration maximum at d = 1.11 gm cm^{-3}, and with coincident maxima for granule membrane marker enzymes such as DBH and cytochrome b_{562} (Figure 1). Granule membrane fractions were also assayed for adenylate cyclase, and the activity was found to distribute in nearly identical fashion to the other marker enzymes (Figure 1). Basal cyclase activity was found to be activated approximately 2-fold by 10^{-4} M GMP-PNP, as reported previously (2,3) and cyclase activity in the gradient fractions were accordingly measured in the presence of this GTP analogue.

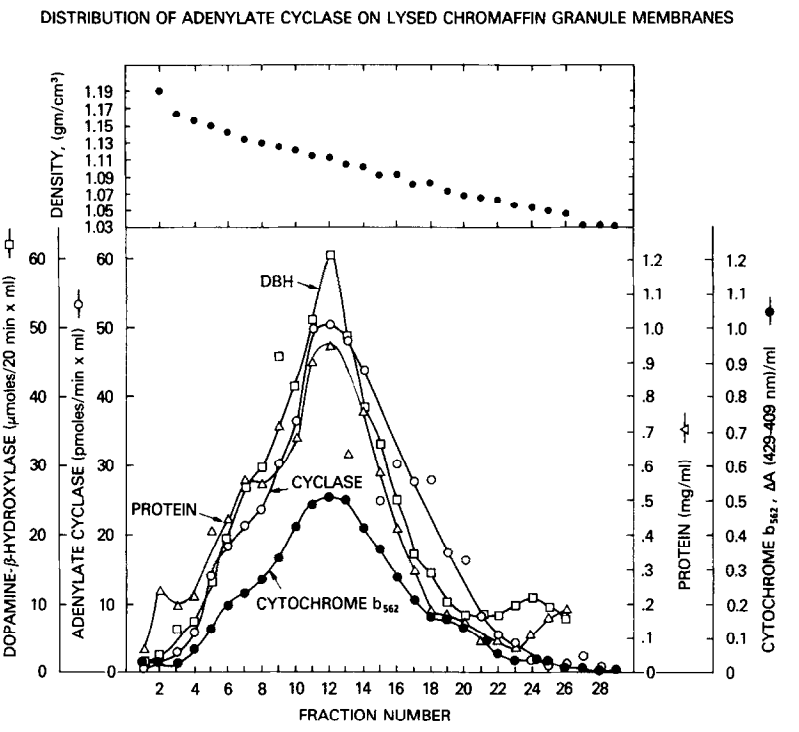

Figure 1. Distribution of adenylate cyclase, dopamine-β-hydroxylase (DBH) and cytochrome b_{562} in equilibrium sucrose gradient of lysed chromaffin granule membranes. Units are as described on the vertical axes. Fractions are one ml each. Analytic details are described in Methods.

The coincidence of activity peaks for DBH, cytochrome b_{562} and adenylate cyclase suggested that the cyclase was a specific granule membrane enzyme. However, the most rigorous test for co-equilibration of granule markers would be observation of a constant ratio of enzyme activities across the activity peak. As shown in Figure 2, the ratios of adenylate cyclase to either cytochrome b_{562} or DBH were constant over the entire gradient.

DISCUSSION: These results clearly demonstrate that adenylate cyclase is an intrinsic chromaffin granule membrane enzyme as are DBH and cytochrome b_{562}. This is an unambiguous example of the localization of adenylate cyclase to a subcellular organelle not directly associated with plasma membranes. This relationship may prove to have some generality in light of recent reports of

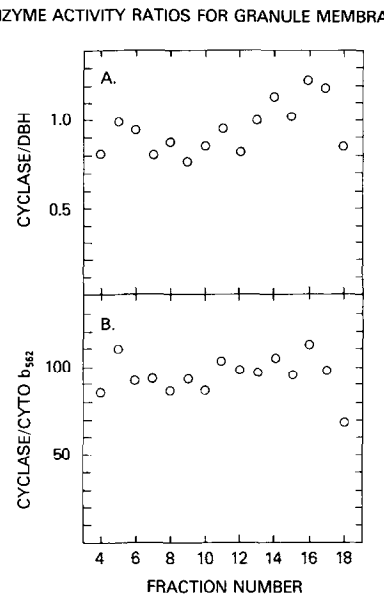

Figure 2. Ratio of adenylate cyclase to dopamine-β-hydroxylase (DBH) and cytochrome b_{562} in the equilibrium sucrose gradient of lysed chromaffin granule membranes. The data are taken directly from enzyme activity values shown in Figure 1.

cyclase being associated with neurosecretory granule membrane preparations from pituitary gland (5). We offer no explanation for Wilson and Kirshner's (4) inability to detect adenylate cyclase activity in chromaffin granules or granule membranes.

The granule cyclase activity represents only 10-15% of the total cyclase activity of the adrenal medulla. Much of the remainder is associated with a fraction enriched in plasma membranes. In separate studies on this latter fraction we have found that adenylate cyclase equilibrated at d = 1.14 gm cm^{-3} in association with 5-nucleotidase and other plasma membrane markers (11). Isolated chromaffin granules also have other plasma membrane-like properties, such as a high cholesterol content (11,12), as well as sialic acid (11) and wheat germ agglutinin receptors (13) on their external surface. These plasma-membrane-like properties of chromaffin granules may be related to the close

structural relationship that granules and plasma membranes eventually enjoy during exocytosis.

The physiologic role of the granule cyclase was not addressed directly in this paper. However, it is known that rats exposed to carbachol, a cholinergic agonist, secrete catecholamines from the adrenal medulla and exhibit a 20-fold rise in medullary cyclic AMP (14). Plasma membrane cyclase from adrenal medulla is insensitive to carbachol or acetylcholine (11,14), as is the granule cyclase (3,14). Isolated chromaffin granules also synthesize cAMP with their intrinsic adenylate cyclase during the in vitro release reaction, and it is possible that the tissue response is based on this granule event (1,3).

As an incidental observation to these studies on adenylate cyclase in purified granule membranes, we were also able to calculate that a chromaffin granule contained a minimum of 24 DBH molecules. The relevant physical data, summarized in Reference 15, included the hydrated granule density (1.12 gm cm^{-3}); average granule radius (100 nm); % of granule dry weight as protein (35%); % of wet weight as water (85%). One granule thus weighed 4.69×10^{-15} gm and 1 mg granule protein represented 4.08×10^{12} granules. Assuming that 20% of the granule protein is membrane protein, then each mg of membrane protein represents 20.4×10^{12} granules. The specific activity for DBH in this laboratory is 24-31 μmoles/mg protein x min at 37°C (16), and is similar to values recently reported by others (17,18,19). Assuming a minimum specific activity of 25 and a measured DBH/protein ratio for purified membranes of 3 μmoles/mg protein x min, the membrane is at most, 12.5% DBH and each granule would contain 12 DBH molecules/granule membrane. Since ∿50% of the granule DBH activity is lost upon hypotonic shock, each granule might contain as many as 24 total DBH molecules. The presence of a relatively few DBH molecules per granule is in agreement with the finding of Klein, et al. (19) who calculated that the smaller large dense core vesicles (LDV) from splenic nerve (radius = 37.5 nm) contained between one and nine DBH molecules/vesicle.

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