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SYNEXIN PROTEIN IS NON-SELECTIVE IN ITS ABILITY TO INCREASE Ca^{2+}-DEPENDENT AGGREGATION OF BIOLOGICAL AND ARTIFICIAL MEMBRANES

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SUMMARY: Synexin, a soluble protein which increases the specificity of Ca^{2+} to aggregate isolated bovine chromaffin granules was prepared from bovine adrenal medullary tissue by the method of Creutz, Pazoles and Pollard (J. Biol. Chem. 253, 2858-2866, 1978). We also find that synexin increases both the initial rate and final amplitude of Ca^{2+}- promoted aggregation of granule membranes. This effect is Ca^{2+}-specific. However in contrast to Creutz et al, we find that synexin also potentiates aggregation of adrenal medulla and liver mitochondria and microsomes as well as phosphatidylserine vesicles. This lack of membrane specificity argues against the suggestion of Creutz et al that synexin specifically binds the granule to the plasma membrane prior to exocytosis in vivo.

INTRODUCTION: Exocytotic release of catecholamines, ATP, and protein from the chromaffin granules of adrenal medullary cells in vivo is triggered by the entry of calcium in response to cell depolarization (1). The ability of Ca^{2+} to aggregate and eventually fuse isolated granules (2,3,4,5) has led to the suggestion that these processes may serve as a model for exocytotic release. Ba^{2+}, Sr^{2+}, and the trivalent lanthanides La^{3+}, Tb^{3+}, and Eu^{3+} will aggregate granules but no fusion is seen (5). However Mg^{2+} is about 3-4 fold less effective than Ca^{2+} in fusing granules (3), and equally effective as an aggregating agent (6) Although Mg^{2+} inhibits exocytosis in vivo, Heuser and collaborators (7, 8) have demonstrated that Mg^{2+} and Sr^{2+} seem to "freeze" the exocytosis process at the point in which the secretory vesicle contacts its release site. This suggests that Ca^{2+} may control exocytosis by activating reactions or structural rearrangements which follow adhesion of the granules to its release site on the membrane.

The steady-state binding constant k_d for Ca^{2+}- or Mg^{2+}-promoted dimerization of granule membranes is $\sim$ 2 mM (6). This is well above the expected rest-

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethansulphonic acid; PS, phosphatidylserine.

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ing cell Ca^{2+}-activity, although calculations (9) suggest that transient concentrations at the release site of 10^{-5} M are not unreasonable. Recently Creutz et al (2) have purified a soluble protein from adrenal medullary tissue, named synexin, which will selectively increase the specificity of Ca^{2+}-promoted aggregation of isolated adrenal medullary chromaffin granules. Synexin lowered the binding constant for Ca^{2+}-promoted aggregation of intact chromaffin granules to 150-200 μM. The protein had no effect on Mg^{2+}- or Sr^{2+}-promoted granule aggregation. Additionally it was not active in Ca^{2+}-promoted aggregation of medullary cell mitochondria (2). These observations have led to the suggestion that synexin directly couples Ca^{2+} entry to granule exocytosis by first specifically binding to the granule membrane and subsequently binding the granules to their release site by a Ca^{2+}-specific process of relatively high Ca^{2+}-affinity (2). This report confirms the findings of Creutz et al (2) that synexin aggregates isolated granules by a Ca^{2+}-specific process. In sharp contrast to their results, we find that synexin will also aggregate intact mitochondria as well as mitochondrial, microsomal and phosphatidylserine (PS) membranes. Therefore we question the specificity of its hypothetical role in exocytosis in vivo.

METHODS: Synexin was prepared and purified by chromatography on Ultragel AcA34 by the method of Creutz et al (2) except that the original homogenization of the dissected bovine medullary tissue was performed in 0.26 M sucrose buffered with 10 mM HEPES at pH 7.4. Adrenal medullary chromaffin granules and mitochondria were prepared from the same homogenate by previously described methods (10) as were mitochondria and microsomes from bovine liver homogenates. Lysed chromaffin granule "ghosts" were prepared as described previously (10). These particles had the same aggregation properties as did whole granules. However whole granules readily lyse when exposed to 37°C (2, 11) and the resulting reduction in turbidity competes with the increase in turbidity seen in aggregation (12). Therefore the ghosts make a better substrate for the synexin-mediated aggregation reaction. Mitochondrial and microsome membrane fractions were prepared by the same lysis procedure except that the latter were centrifuged at 40,000 g-max for 30 min. Protein was determined by the method of Bradford (13). Electrophoresis of granule and mitochondrial membrane samples solubilized in sodium duodesyl-sulfate was performed as described previously (10). Bilayer vesicles were formed by sonicating 4.0 mg of bovine PS in 2.0 ml of 10 mM KCl, 10 mM HEPES, pH 7.4 (14).

RESULTS AND DISCUSSION: At lower than saturating Ca^{2+}-concentrations, synexin increased Ca^{2+}-specificity of both the initial rate and amplitude of the aggregation of adrenal medullary granule and mitochondrial membranes, liver mitochondrial and microsomal membranes, as well as unlysed material at both pH 6.0

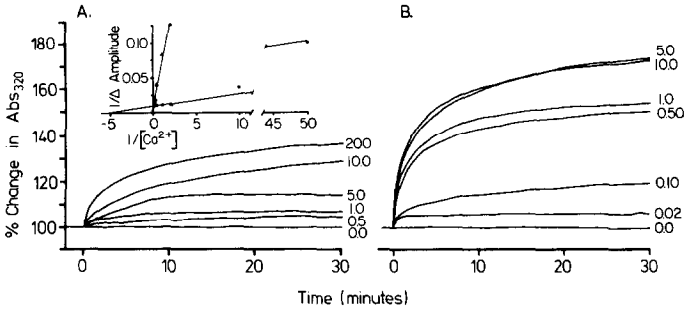

Figure 1. Ca^{2+}-promoted aggregation of chromaffin granule membranes in the (A) absence or (B) presence of synexin. The progress of the reaction was recorded as the time dependent changes in the turbidity (absorbance) measured at 320 nm (cf. reference 12). Fifty µl of a concentrated membrane suspension in 10 mM KCl, 10 mM HEPES, pH 7.4, was added to a cuvette containing 450 µl of 30 mM HEPES, 10 mM KCl, pH 7.4, in a cuvette holder thermostated at 37°C. (If synexin was present, it was added as a 50 µl volume in 10 mM KCl, 30 mM HEPES, pH 7.4 to 400 µl of the HEPES/KCl buffer). The absorbance at 320 nm was recorded as a base line (= 100%). At time 0, 5-10 µl of a concentrated solution of the perturbing cation was added by hand mixing (~ 5 sec) and the change in absorbance recorded as a function of time. Chromaffin granule membrane protein concentration was 8 µg/ml (~ 35 µg/ml whole granule protein). Synexin concentration when present was 10 µg/ml. Final Ca^{2+}-concentrations are noted for each trace. Repetition of experiments gave almost identical traces.
Inset. Reciprocal change in turbidity of chromaffin granule membranes as function of reciprocal Ca^{2+}-concentration measured 30 minutes after addition of the Ca^{2+}. As can be seen the higher $[Ca^{2+}]$-values gave saturating amplitudes; therefore these points were excluded from the least squares fit. See text for further details.
(o) synexin absent (Δ) synexin present

(Histidine buffer; conditions the same as reference 2) and pH 7.4. Figures 1 and 2A show these phenomena for granule and liver mitochondrial membranes at pH 7.4. The change in absorbance is usually complete within 30 minutes (exceptions: high $[Ca^{2+}]$ in the presence of synexin). Therefore the equivalent of a steady-state binding constant can be calculated from Lineweaver-Burk type plots as the reciprocal negative x-intercept of a plot of $1/[Ca^{2+}]$ versus 1/Δ-absorbance, and the maximal change in amplitude as the reciprocal y-intercept (figure 1, Inset). Results from several experiments are tabulated in Table 1, which demonstrates that synexin lowers the Ca^{2+} concentration required for half-maximal aggregation, and except for the microsomal membranes, increases the maximal amplitude of aggregation as well. Lysed mitochondria were better substrates for the reaction than whole mitochondria. A stopped-flow study of the kinetics of synexin-promoted membrane aggregation is in progress.

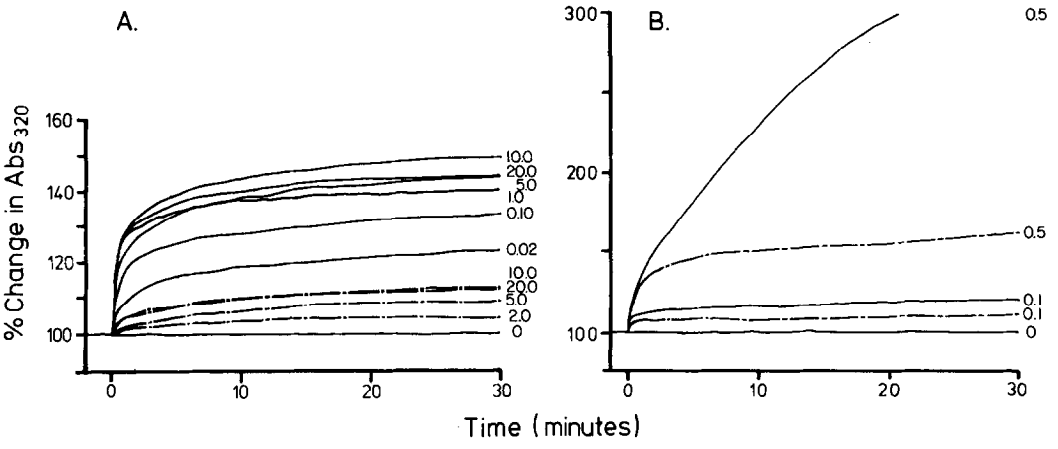

Figure 2. Ca^{2+}-promoted aggregation of (A) liver mitochondrial membranes and (B) phosphatidylserine vesicles in the absence (-··-) and presence (——) of synexin. Final membrane and synexin protein concentrations were 21 and 8 µg/ml for A and 20 µg/ml and 3.6 µg/ml for B respectively. Assays were performed as described for figure 1.

Synexin also enhances Ca^{2+}-promoted aggregation of negatively charged PS vesicles (figure 2B) although no effect was seen with phosphatidylcholine, a neutral lipid (data not shown). It is not possible to calculate K_d's for PS vesicle aggregation since the system never comes to equilibrium but continues to aggregate for more than 1 hour. Synexin activity is located in exactly the same AcA34 column fractions as measured either by PS vesicles or chromaffin granule membranes.

TABLE I: Ca^{2+}-aggregation of various biological membrane fractions in the absence (-) or presence (+) of synexin at pH 7.4. Assay conditions are as described for figure 1 for the membrane and synexin concentrations listed. The steady-state binding constant K_d and the maximal change in amplitude ΔA_{max} were calculated as described in the text (cf. figure 1, Inset).

MEMBRANES	PROTEIN [µg/ml]	SYNEXIN [µg/ml]	K_d* (-)	K_d* (+)	ΔA_{max}** (-)	ΔA_{max}** (+)
ADRENAL MEDULLA:						
CHROMAFFIN GRANULE	8	10	2.77	0.299	38	101
INTACT MITOCHONDRIA	21	8	1.39	0.737	50	76
LIVER:						
MITOCHONDRIA	17	3	6.90	0.017	20	43
MICROSOMAL	55	2	17.1	6.74	197	170

* µM Ca^{2+} ** ((maximal A_{320} - baseline A_{320})/(baseline A_{320})) x 100%

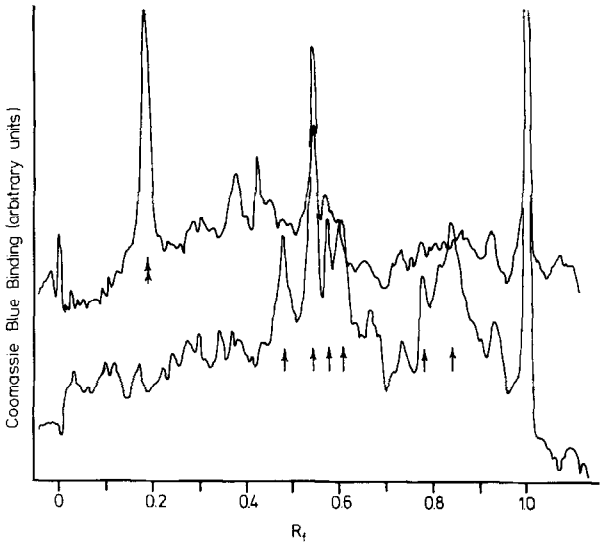

Figure 3. Densitometric tracings of mitochondrial and chromaffin granule membranes electrophoresed on SDS-containing acrylamide gel slabs stained with Coomassie Blue as described in reference 10. Lower trace: mitochondrial membranes; upper trace: granule membranes. No disulphide reagent was included, since this shifts the dopamine-β-hydroxylase dimer (double arrow) of mw ≈ 150,000 to ≈ 75,000 (cf. reference 10). Single arrows denote major bands in the mitochondrial profile.

The activity cannot be due to contamination of liver fractions or PS vesicles by adrenal medullary granule membranes. Contamination of the medullary mitochondria by granules was ruled out by comparing the electrophoretic pattern of granule ghosts with mitochondria lysed by the same method (figure 3). Dopamine-β-hydroxylase (double arrow), a characteristic band for granule membranes is absent from the mitochondria sample. Bands characteristic of the mitochondria (arrows) may be present in the granule fractions, suggesting that if anything, the latter may be contaminating the former.

Although the reaction lacked membrane specificity, Ca^{2+}-specificity for synexin-mediated aggregation was maintained, no increase in rate or amplitude of aggregation promoted by Mg^{2+}, Ba^{2+}, Sr^{2+}, Ni^{2+} or Tb^{3+} was observed. The order of addition of the perturbants had no effect on the outcome of the experiments.

Despite the absence of membrane specificity, the calcium specificity of the aggregation reaction suggests that synexin might belong to the growing number of calcium-modulator proteins such as calsequestrin (15) which regulates free Ca^{2+}

in muscle, calmodulin (16) which regulates enzyme activities, or troponin-C (17) which regulates cytoskeletal structure. Creutz et al. (18) have also shown that Ca^{2+} will specifically promote the self-aggregation of synexin into 50 x 150 Å rods which will further aggregate and elongate into bundles. This latter activity suggests that the protein may also be active in maintenance of the cytoskeleton. Thus synexin could function in exocytosis by rapidly polymerizing during Ca^{2+}-influx, promoting the rearrangement of cytoskeletal elements and allow approach of the granules to their release site. However, synexin enhanced aggregation of PS vesicles suggests that only negative surface charges are necessary to produce the Ca^{2+}-specific effect. Obviously a better understanding of the physiological role of synexin in the cell is necessary before further speculation can be made.

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