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Further Characterization of the Aggregation and Fusion of Chromaffin Granules by Synexin as a Model for Compound Exocytosis

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Synexin was isolated from bovine liver and found to aggregate adrenal chromaffin granules in the same Ca^{2+}-dependent manner as previously described for adrenal synexin. The chromaffin granule aggregating activity of liver synexin was blocked in vitro by the addition of an antibody prepared to the 47,000 molecular weight band extracted from an SDS gel of an adrenal medullary synexin preparation. Chromaffin granules aggregated by synexin fused when exposed to cis-unsaturated fatty acids at concentrations comparable to those released from phospholipids by stimulated secretory cells. The synexin-induced aggregation reaction was blocked by Erythrosin B, a common food coloring, and by the phenothiazine antipsychotics trifluoperazine and promethazine. The aggregation and fusion of chromaffin granules thus appears to be a useful model system for studying synexin from diverse tissues and for testing pharmacologically or toxicologically active substances for effects on secretory systems.

Key words: exocytosis, secretion, synexin, calcium-binding protein, adrenal medulla, chromaffin granule, stimulus-secretion coupling, membrane fusion, arachidonic acid, trifluoperazine, erythrosin B, phenothiazines

Synexin is a Ca^{2+} binding protein, which we have suggested may be an intracellular receptor for Ca^{2+} in the process of exocytosis [1, 2]. specifically, we believe the role of this protein is to seal membranes together and prepare them for fusion: the plasma membrane and the secretory vesicle membrane, or, in the case of compound exocytosis, the membranes of two secretory vesicles. In this report we describe several new observations of the properties of a cell-free model for compound exocytosis that we have developed using synexin and chromaffin granules (secretory vesicles from the adrenal medulla).

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MATERIALS AND METHODS

Materials

Chromaffin granules were prepared from bovine adrenal medullary tissue by differential centrifugation in 0.3 M sucrose as previously described [3]. Synexin was prepared from bovine adrenal medulla or liver by the procedures described earlier: precipitation in 20% ammonium sulfate and gel filtration on LKB Ultragel ACA34 [1, 2]. Fatty acids and other compounds tested for fusogenic activity were obtained from Sigma Chemical Company, St. Louis. Erythrosin B and ethyl eosin were obtained from Aldrich and Rose Bengal from MCB. Trifluoperizine was obtained from Smith, Kline and French and promethazine from Wyeth Laboratories, Philadelphia.

Purification of Synexin by Ca^{2+}-Dependent Affinity Chromatography

Chromaffin granule membranes were prepared by lysing purified chromaffin granules in 25 volumes of distilled water. The lysed membranes were sedimented and resuspended in coupling buffer (0.5 M NaCl, 0.1 $NaHCO_3$) and attached to CNBr-activated Sepharose 4B (Pharmacia) following the manufacturer's recommendations. Briefly, the membranes and gel were mixed and gently agitated for 2 h at room temperature. Following storage at 4°C overnight, the gel was washed with coupling buffer and the remaining active groups on the gel reacted with 1 M ethanolamine at pH 8.0 for 2 h at room temperature. Finally, the gel was washed in column buffer (0.24 m sucrose, 30 mM KCl, 32 mM histidine-HCl pH6.0). The affinity gel was packed in a 15 × 1.5 cm column. A partially purified synexin preparation was applied at room temperature in column buffer containing 2 mM EGTA and 4 mM $CaCl_2$. After washing the column with three volumes of this buffer, synexin was eluted with column buffer containing 2 mM EGTA.

Aggregation and Fusion of Chromaffin Granules

Synexin-induced granule aggregation was carried out under the conditions of the assay for synexin activity. Incubation at 37°C of 1 ml samples containing 5–20 μg of liver synexin, 240 mM sucrose, 30 mM KCl, 32 mM MES-NaOH (pH 6.0), 2.5 mM EGTA and $CaCl_2$ of appropriate concentration to give the desired free Ca^{2+} concentration. The granule suspension had an initial absorbance at 540 nm (A540) of 0.3, corresponding to 70–90 μg/ml of granule protein. For fusion studies, after aggregation by liver synexin for a minimum of 15 min, the fusogen was introduced as follows: Fusogens were stored as 5 mg/ml solutions in ethanol at −20°C and diluted in sucrose-MES buffer to 100 μg/ml prior to each series of experiments; 40–100 μl of the resulting emulsion was added to the granule suspension.

The turbidity (A540) of granule suspensions was monitored on a Gilford 250 recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) equipped with an automatic cuvette positioner which permitted the intermittent monitoring of four simultaneous reactions.

Preparation of an Antisynexin Antibody

A highly purified synexin antigen was prepared by electrophoresis in SDS of synexin partially purified by gel filtration [4]. After brief staining with Coomassie

blue, the major band of molecular weight 47,000 was excised and the protein removed by electrophoresis. The extracted protein was mixed with an equal volume of Freund's adjuvant and injected intradermally into New Zealand white rabbits (∿ 100 μg/animal). After 4 weeks antisynexin activity was detected as the ability of IgG from an immunized rabbit to block the synexin-dependent aggregation of chromaffin granules.

RESULTS AND DISCUSSION

Liver Synexin

Following the procedure for the preparation of adrenal medullary synexin, synexin was prepared from bovine liver with the same yield (10 μg/gm of tissue) and purity (∿80%) as was obtained from the adrenal medulla. Figure 1 illustrates SDS gels of fractions obtained during the isolation procedure. Many of the impurities present in the preparation obtained by gel filtration could be removed by either of two additional purification steps: a repetition of the precipitation of synexin in 20% ammonium sulfate (Fig. 2, lane 8), or Ca^{2+}-dependent affinity chromatography of the synexin on chromaffin granule membranes attached to Sepharose 4B (Fig. 2, lane 7). The apparent molecular weight of the synexin band on the SDS gels was 47,000, identical to that found for adrenal medullary synexin [1].

The Ca^{2+} dependence of the aggregation of chromaffin granules by liver synexin was analyzed by the procedure described previously [1]. Briefly, a titration curve of the turbidity increase induced in a granule suspension by different amounts of liver synexin at a saturating Ca^{2+} concentration (1 mM) was constructed. This curve was used as a reference to determine what fraction of a given amount of synexin appeared to be active at reduced Ca^{2+} levels. The curve of "activated synexin" versus free Ca^{2+} that resulted from this analysis is presented in Figure 3. The curve suggests that Ca^{2+} acts in a positively cooperative fashion to activate synexin with a Hill coefficient of 2.3 and a dissociation constant of 200 μM (pCa 3.7). These characteristics of the titration curve are indistinguishable from those obtained previously for adrenal medullary synexin [1].

Preparation of an Antisynexin Antibody

An antiserum to adrenal medullary synexin was prepared in rabbits by immunization with the extracted 47,000 molecular weight band from an SDS gel of a synexin preparation. As a preliminary test for the presence of the antibody, the IgG fractions of sera were tested for the ability to block the aggregation of chromaffin granules by synexin. Whole serum could not be screened for antisynexin activity by this method since it was found to contain a protein which itself caused the aggregation of granules. The aggregation induced by this factor was not Ca^{2+}- or temperature-dependent and could be inhibited by increasing the ionic strength of the assay medium, characteristics that distinguished its mechanism of action from that of synexin. This factor was soluble in 40% ammonium sulfate and could be purified by chromatography on CM-Sephadex. On SDS gels the factor appeared as a single diffuse band of apparent molecular weight 55,000. On the basis of this molecular weight, and the protein's apparently basic character (affinity for CM Sephadex), we have tentatively identified this protein as the highly basic "histidine-rich glycoprotein" (HRG protein) described by Morgan [5]. Preparation of the IgG fraction of

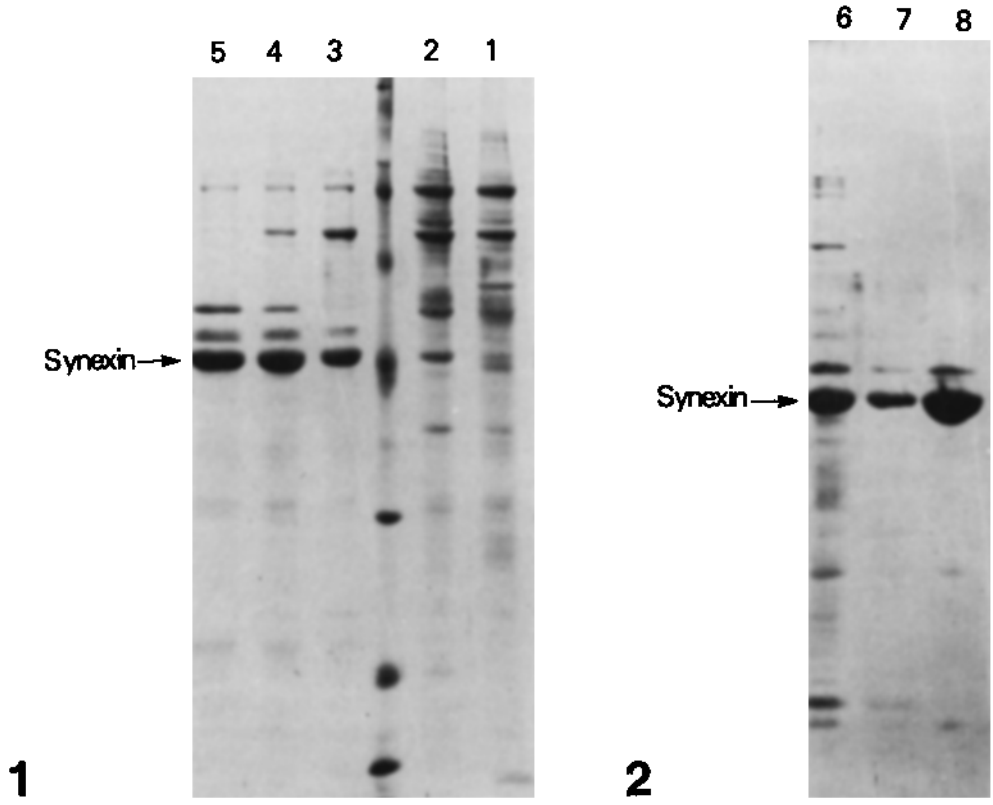

Fig. 1. Ten percent SDS gel of fractions prepared during the isolation of liver synexin. Lane 1, proteins precipitated from the post-microsomal supernatant by 20% ammonium sulfate; lane 2, proteins reprecipitated in 20% ammonium sulfate; unlabeled lane, molecular weight standards, starting from the bottom the major bands represent 14,400, 21,000, 30,000, 45,000, 68,000, and 94,000; lane 3, 4, and 5, fractions across the synexin activity peak from the gel filtration column.

Fig. 2. SDS gels of synexin preparations. Lane 6, pooled synexin from gel filtration column; lane 7, synexin obtained by Ca^{2+}-dependent affinity chromatography on chromaffin granule membranes; lane 8, synexin obtained from the gel filtration peak by reprecipitation in 20% ammonium sulfate.

serum by precipitation in 35% ammonium sulfate removed the HRG protein and eliminated its interference in the assay for antisynexin activity.

The antisynexin antibody was tested for its ability to block granule aggregation induced by either adrenal medullary or liver synexin and was found to block the activity of either protein at a similar concentration (Figs. 4, 5). This suggests an immunochemical similarity of the synexins obtained from the two tissues.

Fusion of Aggregated Chromaffin Granules by Cis-Unsaturated Fatty Acids

We recently found that when a small amount of *cis*-unsaturated fatty acid was added to a suspension of chromaffin granules aggregated as in Figure 6A, the granules rapidly fused to form large vesicular structures (Fig. 6B) [6] that were morphologically similar to the vacuoles that form in the cytoplasm of the chromaffin cell following extensive compound exocytosis [7]. This transition began immediately and was complete in a few minutes. The clusters of fusing granules performed a

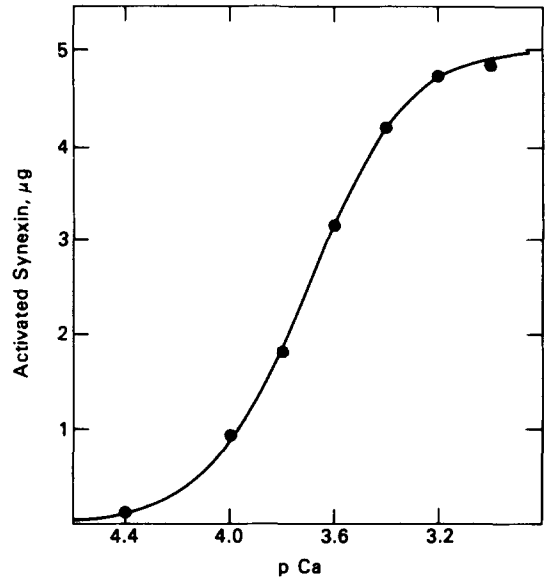

Fig. 3. Ca^{2+} titration of synexin in the chromaffin granule aggregation reaction.

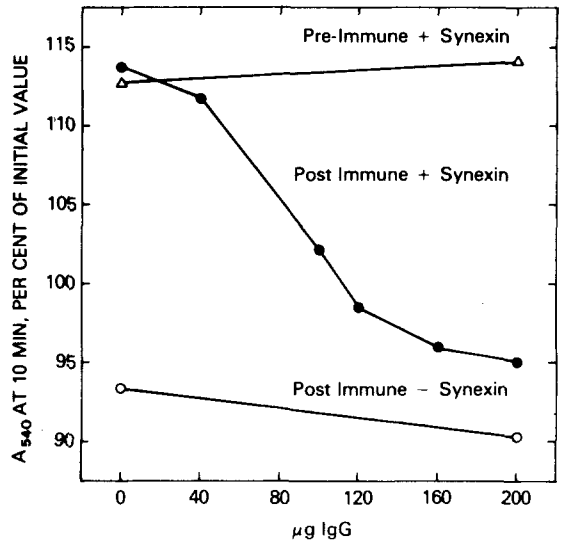

Fig. 4. Inhibition of chromaffin granule aggregation by ~2 μg of adrenal medullary synexin caused by the addition of an antibody to adrenal medullary synexin. The synexin activity is recorded as the turbidity of the granule suspension after 10 min, which increases owing to synexin, or declines slightly in control suspensions without synexin. As indicated, the preimmune serum does not block granule aggregation.

dramatic dance that could be observed in the phase microscope as the large vesicles swelled in sudden and discrete steps from the clusters. The process could also be monitored by recording the decline in turbidity (A540) of the suspension that occurred as fusion took place (Fig. 7).

The fusion reaction was temperature-dependent, occurring at 37°C but not at 0°C. Chemical measurements on supernatants obtained by pelleting the granules

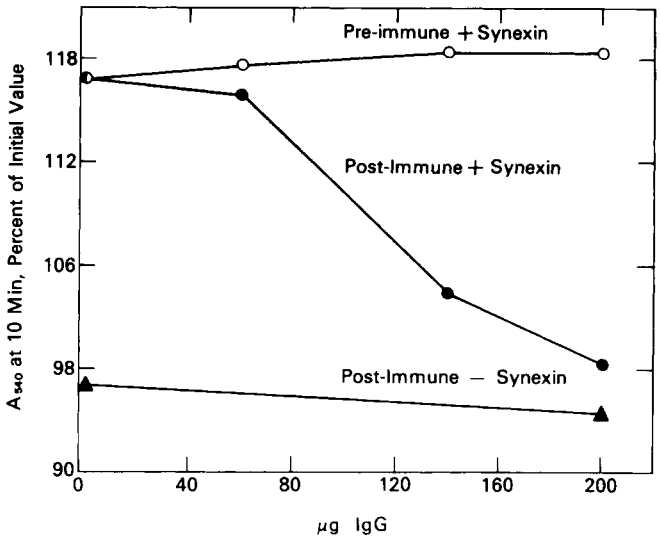

Fig. 5. Cross-reaction of antibody to adrenal medullary synexin with liver synexin. Similar to the analysis presented in Figure 4, the aggregation of chromaffin granules by liver synexin is blocked by the addition of the antibody to adrenal medullary synexin.

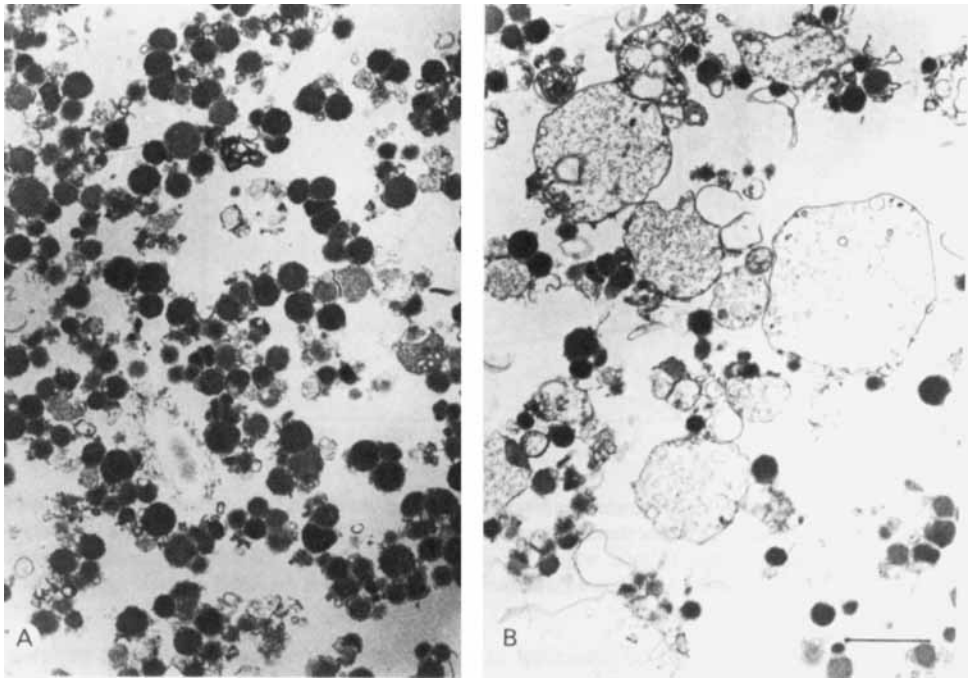

Fig. 6. Fusion of chromaffin granules induced by synexin and arachidonic acid. Suspensions (80 μg/m.) were incubated for 20 min in the presence of synexin (15 μg/ml) and 1 mM Ca^{2+}. In B, 4 μg/ml arachidonic acid was introduced at 15 min. Length of bar = 1 μm.

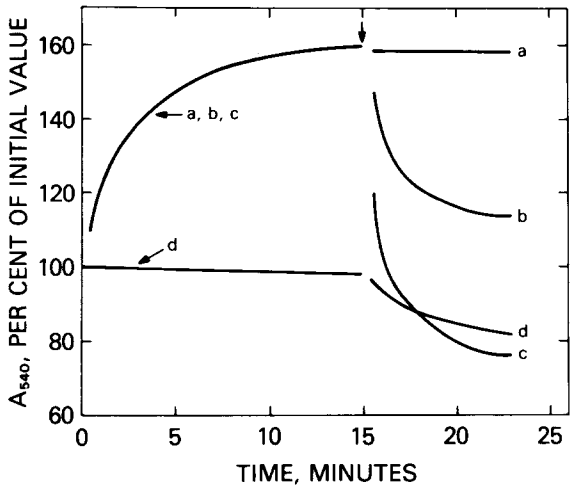

Fig. 7. Turbidity of chromaffin granule suspensions undergoing aggregation and fusion. From 0 to 15 min the granule suspension (A540∿0.3, 80 μg/ml protein) is incubated with (traces a, b, c) or without (trace d) 15 μg/ml of synexin. At 15 min 4 μg/ml of fatty acid is introduced: trace a, elaidic acid (trans 18:1); trace b, oleic acid (*cis* 18:1); traces c and d, arachidonic acid (*cis* 20:4).

after fusion, indicated that during fusion the soluble proteins stored inside the granules were retained, although about 50% of the epinephrine escaped (above control levels of leakage). The rate of fusion could be inhibited 10-fold by raising the pH of the suspension from 6.0 to 7.3. The formation of the large vesicles could be suppressed by increasing the osmotic strength of the medium with sucrose to 800 mOsM. However, the formation of the vesicles did not appear to depend on the chemiosmotic properties of the granule membrane since it was not influenced by ATP, a proton ionophore, (FCCP:carbonyl cyanide p-trifluoromethoxyphenylhydrazone) or an anion transport inhibitor (SITS:4-acetamido-4′ isothiocyanostilbene-2,2′ disulfonic acid).

The fatty acids and other lipids used at high concentrations by Ahkong et al [8] to induce fusion of red blood cells were tested for fusogenic activity in this system. Chromaffin granule fusion was found to require specifically those *cis*-unsaturated fatty acids that are common components of mammalian phospholipids. Arachidonic acid was the most effective fusogen, causing vesicle formation at 2 μg/ml (6 μM), at which concentration it comprised 4% of the total lipid in the suspension. This amount of free arachidonic acid is comparable to the amount, relative to total membrane lipid, that is released from platelet phospholipids during secretion of serotonin by exocytosis [9]. We have recently observed that arachidonic acid is also released from chromaffin cells during exocytosis [10]. Transunsaturated, saturated, or esterified *cis*-unsaturated fatty acids did not induce fusion, nor did lysolecithin or glycerol monoleate. Isomers of oleic acid with the *cis*-double bond moved towards or away from the head group (petroselenic, vaccenic acids) caused little or no fusion. A study with radioactively labeled arachidonic acid indicated that after fusion occurred 93% of the added fatty acid was bound to the granule, but none appeared to be incorporated covalently into phospholipids (separated by thin-layer chromatography after the fusion experiment).

The factors regulating this in vitro process of secretory granule aggregation and fusion—Ca^{2+}, synexin, and unesterified, *cis*-unsaturated fatty acids—may be

available in the cytoplasm of a secretory cell when it is stimulated. Therefore, this fusion event may be the same that occurs between secretory vesicles undergoing compound exocytosis.

Effects of Xanthane Dyes and Phenothiazine Antipsychotics on Chromaffin Granule Aggregation

Using the synexin-induced aggregation of chromaffin granules as a model for membrane interactions in exocytosis, the effects of two classes of compounds were tested. Xanthane dyes have been reported to have effects on neural membranes [11]. Figure 8 illustrates that erythrosin B (FDC Red No. 3) blocks the ability of synexin to aggregate chromaffin granules with half-maximal inhibition occurring at 15 μM. The site of action of the dye is unclear. However, the dye did not block the Ca^{2+}-dependent self-association of synexin, a process that may be necessary for granule aggregation, by synexin [2]. Rose Bengal, another xanthane dye, also blocked aggregation, at 4 μM, and at higher concentrations caused lysis of the chromaffin granules (Fig. 9). Levitan has suggested [11] that the critical factors in the ability of erythrosin B to affect membrane dependent events are the negative charge of the dye and its lipophilicity, as reflected in its octanol–water partition coefficient. However, 5-iodosalicylate, an anionic compound with a partition coefficient similar to that of erythrosin B that Barker and Levitan [12] found was as effective as the dye in modifying the membrane potential and potassium conduction of a molluscoid neural membrane, had no effect on synexin action at a concentration as high as 5 mM. Furthermore, ethyl eosin, an analogue of erythrosin B, which is uncharged, was also an effective inhibitor. Thus the mechanism of action of these dyes in this system remains unknown.

The phenothiazines, particularly trifluoperazine (TFP), were of interest to test because they interact with a hydrophobic portion of another Ca^{2+}-dependent regulatory protein, calmodulin [13]. As indicated in Figure 10, TFP blocked the chro-

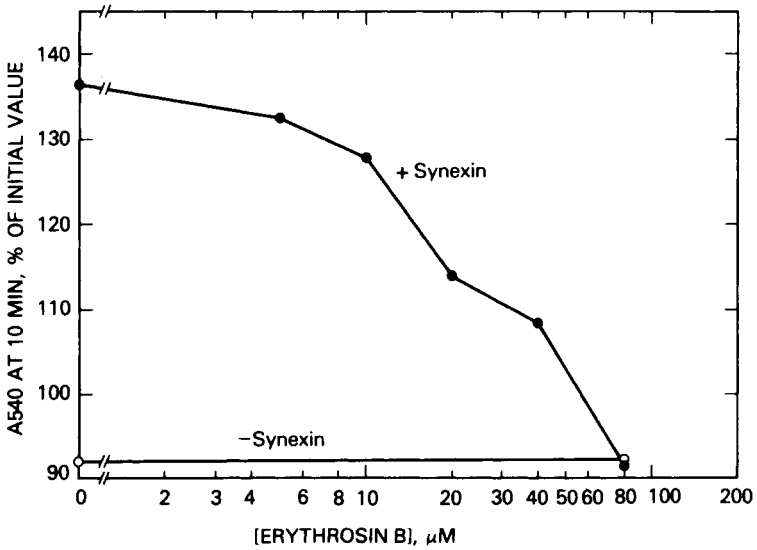

Fig. 8. Inhibition of synexin-induced chromaffin granule aggregation by the food dye erythrosin B. The assay was carried out under the standard conditions described in Materials and Methods. The presence of the dye blocks the increase in turbidity of the granule suspension. (Ca^{2+}) = 400 μM.

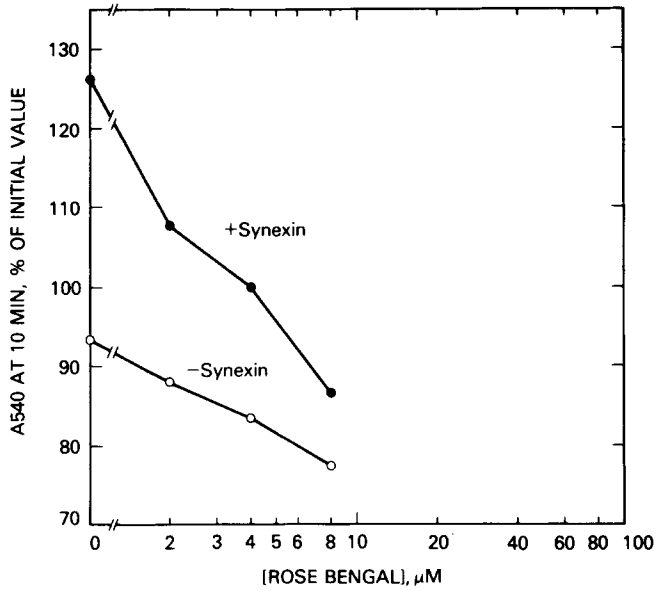

Fig. 9. Inhibition of synexin-induced chromaffin granule aggregation by Rose Bengal. The dye also causes the lysis of the granules (lower curve). $(Ca^{2+}) = 400\ \mu M$.

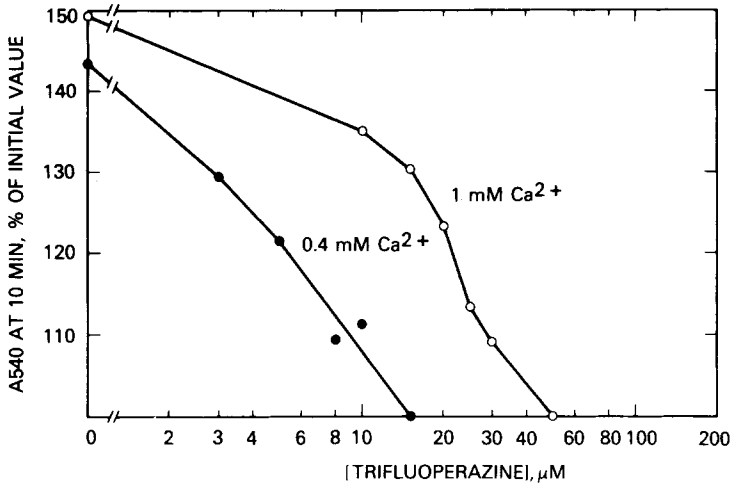

Fig. 10. Inhibition of synexin-induced chromaffin granule aggregation by trifluoperazine at two concentrations of calcium.

maffin granule aggregation reaction. The drug did not, however, block synexin self-association, which was monitored by light scattering measurements on purified synexin samples. The effect of TFP in this system is probably not due to an action on calmodulin (if indeed any calmodulin is present), since a phenothiazine analogue, promethazine, which interacts with calmodulin with only extremely low affinity [13], was also effective at blocking synexin action (Fig. 11).

We have recently observed that both TFP and promethazine block veratridine—or A23187—activated secretion of epinephrine from isolated chromaffin cells

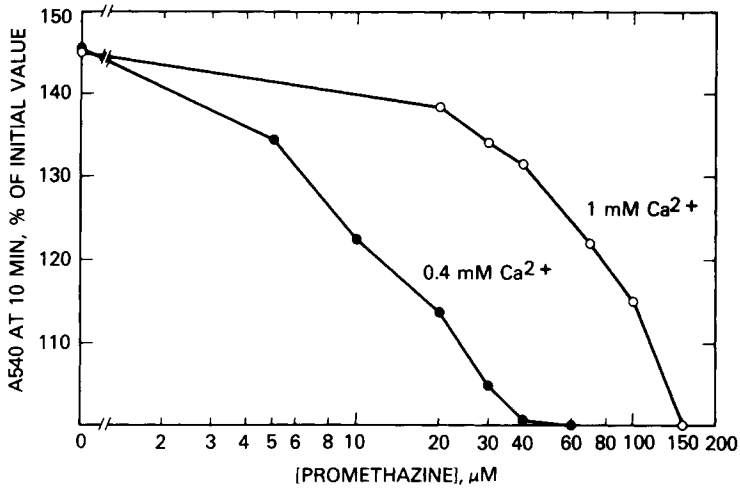

Fig. 11. Inhibition of synexin-induced chromaffin granule aggregation by promethazine at two concentrations of Ca^{2+}.

at a concentration of 25 μM for half-maximal inhibition (H. B. Pollard and C. E. Creutz, manuscript in preparation). The sensitivity of the cells to both drugs indicates that the drugs cannot be acting solely on calmodulin to inhibit secretion. Similar studies on other cell types in which TFP has been demonstrated to block secretion, but analogues have not been tested (eg, [14]), do not provide unequivocal evidence that calmodulin is the mediator of Ca^{2+} action in stimulus secretion coupling.

The concentrations of phenothiazines that inhibited granule aggregation were influenced by the concentration of Ca^{2+} in the medium (figs. 10, 11). This effect was such as to suggest that Ca^{2+} and the phenothiazines acted antagonistically, although we did not demonstrate that the drugs compete directly with Ca^{2+} for the same site.

If the inhibition of secretion from chromaffin cells by the phenothiazines is due to their effects on synexin-mediated membrane interaction, then the level of sensitivity of the cells to the drugs may be an indication that the concentration of Ca^{2+} at the site of membrane interaction during exocytosis is on the order of 400 μM to 1 mM. Presumably this high concentration of free calcium could only occur near the site of Ca^{2+} entry from the extracellular medium.

CONCLUSION

The experiments described in this report demonstrate that synexin can be isolated from liver tissue as well as adrenal medullary tissue and that the proteins from these two sources are indistinguishable in their Ca^{2+}-dependent interaction with chromaffin granules and are equally sensitive to an antiserum prepared to adrenal synexin. Since chromaffin granules are not present in liver, synexin must perform some general function common to more than a single tissue—such as regulation of membrane contact in exocytosis. In addition to membrane contact, if in the stimulated cell a phospholipase causes the release of an unsaturated fatty acid, such as arachidonic acid, then synexin action can lead to actual membrane fusion. The sensitivity of these processes to xanthane dyes and phenothiazine antipsychotics, in ad-

dition to being of toxicological and pharmacological interest, may eventually provide insights into the mechanisms underlying synexin action and exocytosis.

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REFERENCES

1. Creutz CE, Pazoles CJ, Pollard HB: J Biol Chem 253:2858–2866, 1978.
2. Creutz CE, Pazoles CJ, Pollard HB: J Biol Chem 254:553–558, 1979.
3. Pazoles CJ, Pollard HB: J Biol Chem 253:3962–3969, 1978.
4. Lazarides E, Weber K: Proc Natl Acad Sci USA 71:2268–2272, 1974.
5. Morgan WT: Biochim Biophys Acta 538:319–333, 1978.
6. Creutz CE: J Cell Biol 91:247–257, 1981.
7. Pollard HB, Pazoles CJ, Creutz CE: Recent Progress in Hormone Research 37:299–332, 1981.
8. Ahkong QF, Fisher D, Sampson W, Lucy JA: Biochem J 136:147–155, 1973.
9. Marcus AJ: J Lipid Res 19:793–826, 1978.
10. Hotchkiss A, Pollard HB, Scott J, Axelrod J: Fed Proc (in press).
11. Levitan H: Proc Natl Acad Sci USA 74:2914–2918, 1977.
12. Barker JL, Levitan H: J Membrane Biol 25:361–380, 1975.
13. Levin RM, Weiss B: Mol Pharmacol 12:581–589, 1976.
14. Schubart UK, Erlichman J, Fleischer N: J Biol Chem 255:4120–4124, 1980.