Identification and Purification of an Adrenal Medullary Protein (Synexin) That Causes Calcium-dependent Aggregation of Isolated Chromaffin Granules*  

CARL E. CREUTZ, CHRISTOPHER J. PAZOLES, AND HARVEY B. POLLARD  

From the Clinical Hematology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

We have isolated from the adrenal medulla a protein which causes the aggregation of isolated chromaffin granules when incubated in the presence of free calcium at concentrations greater than 6 \( \mu M \). The isolation procedure included precipitation in ammonium sulfate, gel filtration, and hydroxylapatite chromatography. Aggregating activity was assayed using turbidity measurements on granule suspensions. The protein was found to be soluble, heat-labile, and trypsin-sensitive and had an apparent molecular weight of 47,000 when subjected to electrophoresis in gels containing sodium dodecyl sulfate. It did not cause granule aggregation in the presence of magnesium, barium, or strontium, but was activated in a positively cooperative manner by salts of monovalent ions up to 30 mM, and the reaction is also induced by Mg\(^{2+}\), it seems unlikely that this type of aggregation represents the early intracellular events in exocytosis. It has been known for several years that isolated chromaffin granules, the secretory vesicles from the adrenal medulla, aggregate in the presence of high levels of Ca\(^{2+}\) and other divalent cations (7, 8). This aggregation may be verified by light and electron microscopy and can be conveniently assayed by turbidity measurements of granule suspensions. However, since the threshold Ca\(^{2+}\) concentration for this phenomenon is about 2 \( \mu M \), and the reaction is also induced by Mg\(^{2+}\), it seems unlikely that this type of aggregation represents the early intracellular events in exocytosis.

We have named this protein "synexin," from the Greek synexis which means "meeting." We suggest that synexin may be the intracellular receptor for calcium in the process of exocytosis from the adrenal medulla, acting to promote close association of granules both with other granules as well as with plasma membranes prior to secretion.

Exocytosis of neurotransmitters, hormones, and other cell secretory products is generally believed to occur by fusion of secretory vesicles with the plasma membrane following an increase in the intracellular free Ca\(^{2+}\) concentration (1). Although the requirement for calcium has been recognized since 1894 (2), the precise role calcium plays in this process has not been resolved in molecular terms primarily because of the lack of biochemical assays for the morphological events associated with exocytosis. However, there are many examples of cells in which, during stimulation, secretory vesicles fuse not only with the plasma membrane but with one another to form channels to the exterior of the cell (3-6). We therefore believe that the process of vesicle aggregation, which can be studied in vitro, may present a valid model for the initial interaction that occurs between vesicles and plasma membranes during exocytosis. In particular, both kinds of events are likely to be regulated by the same molecular factors.

It has been known for several years that isolated chromaffin granules, the secretory vesicles from the adrenal medulla, aggregate in the presence of high levels of Ca\(^{2+}\) and other divalent cations (7, 8). This aggregation may be verified by light and electron microscopy and can be conveniently assayed by turbidity measurements of granule suspensions. However, since the threshold Ca\(^{2+}\) concentration for this phenomenon is about 2 \( \mu M \), and the reaction is also induced by Mg\(^{2+}\), it seems unlikely that this type of aggregation represents the early intracellular events in exocytosis.

We now report the discovery and isolation from the adrenal medulla of a protein that initiates *in vitro* chromaffin granule aggregation when the Ca\(^{2+}\) concentration is raised above 6 \( \mu M \). The regions of contact between granules in the aggregates formed by this protein are morphologically similar to the contacts formed between vesicles and the plasma membrane when exocytosis occurs in secretory cells. Because of its ability to bring granules together, we have named this protein "synexin" from the Greek synexis which means "meeting." Synexin may be the intracellular receptor for Ca\(^{2+}\) in the process of exocytosis from the adrenal medulla.

**Experimental Procedures**

Preparation of Chromaffin Granules - Chromaffin granules were prepared from bovine adrenal medullary tissue by a modification of the differential centrifugation procedure of Taugner (9) as described by Pollard et al. (10).

Assay of Synexin Activity - Synexin activity was assayed by measuring the change in turbidity (absorbance at 540 nm) induced in a dilute suspension of chromaffin granules. Absorbance measurements were made on a Gilford 2000 recording spectrophotometer equipped with a water-jacketed sample chamber for temperature control (\( \pm 1^\circ\)). The standard aggregation assay was conducted for 15 min at 37°C on a granule suspension having an absorbance at 540 nm...
of 0.3 (approximately 0.1 mg of protein/ml). The reaction mixture contained 240 mM sucrose, 30 mM KCl, 28 mM histidine/HCl (pH 6.0) or 28 mM Hepes/NaOH (pH 7.3), and a calcium buffer consisting of 2.5 mM CaCl₂ and 0.5 mM CaC₂O₄. Most turbidity experiments were carried out at pH 6.0 so that EGTA would buffer calcium effectively in the range of pCa 5.4 to 3.4. To analyze the system at lower calcium concentrations, a pH of 7.3 was selected. The characteristics of the synexin reaction appeared to be the same at these two values of pH. The reaction mixture was prepared in 1-ml cuvettes by making additions of prewarmed solutions in the following order: 20 μl of calcium/EGTA buffer; 200 μl of 150 mM KCl; 400 μl of 300 mM sucrose, 40 mM histidine (sucrose/histidine solution); and 400 μl of a mixture of (sucrose/histidine solution at 37°); chromaffin granules (A₅₀₂, 600 μg/mg). At the completion of the aggregation reactions seen in Figs. 1 and 2.

Isolation of Synexin - Ten to twelve pairs of bovine adrenal glands were obtained at a slaughter house and immediately chilled on ice. Within 2 h the medullae were dissected free of cortical tissue and placed in 10 volumes of unbuffered 0.3 M sucrose. All subsequent procedures were carried out at 0 to 4°C. The tissue was blended for 5 s in a Waring Blender and then homogenized in a cylindrical glass homogenizer with a loose-fitting motor-driven Teflon pestle. After filtering through four layers of gauze, the unbroken cells and nuclei were removed by centrifugation at 500 × g for 10 min. The supernatant was then centrifuged at 20,000 × g for 30 min and chromaffin granules were prepared from the resulting pellet (10). The supernatant was then centrifuged at 100,000 × g for 90 min to remove microsomes. The soluble protein fraction was then brought to 20% of saturation in (NH₄)₂SO₄ and centrifuged at 50,000 × g for 10 min at 0°C. The resulting pellet was resuspended in 16 ml of sucrose/histidine solution with the aid of a Dounce homogenizer. The suspension was then clarified by centrifugation at 50,000 × g for 30 min. The supernatant was brought to 20% of saturation in (NH₄)₂SO₄ and centrifuged at 50,000 × g for 90 min. The resulting pellet was resuspended in 20 ml of sucrose/histidine solution at pH 6.0. The clear supernatant was then applied to a column (1.5 × 30 cm) of Ultragel AcA34 equilibrated and then eluted with sucrose/histidine solution. Synexin activity (measured as per cent change in 8 min of the absorbance at 540 nm of a chromaffin granule suspension) was detected in the final third of the elution volume. The fractions containing synexin were pooled and applied to a column (0.9 × 30 cm) of hydroxyapatite (Hypatite C from Clarkson Chemical Co., Williamsport, Pa.) equilibrated with sucrose/histidine solution and the column was eluted with a 0 to 1 M linear sodium phosphate gradient in sucrose/histidine solution. Synexin activity was detected in fractions containing between 0 to 0.5 M phosphate. The synexin fractions were pooled, dialyzed against a column (1.5 × 30 cm) of Sephadex G-25 equilibrated with sucrose/histidine solution in order to remove phosphate.

Binding of Synexin to Chromaffin Granules - Samples of synexin were incubated with various concentrations of chromaffin granules in the standard aggregation assay mixture. The granules were then centrifuged at 20,000 × g for 10 min at 37°. The unbound synexin activity remaining in each supernatant was assayed by incubating 600 μl of the supernatant in a 1-ml cuvette with 400 μl of a suspension of chromaffin granules in 240 mM sucrose, 30 mM KCl, 40 mM histidine/HCl (pH 6.0), 2.5 mM calcium/EGTA buffer (pCa 3.4 or 5.4) plus additional CaCl₂ to adjust the final free Ca²⁺ concentration to pCa 3.4.

Electron Microscopy - Chromaffin granule suspensions were fixed at 37° during the standard aggregation assay by adding 100 μl of 10% glutaraldehyde, pH 7, to the 1-ml cuvette. After 2 to 4 min, the contents of the cuvette were removed free of free phosphate on ice and chilled in iced phosphate on ice. The granules were then pelleted in a Beckman microfuge and the supernatant prepared from the homogenate. Under the phase microscope granules suspended in this supernatant were seen to aggregate when the pCa was raised above 5.0. The aggregating factor, subsequently called synexin, was found to be inactivated by heat (60° for 5 min) or trypsin and we concluded that it might be protein in nature. We then attempted to purify the presumed protein using turbidity changes in granule suspensions as an assay for its activity.

Quantitation of Synexin Activity - During the purification of synexin we found that turbidity changes in granule suspensions produced by synexin at any degree of purity followed a similar time course and had the same Ca²⁺ dependence. It was, therefore, possible to assess the relative activity of different fractions by measuring the percent turbidity change occurring at a constant Ca²⁺ concentration in a given time. Examples of time courses of turbidity change as a function of synexin concentration and of Ca²⁺ concentration are shown in Figs. 1 and 2, respectively. In this report, most of the data on the aggregation reaction is presented in terms of the turbidity change at a particular time point, 8 min, which is near completion of the aggregation reactions seen in Figs. 1 and 2.

As can also be seen in Fig. 1, a suspension of chromaffin granules incubated at 37° in the absence of synexin showed a gradual decline in turbidity which was presumably due to granule lysis. Accordingly, in each experiment the turbidity change induced by synexin was corrected for this "base-line" change for granules alone (which was insensitive to the calcium concentration) according to the following equation: percent of initial value, corrected = per cent of initial value for granules + synexin – per cent of initial value for granules alone × 100.

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis[p-aminoethylether]N,N',N'-tetraacetic acid; pCa, -log [Ca²⁺]; sucrose/histidine solution, 300 mM sucrose, 40 mM histidine/HCl, pH 6.0; SDS, sodium dodecyl sulfate.

Some Epon sections of granule pellets were examined in the light microscope after staining with 1% methylene blue, 1% azure II (1:1). Analytical Methods - Electrophoresis was carried out in 20-cm slab polyacrylamide gels containing 10 or 15% acrylamide using the discontinuous buffer system containing SDS described by Laemmli (12). Protein concentrations were determined by the method of Bradford (13) using crystalline ovalbumin as standard.
Synexin: Chromaffin Granule Aggregating Protein

Fig. 1. Time course of turbidity changes induced in a chromaffin granule suspension by addition of various amounts of synexin. Initial absorbance ~0.3. Synexin obtained from the Ultragel AcA34 column (Fraction III). pCa 3.4, pH 6.0.

Fig. 2. Time course of turbidity changes induced by synexin in a chromaffin granule suspension in the presence of different concentrations of free calcium. Initial absorbance =0.3. Synexin (3.4 μg) obtained from the Ultragel AcA34 column (Fraction III), pH 6.0.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>S.A.*</th>
<th>T.A.</th>
<th>n°</th>
<th>M°</th>
<th>K°°</th>
<th>n°°</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. First (NH₄)₂SO₄, ppt</td>
<td>10.778</td>
<td>0.0991</td>
<td>459.9</td>
<td>9.96 ± 0.33</td>
<td>81.8 ± 4.7</td>
<td>3.80</td>
<td>3.68 ± 0.76</td>
</tr>
<tr>
<td>II. Second (NH₄)₂SO₄, ppt</td>
<td>5.677</td>
<td>0.0943</td>
<td>535.4</td>
<td>2.55 ± 0.56</td>
<td>79.6 ± 5.7</td>
<td>3.86</td>
<td>4.20 ± 0.53</td>
</tr>
<tr>
<td>III. Ultragel AcA34 synexin fraction</td>
<td>451.9</td>
<td>0.769</td>
<td>347.6</td>
<td>2.39 ± 0.26</td>
<td>75.5 ± 3.2</td>
<td>3.86</td>
<td>4.01 ± 0.44</td>
</tr>
<tr>
<td>IV. Hydroxylapatite synexin fraction</td>
<td>79.0</td>
<td>0.025</td>
<td>49.4</td>
<td>2.40 ± 0.08</td>
<td>91.2 ± 18.8</td>
<td>3.77</td>
<td>4.01 ± 0.77</td>
</tr>
</tbody>
</table>

* Specific activity, units/μg of protein, taken as the reciprocal of the number of micrograms which give a half-maximal absorbance change on the fitted protein titration curve (Fig. 3).
* Total activity (specific activity × μg of protein).
° Hill coefficient for the protein titration curve.
°° Fitted maximum absorbance change for the protein titration curve, in per cent change.
°° Value of pCa giving half-maximal absorbance change on the fitted calcium titration curve (Fig. 4).
°°° Hill coefficient for calcium titration curve.

Table I shows the handling of the dilute synexin fractions in the hydroxylapatite and Sephadex G-25 chromatographies resulted in a great loss of synexin (~80%). Since the nature of the aggregation induced by this very highly purified fraction was not detectably different from that induced by the fraction obtained from the gel filtration step (which the SDS gel indicates was 70 to 80% pure) the latter fraction (Fraction III) was generally used for the further characterization of synexin induced aggregation reported here.

Partial Characterization of Synexin and the Aggregation Reaction—Synexin did not cause any change in absorbance of a granule suspension in the presence of 1 mM MgCl₂, 1 mM BaCl₂, or 1 mM SrCl₂, whereas a maximal change was observed with 1 mM CaCl₂.

The aggregation was found to be strongly temperature-dependent between 0°C, where synexin was completely inactive, and 25°C with a Q10 of ~2. Between 25°C and 37°C the reaction was virtually independent of temperature.
FIG. 3. Dose-response curves for fractions containing synexin. I, first (NH₄)₂SO₄ precipitate; II, second (NH₄)₂SO₄ precipitate; III, Ultragel AcA34 column fraction; IV, hydroxylapatite column fraction. Synexin activity is given as the absorbance at 540 nm of a chromaffin granule suspension after incubation with synexin for 8 min at pCa 3.4, pH 6.0.

FIG. 4. Calcium titration of aggregating activity in fractions containing synexin. Roman numerals refer to fractions as defined in the legend to Fig. 3. The amounts of protein used in the assays were: I, 104.1 μg; II, 27.3 μg; III, 3.02 μg; IV, 1.35 μg, pH 6.0.
Synexin: Chromaffin Granule Aggregating Protein

FIG. 5. Analysis by electrophoresis in SDS of fractions prepared during the isolation of synexin. A, 15% polyacrylamide gel. a, molecular weight standards: 1, β and β' subunits of RNA polymerase from E. coli, 155,000 and 165,000; 2, bovine serum albumin, 68,000; 3, α subunit of RNA polymerase, 39,000; 4, soybean trypsin inhibitor, 21,500. b, initial 100,000 × g supernatant; c, supernatant from b after 20% (NH₄)₂SO₄ cut; d, proteins precipitating from b in 20% (NH₄)₂SO₄; e, proteins remaining in solution after clarification of d by centrifugation (Fraction I); f, supernatant from e after 20% (NH₄)₂SO₄ cut; g, proteins precipitating from e in 20% (NH₄)₂SO₄, after clarification by centrifugation (Fraction II); h, pooled synexin-containing fractions from Ultragar AnA34 column (Fraction III). The asterisk marks the position of synexin in Gels g and h. B, 10% polyacrylamide gel. a, molecular weight standards (see f below for molecular weights); b, pooled synexin-containing fractions from Ultragar AcA34 column (Fraction III); c, d, and e, three consecutive fractions comprising the peak of synexin activity in the effluent from the hydroxylapatite column; the asterisk marks the solitary synexin band. f, molecular weight standards: 1, phosphorylase B, 94,000; 2, bovine serum albumin, 68,000; 3, actin, 42,000; 4, tropomyosin, 35,000. C, interpolation of synexin molecular weight, 47,000, from molecular weight standards on Gel B. Numbers refer to the standards defined in f above.

The aggregation was stimulated by salts of monovalent ions. The reaction was stimulated 2- to 3-fold between 0 and 30 mM, where the effect saturated. KCl, NaCl, potassium isethionate, and choline chloride were equally effective as activators, indicating that the effect was not specific to a particular monovalent ion.

Synexin was functionally distinguishable from muscle actin. It has been demonstrated that muscle actin binds to chromaffin granule membranes (14). Because the molecular weight of synexin was close to that of actin, it seemed important to determine if synexin was in fact actin. Actin containing fractions were prepared from rabbit skeletal muscle following the procedures of Spudich and Watt (15). Both the initial extract from an acetone powder (containing G-actin) and the once polymerized and pelleted F-actin were assayed for synexin activity. Both extracts contained the Ca²⁺-sensitizing tropomyosin troponin complex, but neither showed any aggregating activity. In another test, incubation of synexin and chromaffin granules in the presence of 100 μg/ml of cytochalasin B, which disrupts the structure of actin filaments, had no effect on the aggregation reaction.

The aggregation reaction was not stimulated by the addition of ATP or MgATP.

The reaction was specific for chromaffin granules. Synexin did not induce a change in the absorbance at 540 nm of a suspension of adrenal cortical mitochondria prepared from a cortical tissue homogenate by differential centrifugation in 0.3 M sucrose. Meyer and Burger (16) have demonstrated that of five different lectins, only wheat germ agglutinin will aggregate chromaffin granules. This result indicated that the variety of carbohydrate structures on the granule surface is somewhat limited. If synexin acts by binding to carbohydrates on the granule surface, the wheat germ agglutinin binding site may be involved in the synexin binding. However, 50 mM N-acetylglucosamine, the hapten sugar for wheat germ agglutinin, was ineffective as an inhibitor of the aggregation reaction. However, 300 μg/ml of fetuin, a glycoprotein bearing many carbohydrate structures, was similarly ineffective as an inhibitor in the presence of 3 μg/ml of synexin.
was comparable at either value of pH. As anticipated from the turbidity studies, the omission of synexin at a high calcium concentration. This result suggests that synexin causes aggregation by effecting a change in their surface properties so that they spontaneously aggregate, or the synexin molecule itself may attach to the granule surface and form bridges between granules. To determine whether synexin was bound, granules were incubated with synexin under the conditions of the standard aggregation assay, with a pCa of 3.4 or 5.4. The granules were then sedimented by centrifugation and the supernatant was assayed for synexin.

Any reduction in the amount of synexin in the supernatant was assumed to be due to the binding of the protein to the chromaffin granules. As illustrated in Fig. 6, synexin activity bound to granules only in the presence of the higher calcium concentration. This result suggests that synexin causes aggregation by binding to granules and that aggregation does not occur at low calcium concentrations because in that case synexin remains unbound. Whether synexin itself forms an intergranular bridge, or whether by, or after, binding it changes the surface properties of the granule remains to be determined.

**Morphology of Chromaffin Granule Aggregates —** Electron microscopy of granule suspensions treated with synexin and calcium revealed frequent granule aggregates at both pH 6.0 and 7.3. This was consistent with our observation that the influence of synexin on the turbidity of a granule suspension was comparable at either value of pH. As anticipated from the turbidity studies, the omission of synexin at a high calcium concentration (pCa 4.0) (Fig. 7A) or a low calcium concentration (pCa 6.0) in the presence of synexin (Fig. 7B) lead to no aggregate formation. However, when granules were incubated in the presence of synexin at pCa 4.0, numerous aggregates were noted (Fig. 7C). Similar aggregates, although reduced in number and size, were formed at pCa 5.2 (data not shown), and we concluded that calcium-dependent formation of aggregates by synexin begins between pCa 6 and pCa 5.2 (1 and 6 $\mu$M Ca$^{2+}$, respectively). The dramatic changes in turbidity centered at pCa 3.8 (see the calcium titration curves of Fig. 4) apparently were not due to the initial formation of aggregates by synexin. Instead, these changes resulted from the formation of distinctly larger aggregates which were not easily recognized on the scale of the micrographs in Fig. 7. However, in Fig. 8 are shown two light micrographs of the Epon sections prepared for electron microscopy at pCa 5.2 and 3.6. The difference in aggregate size, and the effect of this on the optical density of the suspension is suggested by these pictures.

Higher magnification of the chromaffin granule aggregates, as in Fig. 9, revealed that individual granules had not fused to the point of having common lumina. Instead, the granules were in close contact, frequently with some distortion to maximize the area of contact. In some places the contact between the trilaminar membranes was close enough that the cross-section of the contact area revealed a pentilaminar structure. This was particularly evident where empty membranes (which probably arose from lysis of granules during the incubation period) were attached to granules or to one another (Fig. 9B).

**Discussion**

In these experiments we have made use of turbidity measurements to assay a supramolecular event, the aggregation of secretory vesicles, that correlates with morphological findings in studies of whole cells undergoing exocytosis. The approach has proven to be a powerful one, providing evidence that adrenal medullary tissue contains a protein, synexin, which can induce the in vitro aggregation of chromaffin granules in the presence of free calcium, and which we infer may be involved in the regulation of exocytosis in vivo.

The aggregating activity of synexin was found to depend absolutely upon Ca$^{2+}$ at concentrations that are in the range normally regulated in the cytoplasm. There are reports of other vesicular systems, pancreatic $\beta$ granules (17) and golgi-derived liver secretory vesicles (18), in which aggregation is induced by similarly low levels of Ca$^{2+}$ without a requirement for a cofactor such as synexin. In these systems, a synexin-like protein may already be bound to the isolated vesicles. In any event, the chromaffin granule system provides an advantage in that the active principle, synexin, can be easily separated, isolated, and subjected to analysis.

The calcium titration curves for the turbidity changes induced by synexin showed positive cooperativity with a Hill coefficient of about 4 (Table I). This is of interest because 4 is also the approximate value of the coefficient reported in a physiological study of exocytosis: titration of the magnitude of the evoked end plate potential in the frog neuromuscular junction (19). The magnitude of the end plate potential is probably directly related to the number of contacts between secretory (synaptic) vesicles and the plasma membrane in this system. However, the light scattering changes of aggregating chromaffin granules are not necessarily a direct measure of the number of contacts between granules. Therefore, a mean-
FIG. 7. Electron micrographs of chromaffin granule suspensions incubated with synexin. Incubation was carried out for 12 min before fixation as described under "Experimental Procedures." x 17,500. A, control suspension incubated in the absence of synexin; pH 7.3, pCa 4.0. B, suspension incubated in the presence of synexin; pH 7.3, pCa 4.0. C, As in B, but with pCa 4.0. Note that almost all of the granules have formed contacts with other granules. The arrowheads point to some examples.

One way of further analyzing the role of Ca\textsuperscript{2+} in activating the synexin reaction is to use the titration curves of turbidity change versus calcium concentration (as in Fig. 4) and of turbidity change versus synexin concentration (as in Fig. 3) to construct a titration curve of "activated synexin" versus calcium concentration. This has been done in Fig. 10, and the resulting curve shows that Ca\textsuperscript{2+} acts in positively cooperative manner to activate the synexin reaction with a Hill coefficient of approximately 2. In making this analysis, only the data of synexin which gave maximum aggregation at high Ca\textsuperscript{2+} concentrations; pH 7.3, pCa 6.0. Note that at this pCa no aggregation is seen. C, As in B, but with pCa 4.0. Note that almost all of the granules have formed contacts with other granules. The arrowheads point to some examples.

The results of the binding experiment suggest that synexin may cause aggregation by binding to granules in the presence of Ca\textsuperscript{2+}. At a pCa of 5.4, where aggregation of chromaffin granules was not detected by turbidity measurements, no binding of synexin was detected. This pCa corresponds to a calcium concentration slightly less than that at which a small
degree of granule aggregation was detected by electron microscopy (pCa 5.2). The limited amount of aggregation occurring at such a low level of calcium may be due to the binding of small amounts of synexin that cannot be detected by the binding assay we used. It is not known whether, in binding, synexin itself forms a bridge between granules or, after binding, leads to some change in the surface properties of the granules. It is also not known if Ca\(^{2+}\) acts by binding to synexin, or to a site on the chromaffin granule, or both. The presence of carbohydrate groups on the outer surface of the chromaffin granule has been demonstrated (15). One possible role for these groups would be to act as synexin-binding sites, in which case synexin would be an intracellular lectin. However, so far we have not been able to show that this is the mechanism of synexin binding by competition experiments with various carbohydrates.

Synexin was found to cause chromaffin granule aggregation at remarkably low concentrations. A half-maximal turbidity change in a suspension of chromaffin granules containing 0.13 mg of protein occurred in the presence of 1.3 \(\mu\)g of synexin. From the density (1.12 gm/cm\(^3\)) and size (200 nm diameter) of a granule, its mass can be calculated to be \(4.69 \times 10^{15}\) g. Given that 35% of the dry weight of a granule is protein and that a hydrated granule is 85% water (20), a suspension of 1 mg of granule protein contains \(4.08 \times 10^{15}\) granules. Since the molecular weight of synexin is 47,000, there would be \(1.28 \times 10^{13}\) synexin molecules/\(\mu\)g of synexin. Therefore, in our experiments a half-maximal turbidity change occurred at a ratio of 31 synexin molecules/granule. In the binding study, at the concentration of granules removing half the synexin from the supernatant, 89 molecules of synexin were bound/granule. This should represent the number of binding sites per granule. Therefore, the data suggest that a half-maximal turbidity change was induced when approximately one-third of the synexin binding sites on the granules were occupied.

Aggregation of granules by synexin produced the first readily observable changes in turbidity at a ratio as low as 10 synexin molecules/granule. At this concentration there is, on the average, only 1 synexin molecule/10,000 nm\(^2\) of granule surface. Therefore, it seems unlikely that the synexin molecules cause aggregation simply by reducing the granule surface charge with their intrinsic charge, as is a possible mechanism for granule aggregation caused by high concentrations of divalent cations.
The morphological observations on the synexin-granule complexes provided several important results. First they demonstrated that the observed light scattering changes were due only to aggregation of granules, along with some distortion of granules as regions of contact between them were maximized. The micrographs also indicated that the aggregates formed at lower concentrations of Ca\(^{2+}\) (down to 6 \(\mu\)M) than might have been suspected on the basis of the turbidity measurements alone. The highly magnified images of aggregates revealed that the contacts between granules and between granules and membranes were as intimate as seen in secreting cells when vesicles initially attach to one another or to the plasma membrane to form a pentalaminar region of contact (21, 22).

However, the vesicles joined by synexin do not completely fuse to form larger vesicles with a common interior space, as would be expected if the entire process of exocytosis was occurring in this model system. What, then, could be the precise role of synexin in exocytosis? Previous studies (23, 24) have demonstrated that isolated chromaffin granules undergo osmotic lysis when incubated in the presence of magnesium, ATP, and high concentrations (~90 mM) of permeant anions such as Cl\(^{-}\). Intracellular Cl\(^{-}\) concentrations, ~30 mM, are not high enough to stimulate this lysis although extracellular concentrations are. Anion transport blocking drugs such as 4-acetamido-4'-isothiocyanostilbene-2'-disulfonic acid disodium, probenecid, and pyridoxal phosphate block the lysis (25) and they also block exocytosis from cellular systems (25, 26).

Therefore, synexin may function by bringing a vesicle into contact with the plasma membrane or with the membrane of a previously opened vesicle so that transport sites in the vesicle membrane are exposed to the large gradient between extra and intracellular Cl\(^{-}\) concentrations. The union would then be translocated into the vesicle leading to the solubilization of the granule contents and subsequent osmotic lysis of the vesicle. The lysis of the vesicle membrane would occur at the (hypothetically) less stable region of contact with the plasma membrane, leading to release of the vesicle contents to the exterior of the cell. In this model, Ca\(^{2+}\) regulates the process by its action on synexin (or the synexin binding site).

It has been suggested that Ca\(^{2+}\) may regulate exocytosis by acting on the tropomin troponin complex of an actomyosin system in the cytoplasm of a secretory cell (27). Indeed, myosin has been isolated from adrenal medullary tissue and its presence in the chromaffin cell has been verified by immunofluorescence (28). However, the actomyosin complex is not included as part of the model above. Its action may precede the action of synexin. By controlling the consistency of the cytoplasm, actomyosin, under Ca\(^{2+}\) regulation, may permit or accelerate the movement of granules toward the plasma membrane where synexin would then act to cause fusion.

If synexin is universally important for exocytosis then it should be present in other secretory tissues. However, since membrane fusion is probably a normal event in all cells, occurring during such processes as plasma membrane regeneration, synexin may be present in nonexocytotic tissues as well. Preliminary results in our laboratory indicate that proteins with the properties of synexin are also present in brain and in adrenal cortical tissue.

REFERENCES
2. Locke, R. S. (1894) Zbl. Physiol. 8, 166–167
Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules.

C E Creutz, C J Pazoles and H B Pollard