Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1164-1171

SPECIFICITY OF SYNEXIN-INDUCED CHROMAFFIN GRANULE AGGREGATION

Michael Dabrow, Samuel Zaremba, and Ruth A. Hogue-Angeletti

Laboratory of Neuropathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received August 26,1980

ABSTRACT

Preparations of synexin (1) exhibit a self-interaction in the absence of chromaffin granules as evidenced by an increase in absorbance at the wavelength used for observing granule aggregation (2). We incorporated this observation into a new formula for calculating the synexin-induced chromaffin granule aggregation. According to this amended analysis, synexin-induced aggregation is specific for chromaffin granules or their membranes. Treatment of intact chromaffin granuleswith trypsin or pronase renders the granules unresponsive to synexin.

An adrenal medulla cytoplasmic protein has been reported (1) to induce aggregation of isolated chromaffin granules. This protein, called synexin, was postulated to play a role in the events leading up to fusion of the granule with plasma membrane, a step required for exocytosis (3-7). However, Morris and Hughes (8) recently reported that synexin induces aggregation of foreign membrane systems. We have developed an amended analysis of granule aggregation which we feel is more rigorous than those previously described. According to our analysis, but not according to the earlier one, synexin-induced aggregation is probably specific for the chromaffin granule system and does not occur with non-secretory organelles. As a part of our study of the topography of the chromaffin granule membrane, we found that proteolytic treatment of the outer surface of intact granules renders the granules incapable of aggregation with synexin.

MATERIALS AND METHODS

Synexin was prepared from fresh adrenal medullae according to the method of Creutz et al (1), except that a 25% saturated ammonium sulfate fraction was taken in precipitates I and II. Aggregation assays were carried out using either the clarified second ammonium sulfate precipitate which had been dialyzed overnight against 40 mM histidine/HCl pH 6.0, 300 mM sucrose (sucrose-histidine),or synexin pooled from active fractions of a 1.5 x 93 cm column of AcA 34 developed in sucrose-histidine (crude and purified synexin, respectively). Chromaffin granules

0006-291X/80/191164-08$01.00/0

were prepared from fresh adrenal medulla tissue dissected free of cortex according to Bartlett and Smith (9). The final granule pellet was resuspended in 10 mM Tris-Cl pH 7.5, 0.32 M sucrose (Tris-sucrose) and washed several times in the same buffer at 12,000 g for 10 minutes in a Sorvall RC-2B centrifuge. These granules were 95-98% intact as assessed by distribution of catecholamines and were still a minimum of 75-80% intact after standing two hours at room temperature. Sheep red blood cells were suspended in 10 mM acetate buffer pH 5.4 and pelleted by centrifugation as above. The pellet was rewashed several times and ghosts were finally resuspended in sucrose-histidine. Mitochondria were prepared from two separate sources: bovine adrenal cortex and bovine adrenal medulla. Fresh cortices were cut up and homogenized in 0.25 M sucrose and the mixture passed through cheesecloth. The filtrate was centrifuged to remove cellular debris and the supernatant centrifuged for 30 minutes at 11,000 g, generating a crude mitochondrial pellet. The procedure was repeated and the mitochondria were washed several times at 11,000 g to yield a final pellet (10). The source material for medullary mitochondria was an upper layer from a Percoll gradient used in the preparation of chromaffin granules (a gift from S. Carty). The layer was aspirated off, diluted with 0.25 M sucrose and washed four times in the same solution by centrifuging at 11,000 g to yield a final pellet. Mitochondrial pellets were suspended in sucrose-histidine for use in aggregation assays.

Fresh, intact granules prepared as above were suspended in Tris-sucrose and incubated at room temperature with buffer (control), trypsin or pronase (0.1 mg) for 90 minutes. At the end of incubation, suspensions were washed twice at 12,000 g with 1 ml soybean trypsin inhibitor (0.1 mg) in Tris-sucrose. Final pellets were resuspended in 1 ml Tris-sucrose for use in the aggregation assay.

Assays were carried out in 1 ml volumes in spectrophotometer cuvettes at 37° by measuring absorbance at 540 nm. Granules or other systems were incubated in 28 mM histidine-HCl pH 6.0, 240 mM sucrose, 30 mM KCl, 3 mM $CaCl_2$, 2.5 mM EGTA (sucrose-histidine-Ca buffer) to 37° and synexin containing material was then added (1). Absorbance at 540 nm at zero time and at 8 minutes was recorded. Background controls were examined in each series in which the change in absorbance due to the granules alone or the synexin preparation alone were monitored.

The percentage of initial absorbance that was observed after 8 minutes (% A_i) of incubation in the complete reaction (granules + synexin) can be expressed without considering any changes in absorbance contributed by the individual components.

$$\%\ A_i \text{ unadj.} = (A_{8\ min}/A_{zero})_{\text{synexin + granules}} \times 100$$

Creutz and coworkers (1) observed that in the absence of synexin (syn), suspensions of intact granules (gran) showed a decrease in absorbance as a function of time. They included a term for this observation in their expression for synexin-induced aggregation.

$$\%\ A_i \text{ Creutz} = \frac{A_{8\ min,\ syn\ +\ gran} - (A_{8\ min} - A_{zero})_{gran}}{A_{zero,\ syn\ +\ gran}} \times 100$$

We observed that all our synexin preparations exhibited a significant increase in absorbance in the absence of granules. We added a term to the equation to include this observation.

$$\%\ A_i \text{ Dabrow} = \left\{ \frac{A_{8\ min,\ syn\ +\ gran} - [(A_{8\ min} - A_{zero})_{gran} + (A_{8\ min} - A_{zero})_{syn}]}{A_{zero,\ syn\ +\ gran} \right\} \times 100$$

The intactness of granule preparations was determined by separating granule suspensions according to the centrifugation above and then assaying the resulting supernatant and pellet for catecholamines according to von Euler and Floding (11) Immunodiffusion was according to Ouchterlony (12) using antiserum directed against purified dopamine β-monooxygenase (E.C. 1.14.17.1), the major protein in chromaffin granules.

Trypsin (TP-TPCK) was obtained from Worthington Biochemicals. Pronase, a nonspecific bacterial protease from S. Griseus, and soybean trypsin inhibitor were obtained from Sigma Chemical Corporation. AcA 34 for gel filtration was obtained from LKB Instruments. Sheep red blood cells were obtained as a washed 10% suspension from Cappel Laboratories (Downingtown, PA). All other chemicals were laboratory stock. Water used throughout was doubly distilled, deionized, and charcoal filtered water (Hydro Services).

RESULTS

Comparison of formulae in test reactions. In attempts to consider all possible contributions to the synexin aggregation assay, we defined the per cent of initial absorbance ($\%A_i$) as previously stated in Methods . Table I shows both the raw data for the granule aggregation assay and the calculated results

TABLE I

AGGREGATION OF CHROMAFFIN GRANULES WITH SYNEXIN - ANALYSIS OF DATA

		A_{540} start	A_{540} 8 minutes	$\%A_i$ unadj.	$\%A_i$ Dabrow	$\%A_i$ Creutz
a.	SYNEXIN ALONE	0.023	0.052			
	GRANULES ALONE	0.285	0.279			
	SYNEXIN + GRANULES	0.285	0.381	134	126	136
	SYNEXIN ALONE	0.026	0.054			
	GRANULES ALONE	0.150	0.140			
	SYNEXIN + GRANULES	0.150	0.206	137	125	144
b.	SYNEXIN ALONE	0.027	0.071			
	GRANULES ALONE	0.257	0.245			
	SYNEXIN + GRANULES	0.207	0.280	135	120	141
		0.257	0.338	132	119	136
		0.258	0.343	133	121	138
		0.266	0.345	130	118	134
		0.206	0.278	135	119	141
					119.4 ± 0.88	

Aggregation assay using crude synexin and freshly prepared chromaffin granules. Cuvettes contained granules and sucrose-histidine-Ca buffer at 37°. Crude synexin (20-25 µl) was added, making a total volume of 1 ml and absorbance at 540 nm was monitored. Controls contained synexin alone or granules alone in buffer. The value % A_i Dabrow is the degree of aggregation according to our formula. The value % A_i Creutz represents the degree of aggregation using our experimental data according to the formula offered in REF. 1.

TABLE II

SPECIFICITY OF SYNEXIN INDUCED AGGREGATION: EXPERIMENTS WITH NON-SECRETORY ORGANELLE MEMBRANES

	$\%A_i$ unadj.	$\%A_i$ Dabrow	$\%A_i$ Creutz
CHROMAFFIN GRANULES	119	116	121
ERYTHROCYTE GHOSTS	118	100	118
ADRENAL CORTEX MITOCHONDRIA	103	99	104
ADRENAL MEDULLA MITOCHONDRIA	108	105	110

Standard synexin assay using various non-secretory organelle membrane systems in place of chromaffin granules. Cuvettes contained 100 µl erythrocyte ghosts or 5 µl of either mitochondrial suspension in buffer. Conditions and controls were as described in the legend in TABLE I.

according to the three formulations. Assays were performed with either crude synexin or with synexin purified from the AcA 34 column described above. Within a given preparation, the results were reproducible. The increase in absorbance seen in the synexin background reaction is probably due to synexin self-aggregation. (2). Comparison of the three formulations in the test assays yields lower values for our formulation because this factor is taken into account. As described below, use of the other formulations which ignore the synexin contribution can lead to misleading conclusions about the reactivity of synexin with other membrane systems.

Synexin aggregation of other membrane systems. When erythrocyte ghosts were incubated together with synexin, the absorbance at 540 nm increased significantly (Table II). However, in the same series of experiments, when crude synexin was incubated alone, it exhibited an absorbance increase almost precisely equal to that observed with the full system. Therefore, if the results are portrayed without taking into consideration the synexin contribution ($\%A_i$ unadj or $\%A_i$ Creutz), one would conclude that synexin induces aggregation of erythrocyte membranes. However, if this factor is taken into account ($\%A_i$ Dabrow), it can be seen that the erythrocyte membranes do not aggregate at all.

Similarly, when purified synexin was incubated with mitochrondria from adrenal cortex (Table II), the absorbance increases detected in the complete reaction could be fully accounted for by the increases seen in the synexin-

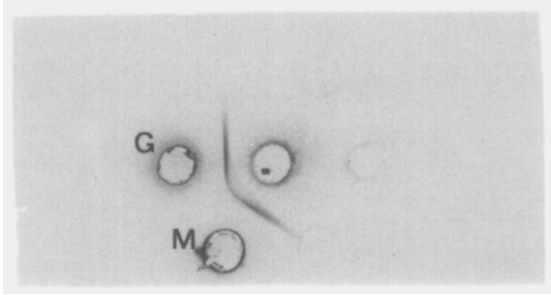

FIGURE 1 Ouchterlony immunodiffusion of mitochondrial suspensions and chromaffin granules. Center well contained rabbit antiserum raised against purified dopamine ß-monooxygenase from the soluble portion of chromaffin granules. G - 1% triton x-100 extract of granules. M - 1% triton x-100 extract of adrenal medulla mitochondria.

preparation alone. However, when the source of mitochondria is the adrenal medulla, an apparent synexin-specific mitochondrial aggregation can be detected, as reported by other investigators (8). When this preparation of mitochondria was examined by immunodiffusion against antiserum to purified dopamine ß-monooxygenase, a positive reaction was obtained (Figure 1). Therefore, the adrenal medulla mitochondrial preparation is contaminated with significant amounts of chromaffin granule membranes which could account for the observed aggregation. The observation that cortical mitochondria do not aggregate in the presence of synexin, reinforces the notion that the synexin reaction is specific. However, these results are clear only when a more rigorous analysis is made of the factors contributing to the absorbance changes in the reaction mixture.

Proteolytic digestion of chromaffin granules. Table III presents the results of typical synexin assays in which the chromaffin granules have been treated with proteases. Control granules incubated for 90 minutes can still be aggregated by crude or purified synexin. However, trypsin and pronase treated granules have lost the ability to be aggregated by synexin. Thus, it appears that the synexin-specific granule aggregation is mediated through a protease-sensitive factor present on the cytoplasmic face of the granule membrane. This protein appears to be particularly susceptible to tryptic cleavage.

TABLE III

EFFECT OF PROTEOLYTIC TREATMENT OF GRANULES ON SYNEXIN INDUCED AGGREGATION

	$\%A_i$ Dabrow	
CONTROL GRANULES	118.3 ± 0.43	(3)
TRYPSIN - TREATED GRANULES	102.5 ± 0.5	(2)
PRONASE - TREATED GRANULES	101.6 ± 2.2	(3)

Fresh intact chromaffin granules were incubated for 90 minutes at room temperature with buffer (control) or proteolytic enzymes (0.1 mg/ml). Granule concentration was 1.35 mg membrane protein/ml. At end of incubation, suspensions were washed twice with 1 ml (0.1 mg) soybean trypsin inhibitor in buffer and resuspended for use in standard aggregation assay. Controls and conditions were as presented in the legend to TABLE I. Numbers in parentheses indicate the number of assays in each category.

DISCUSSION

In an effort to further characterize the nature of the synexin-granule interaction (1) we found it necessary to re-evaluate the analysis of granule aggregation described by these authors, taking into account the synexin self-aggregation reaction (2). To assess the possible effect of this property on the observed reaction with granules, we measured the absorbance changes of synexin preparations alone under the same reaction conditions; that is, 37° and sucrose-histidine-Ca buffer. An increase in absorbance at 540 nm of the synexin alone was always observed, but was particularly marked as the preparations aged. It could be minimized by using freshly prepared material, but the contribution of the synexin alone could not be ignored. We therefore constructed our new formulation for absorbance change due to synexin-specific aggregation. When erythrocyte ghosts or cortical mitochondria were used as substrates for synexin binding, our data indicate no aggregation. The data interpreted according to earlier conventions would show significant increases. However, in these cases the increases are accounted for by increases in the synexin preparation alone. We interpret these data as support for the specificity of synexin for chromaffin granule aggregation.

Mitochondrial suspensions prepared from adrenal medulla apparently aggregate with synexin, but there is ample reason to believe that this is due to the contamination of this preparation by chromaffin granules.

In a recent report (8), synexin was found to induce aggregation of various other subcellular organelles. It was thereby concluded that synexin could not be a specific factor in secretion in the cell. However, these authors do not report considering the absorbance increases in the synexin preparation alone as we have done. In addition, certain subcellular fractions of adrenal medulla may be contaminated with chromaffin granules. It is possible that if these factors are considered, they would account for the observed increases in absorbance.

In order to further determine the specificity and nature of the reaction, chromaffin granules were subjected to protease digestion. The clear difference between control and treated granule aggregation argues for the existence of a protein receptor. The lack of distinction between trypsin-and pronase-treated granules in the aggregation assay suggests that cleavage at a tryptic site alone releases the receptor from the membrane.

The digestion data in conjunction with the foreign membrane systems data, argues for the specific protein-mediated interaction of synexin with chromaffin granules. Proteolytic digestion experiments have indicated the presence of other proteins on the cytoplasmic face of granules membranes (13-15). Logically, a receptor involved in granule aggregation could be expected to be located on the cytoplasmic surface.

ACKNOWLEDGMENT

This work was supported by NIH Grant NS-13201 and NS-00201.

REFERENCES

1. Creutz, C.E., Pazoles, C.J., and Pollard, H.B. (1978) J. Biol. Chem. 253, 2858-2866.
2. Creutz, C.E. Pazoles, C.J., and Pollard, H.B. (1979) J. Biol. Chem. 254, 553-558.
3. Douglas, W.W. (1974) Biochem. Soc. Symposium 39, 1-28.
4. Winkler, H. (1977) Neurosciences 2, 657-683.
5. Hogue-Angeletti, R.A., Roda, L.G., Nolan, J.A., and Zaremba, S. (1980)in Proteins in the Nervous System (ed.) D. Schneider and R.A. Bradshaw, Raven Press, 257-282.

6. Kirshner, N.K., and Viveros, O.H. (1974) Pharm. Reviews 24, 385-398.
7. Margolis, R.K., Janus, S.D., and Margolis, R.V. (1973) Mol. Pharm. 9, 590-594.
8. Morris, S.J. and Hughes, J.M.X. (1979) Biochem. and Biophys. Res. Comm. 91, 345-350.
9. Bartlett, S.F. and Smith, A.D. (1974) Methods in Enzymology XXXI 379-389
10. Dr. H. Schleyer, personal communication
11. Von Euler, U.S., and Floding, I. (1955) Acta Physiol. Scand. 33 (supp.) 45-56.
12. Ouchterlony, O. (1958) Progress in Allergy 5, 1-10.
13. Abbs, M.T. and Phillips, J.H. (1980) Biochim. Biophys. Acta 595, 200-221.
14. Meyer, D.I., and Burger, M.M. (1976) Biochim. Biophys. Acta 443, 428-436.
15. Zaremba, S., and Hogue-Angeletti, R.A. manuscript in preparation.