Isolation of a Protein from the Plasma Membrane of Adrenal Medulla which Binds to Secretory Vesicles*  

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Solubilized proteins of the plasma membrane of bovine adrenal medulla were fractionated on the basis of their affinity for secretory vesicles. The isolation procedure included preparation of a highly purified fraction of plasma membranes, its solubilization in detergent, and application to a column prepared from glutaraldehyde-fixed chromaffin granules. Using this technique, one major polypeptide (80% of the material bound) was isolated. 

This protein has been shown to originate from the plasma membrane and has no affinity for fixed bovine adrenal medullary mitochondria or lysosomes. It is eluted most effectively by low pH (3.0) and can be rebound and re-eluted from fixed secretory granules. In sodium dodecyl sulfate and β-mercaptoethanol it has an apparent molecular weight of 51,000. In addition, two minor components, comprising about 20% of the material bound were detected having apparent molecular weights in sodium dodecyl sulfate of 14,000 and 62,000.

It is suggested that such a molecule could function as a plasma membrane-located receptor for chromaffin granules during the secretory process.

It is generally accepted that secretion of catecholamines from the adrenal medulla occurs via the fusion of chromaffin granules with the plasma membrane (exocytosis). This phenomenon has been studied extensively in a variety of secretory cells at the morphological level by electron microscopy of thin sections (1, 2), and also by freeze fracture technique (1, 4). What is evident is that the membrane of the secretory granule becomes closely apposed to the plasma membrane and the two membranes break down and fuse with one another. As yet there are few biochemical correlates that serve to explain this apposition and fusion at the molecular level. What molecules take part in exocytosis, the exact role of calcium or other regulatory agents, and the fate of fused membrane are still unresolved issues.

There appears to be a certain amount of specificity involved in the fusion of secretory granules, i.e. they fuse principally with the plasma membrane. On the basis of this, it is valid to assume the presence of molecules located in chromaffin granules and plasma membranes that mediate this specificity. What we hypothesize, and present data to support, is the existence of a protein molecule present in the cell membrane that possesses an affinity for chromaffin granules.

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1 The abbreviations used are: SME buffer, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 0.9 mM CaCl₂, 0.5 mM MgCl₂, WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate.
Solubilization of Plasma Membranes—Plasma membranes were washed once in SME buffer and once in NaCl/PO. The membrane pellet was then resuspended in 1.0% Triton X-100 in NaCl/PO (2 to 4 mg of protein/ml) by 10 strokes of a Dounce homogenizer with tip-fitting pestle. The partially solubilized membranes were then fully solubilized in the presence of 5- to 10-mg sonication in a bath-type sonicator (Transsonic T400, Hans Schmidbauer, Singen, Federal Republic of Germany). Insoluble material (less than 5% of the total) was removed by centrifugation at 100,000 g for 1 h.

Radiolabeling of Plasma Membranes—The solubilized membranes were iodinated with 125I by the lactoperoxidase-catalyzed reaction. For each milliliter of membrane solution, 100 to 250 μCi of 125I (500 μCi/μl) and 10 μl (25% units) of lactoperoxidase (Calbiochem, La Jolla, Cal.) were used. The reaction was initiated by the addition of 20 μl of membrane solution of 0.003% H2O2, which was added in the same quantity again at 2 and 5 min. The iodination was carried out at 25°C for 10 min. The reaction was terminated by the addition of 100 μl of 0.5 M KI. The solution was dialyzed three times against 100 to 200 volumes of 1% Triton X-100 in NaCl/PO for 6 h. The specific radioactivity of such a preparation ranged from 5.6 to 7.4 × 10^6 cpm/mg of protein.

Preparation of the Affinity Column—Columns for affinity chromatography were routinely made from 10-ml disposable plastic syringes (1.5 × 6 cm). The first 1 ml of the column was filled with Bio-Gel P-30, 50 to 100 mesh (polyacrylamide beads, Bio-Rad Laboratories, Richmond, Cal.) which served as a support for the affinity adsorbent mixture. A 50% (v/v) slurry of fixed chromaffin granules in 1% Triton X-100 was mixed in a 1:1 ratio with a 50% (v/v) slurry of Bio-Gel P-30, 400 mesh, in the same buffer and poured onto the 100 mesh support. The fixed granule-bead mixture was overlaid with another 1 ml (packed volume) of 50 to 100 mesh beads. The column was extensively washed in 1% Triton X-100 in NaCl/PO, then with the elution medium, and re-equilibrated with 1% Triton X-100 in NaCl/PO.

Preparation of 125I-Labeled WGA—WGA was prepared by the method of Blob and Burger (5). Lactoperoxidase labeling was carried out as described above for plasma membrane proteins, and the iodinated lectin purified by affinity chromatography on chitin.

Electron Microscopy—Samples were fixed with an isotonic solution of glutaraldehyde (2%) in sodium cacodylate buffer, pH 7.0, for 2 to 3 h at 4°C, pelleted in an Eppendorf microfuge, postfixed with 1% osmium tetroxide, and embedded in Epon. Sections were stained with 2% uranyl acetate.

Analytical Methods—The activities of the following marker enzymes were measured according to the methods referenced. Acetylcholinesterase, EC 3.1.1.7 (6); (Na’-K’)-ATPase, EC 3.6.1.3 (7); 5’-nucleotidase, EC 3.1.3.5 (8); cytochrome c oxidase, EC 1.9.3.1. (9); monoamine oxidase, EC 1.4.3.4 (10); dopamine-β-hydroxylase, EC 1.14.17.1 (11); glucose-6-phosphatase, EC 3.1.3.12 (12); and acid phosphatase, EC 3.1.3.2-15 were measured fluorometrically according to the method of Westerink and Korf (14), and inorganic phosphate by the method of Ames (15).

Protein measurements were carried out according to the method of Lowry et al. (16), using bovine serum albumin as a standard. When catecholamines were present, samples and standards were first precipitated in 10% trichloroacetic acid. Protein assays were performed in the presence of 2.5% (w/v) SDS when samples contained Triton X-100.

Polyacrylamide gel electrophoresis was performed according to the method described by Laemmli (17).

RESULTS

Isolation and Fixation of Chromaffin Granules—In order to prepare chromaffin granules for affinity chromatography which were as pure and as free from damage as possible, step gradients of Percoll were used in the isolation procedure. Such a method also increased the yield when compared to conventional techniques (Table I). It did, however, eliminate the necessity of the granules being exposed to extreme hypertonic conditions during isolation, and the relative hypothonic state brought about during their resuspension for further use. The data in Table 1 also show the granules isolated in this way to be significantly more stable (as determined by lysis resistance) than those isolated through barriers of sucrose. Furthermore, a good deal of time was saved in preparing since ultracentrifugation was eliminated altogether. Electron microscopy (Fig. 1) demonstrates the purity of Percoll-isolated granule fractions which are composed of adrenal-containing (less dense) and nonadrenal-containing (dense core) vesicles. Morphometric measurements of granules isolated in this way revealed one mitochondrion per 90 to 100 chromaffin granules.

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Protein</th>
<th>Catecholamines</th>
<th>Contamination</th>
<th>Mitochondria</th>
<th>Lysosomes</th>
<th>Stability</th>
<th>Protein (μg/mg granule protein)</th>
<th>Catecholamines (μg/mg granule protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.7 ± 0.2</td>
<td>14.7 ± 3.9</td>
<td>32.6 ± 9.0</td>
<td>2.8 ± 0.3</td>
<td>3.8 ± 0.5</td>
<td>2.1 ± 0.4</td>
<td>22.3 ± 5.0</td>
<td>231 ± 36</td>
</tr>
</tbody>
</table>

* Values are expressed as a percentage of the total protein or catecholamines found in the whole homogenate.

‡ Values represent the percentage of total activity of cytochrome oxidase (mitochondria) and acid phosphatase (lysosomes) found in the whole homogenate.

Table I

Comparison of chromaffin granule fractions

Chromaffin granules were isolated from crude granule fractions either through barriers of 1.6 M sucrose (18) or on discontinuous gradients of Percoll as described under “Experimental Procedures.” All values represent the average of three separate experiments ± S.D.

† Percoll has been used previously to isolate chromaffin granules using a continuous gradient system and ultracentrifugation (M. Gratzl, personal communication).

Plasma Membrane Isolation—The procedure employed for membrane purification (Fig. 2) provided a fraction rich in plasma membranes. An analysis of the activities of marker enzymes (Table II) showed that the highest degree of enrichment of plasma membrane-associated enzyme activity could be obtained in the 32 to 36% sucrose interface. Although all of the fractions obtained in the sucrose step gradients contained both mitochondrial and plasma membrane markers, the 32 to 36% interface yielded the most highly purified plasma membrane population (based on acetylcholinesterase, 5’-nucleotidase, and (Na’-K’)-ATPase activity) and the relatively lowest mitochondrial contamination (based on the activities of monoamine oxidase and cytochrome c oxidase). Most relevant for the next step, in which solubilized membrane fractions were passaged over fixed chromaffin granules, was the relatively low level of contamination by chromaffin granule membranes (based on dopamine-β-hydroxylase activity).

Electron microscopy (Fig. 3) of the 32 to 36% interface showed a population of smooth, relatively empty vesicles of varying sizes. The material in this field is free of mitochondrial contamination. Some fuzzy material can be observed attached to the membranes, some of which is probably due to typical cell surface-associated substances. In some cases, the membranes appear to be sectioned obliquely, not normal to the plane of the membrane.

Affinity Chromatography using Fixed Chromaffin Granules—In order to determine the effectiveness of the experi-
Fig. 1. Electron micrograph of unwashed chromaffin granules derived from the 40 to 60% interface of a discontinuous gradient of Percoll (see "Experimental Procedures" for details). Magnification: a, × 10,000; b, × 45,000. (The small dense grains are Percoll particles.)

Fig. 2. Scheme of plasma membrane isolation. Details are given under "Experimental Procedures."

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TABLE II

Specific activities of marker enzymes from sucrose gradients during plasma membrane isolation

The principal value given is the purification factor arrived at by dividing the specific activity of the fraction by the specific activity of the whole homogenate. All specific activities (indicated in parentheses) are given in micromoles of substrate converted per h per mg protein except for dopamine-β-hydroxylase which is given in nanomoles per h per mg of protein and monoamine oxidase which is given in arbitrary units per h per mg of protein. Values represent the average of three separate determinations ± S.D.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acetylcholinesterase</th>
<th>(Na+–K+)-ATPase</th>
<th>5’-Nucleotidase</th>
<th>Mg2+-ATPase</th>
<th>Monoamine oxidase</th>
<th>Glucose-6-phosphatase</th>
<th>Dopamine-β-hydroxylase</th>
<th>Cytocrome c oxidase</th>
<th>Acid phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>32–36% Interface</td>
<td>10.2</td>
<td>16.9</td>
<td>6.3</td>
<td>4.2</td>
<td>2.0</td>
<td>2.5</td>
<td>0.3</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>(13.6 ± 0.75)</td>
<td>(93.9 ± 8.6)</td>
<td>(3.03 ± 0.3)</td>
<td>(31.4 ± 3.5)</td>
<td>(0.228 ± 0.02)</td>
<td>(1.24 ± 0.10)</td>
<td>(8.8 ± 0.7)</td>
<td>(56.2 ± 4.4)</td>
<td>(0.44 ± 0.08)</td>
</tr>
<tr>
<td>36–40% Interface</td>
<td>6.3</td>
<td>6.7</td>
<td>6.1</td>
<td>3.9</td>
<td>3.1</td>
<td>5.5</td>
<td>0.2</td>
<td>4.2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>(8.4 ± 0.6)</td>
<td>(37.2 ± 3.6)</td>
<td>(2.94 ± 0.2)</td>
<td>(28.5 ± 2.5)</td>
<td>(0.353 ± 0.04)</td>
<td>(7.25 ± 0.20)</td>
<td>(4.9 ± 0.5)</td>
<td>(147 ± 7.6)</td>
<td>(1.03 ± 0.1)</td>
</tr>
<tr>
<td>Pellet</td>
<td>4.1</td>
<td>2.4</td>
<td>3.8</td>
<td>3.9</td>
<td>5.5</td>
<td>9.1</td>
<td>2.2</td>
<td>8.2</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>(5.4 ± 0.2)</td>
<td>(13.3 ± 1.1)</td>
<td>(1.83 ± 0.1)</td>
<td>(28.5 ± 3.5)</td>
<td>(0.627 ± 0.04)</td>
<td>(4.51 ± 0.4)</td>
<td>(64.2 ± 5.1)</td>
<td>(291 ± 12.0)</td>
<td>(2.99 ± 0.5)</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.33 ± 0.75</td>
<td>5.56 ± 0.5</td>
<td>0.485 ± 0.03</td>
<td>7.32 ± 1.4</td>
<td>0.114 ± 0.02</td>
<td>0.49 ± 0.04</td>
<td>(28.3 ± 1.1)</td>
<td>(35.2 ± 1.7)</td>
<td>(0.49 ± 0.06)</td>
</tr>
</tbody>
</table>

**FIG. 3.** A typical field from a plasma membrane preparation as seen in thin section. The sample was prepared as described under “Experimental Procedures.” Magnification: × 60,000.

The column is proportional to the content of plasma membrane in the fraction being studied. Based on trichloroacetic acid-precipitable radioactivity, exactly twice as much material (8.6% compared to 4.3% of total counts per min) could be eluted from the column using 32 to 36% interface membranes as compared to ones from the 36 to 40% interface. Such a value agrees well with the 1.6- to 2.5-fold purification factor based on marker enzyme activities. A further control was undertaken to ascertain the specificity of the protein derived from solubilized plasma membranes for chromaffin granules per se. When columns were prepared from fixed bovine adrenal medullary mitochondria and lysosomes, instead of chromaffin granules, labeled protein was not bound and all of the radioactivity was recovered in the void volume (Fig. 5C). Lastly, the plasma membrane-derived material which was bound and subsequently eluted from fixed granules (as in Fig. 5A), retained, to a large extent, its capacity for being bound and eluted following pH neutralization (Fig. 5D).

**Polyacrylamide Gel Electrophoresis of Eluted Materials—Gel electrophoresis (Fig. 6, a to c) of the plasma membrane protein bound to the affinity column showed it to be composed**

**FIG. 4.** Affinity chromatography of 125I-WGA from a column of fixed chromaffin granules. One milligram of 125I-labeled WGA (specific activity: 300,000 cpm/mg) was applied to a 10-ml column of fixed granules as indicated under “Experimental Procedures.” Elution was carried out using 0.05 N HCl in 1% Triton X-100 (pH 1.5). The pH was measured in each fraction and is shown below the abscissa.

**FIG. 5.** Affinity chromatography of solubilized plasma membranes. A, 10 mg of 125I-labeled plasma membrane protein (specific activity: 6 × 106 cpm/mg of protein) was chromatographed on a 10-ml column of fixed chromaffin granules. The arrow indicates here, as well as in B, C, and D, the point at which the pH of the effluent reached 3.0. Sample application and elution is described under “Experimental Procedures.” B, 10 mg of solubilized membranes from the 36 to 40% interface were passed over a column identical with that used in A. C, chromatography of solubilized plasma membranes on a 10-ml column of glutaraldehyde-fixed mitochondria. D, the material eluted at low pH from A was reapplied to the affinity column of fixed chromaffin granules. All the data shown here are representative of at least three experiments performed identically.
of one major polypeptide with an apparent molecular weight of about 51,000 in the presence of SDS and β-mercaptoethanol. The autoradiogram (Fig. 6d) indicated that this was also the major source of the labeled material observed in the elution patterns of Fig. 5, and that there is a lesser component of about 14,000 which either stains poorly with Coomassie blue, or is highly iodinated by lactoperoxidase. In addition, a minor component can be seen in the stained gel at about 62,000 which is not significantly iodinated. When the bands were sliced out of the gel and assayed for radioactivity, the 62,000, 51,000, and 14,000 molecular weight species possessed 5%, 80%, and 15% of the total, respectively.

Two prominent proteins in secretory cells are actin (Mr = 42,000) and tubulin (Mr = 53,000 to 55,000). Isolated plasma membranes have also been shown to contain actin and this protein has been found to be associated (22) or capable of reassociating (23, 24) with chromaffin granules. To rule out the possibility that either of these two ubiquitous cellular proteins represents the major component possessing an affinity for chromaffin granules, iodinated G-actin and tubulin were electrophoresed together with the granule-bound material on polyacrylamide gels. Fig. 6e indicates that G-actin clearly migrates ahead of the granule-binding protein while a similar study showed tubulin migrating more slowly (data not shown).

**DISCUSSION**

The use of cellular organelles as affinity adsorbents has led to the purification of one major and two minor polypeptides whose further characterization and possible role in exocytosis can now be evaluated. The maintenance of isotonic conditions throughout the subcellular fractionation procedure yielded highly purified and stable chromaffin granules. This lessened the likelihood that proteins from the granule matrix, released upon lysis, could be covalently linked to the surface of the vesicles (during glutaraldehyde fixation) which could either result in the blocking of existing affinity sites, or in the creation of new ones.

Plasma membrane-enriched fractions were purified to a level which was in good agreement with previously published results. There was a slight improvement using our technique over the procedures described by Zinder et al. (25), Wilson and Kirschner (26), or Nijar and Hawthorne (27), although the procedures and the enrichment in marker enzyme specific activity differed in each case.

The use of fixed chromaffin granules and solubilized plasma membranes is not entirely free from the criticism that additional proteins possessing affinity for chromaffin granules might exist. First, solubilization in detergents, although it does allow the isolation of basically one active species, may be too selective and a similarly active, but poorly solubilized species would be overlooked. In addition, other proteins with an affinity for the granules may lose their activity upon solubilization of the membrane. Second, fixation of the granule may result in the inability to isolate a protein (possibly multivalent) whose affinity would be enhanced by the presence of freely mobile receptor sites within the granule membrane capable of forming patches or clusters. Despite these criticisms, the approach presented here and the results obtained comprise an essential first step in the examination of exocytosis at the molecular level. On the basis of its affinity for the granule surface, the binding protein can now be used as an affinity adsorbent in the isolation of its receptor from granule membranes, and piece by piece, an exocytotic system can be reconstituted.

The exact mechanism of binding of the protein to the granules is unclear. We have previously demonstrated, using plant lectins and neuraminidase, that there is carbohydrate-containing material on the cytoplasmic surface of chromaffin granules (19). For the sake of simplicity, it was hoped that such structures would serve, based on the specificity conferred by their oligosaccharides, as the receptors for the binding protein on the chromaffin granule. We have not yet been able to demonstrate such a specificity. The protein, when bound to fixed granules, could neither be eluted by a variety of glycopeptides derived from a pronase digest of whole chromaffin granules.

Creutz et al. (28) have demonstrated the existence of another protein, synexin, in the soluble phase of fractionated adrenal medulla. In our hands, this protein, capable of aggregating chromaffin granules in the presence of Ca++, binds very weakly, if at all, to columns of fixed granules. This is consistent with previous studies in which it was shown that aggregation by synexin was inhibited by prior fixation of the granules in glutaraldehyde (28). Furthermore, synexin has a lower apparent molecular weight in SDS than the protein described here, 47,000 as compared to 51,000 (28).

We would like to propose that the protein isolated in this study serves to bring about the anchoring of the chromaffin granule to the plasma membrane during exocytosis. Morphological studies on a variety of secretory systems have shown the granules to be in extremely close contact with the cell membrane immediately prior to fusion (1–3). In certain protozoans, intramembranous particles have been seen to be aggregated into rosettes to which secretory granules are bound...
prior to and during release of their contents (29, 30). In the last step prior to the breakdown of the two membranes during exocytosis the fusion site is devoid of intramembranous particles on faces belonging to either granules or plasma membranes (2, 3). One model, consistent with these morphological data, postulates that upon stimulation of the secretory cell the binding protein becomes available to the granule surface. The granules, arriving at the membrane by either a passive (e.g. Brownian movement) or an active (e.g. contractile elements) mechanism, are bound to the cell membrane by the formation of a binding protein-receptor complex. The next step would involve the clearance of the fusion site of the elements corresponding to intramembranous particles, in this case, the binding protein-receptor complex. Lastly, fusion occurs in particle-free areas perhaps as a result of the high lysophosphatidylcholine content of granule membranes (19, 31).

Preliminary experiments on functional aspects of this protein have been carried out in a system where chromaffin granules can be observed to fuse with one another in a fashion similar to that observed in the phenomenon of compound exocytosis. The extent of fusion could be determined morphometrically from electron micrographs of freeze-fractured membranes (2.3). One model, consistent with these morphological data, postulates that upon stimulation of the secretory cell the binding protein becomes available to the granule surface. The granules, arriving at the membrane by either a passive (e.g. Brownian movement) or an active (e.g. contractile elements) mechanism, are bound to the cell membrane by the formation of a binding protein-receptor complex. Lastly, fusion occurs in particle-free areas perhaps as a result of the high lysophosphatidylcholine content of granule membranes (19, 31).

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