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IDENTIFICATION OF A SECOND SYNEXIN-LIKE ADRENAL MEDULLARY AND LIVER PROTEIN THAT ENHANCES CALCIUM-INDUCED MEMBRANE AGGREGATION

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SUMMARY: Synexin, an ~ 47,000 M_r soluble protein isolated from adrenal medulla or liver, shows Ca^{2+}-specific enhancement of the aggregation of chromaffin granule or other negatively charged biological or artificial membranes. We report the identification of second synexin-like protein (M_r ~ 56,000) from the same sources with similar Ca^{2+}-specific membrane aggregation activities. However, the molecular weight, aggregation kinetics, susceptibility to protease inactivation and peptide maps of the two synexins are quite different, suggesting that they are entirely different proteins, and that the aggregation assay is only a convenient method for identifying a large number of Ca^{2+}-specific proteins with diverse, yet to be defined activities.

Synexin is the name suggested for a soluble ≈ 47,000 M_r protein originally isolated from adrenal medullary homogenates (1) and later from liver (2), which enhances aggregation of adrenal chromaffin granule, mitochondrial and negatively charged artificial phospholipid membranes by a calcium-specific mechanism (1, 3). Recently, relatively pure synexin has been shown to facilitate both aggregation and fusion of specific phospholipid membranes (4-7). Synexin also self-aggregates in the presence of calcium (8). This self-association has recently been employed to enhance the purity of the 47,000 M_r protein without appreciable loss of activity (6).

In this report, we demonstrate the presence of a second synexin-like protein (Synexin II) in adrenal medullary and liver homogenates. Synexin II enhances calcium but not magnesium-induced chromaffin granule membrane or

Abbreviations: PS, phosphatidyl serine; PC, phosphatidyl choline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacitic acid.

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phosphatidylserine (PS) liposome aggregation. The kinetics of synexin II-enhanced calcium-induced aggregation differ greatly from that of the previously described synexin (Synexin I). Aggregation activity of synexin I is abolished by both chymotrypsin and Staphlococcus aureus V-8 protease while synexin II is highly resistant to V-8 protease. The two proteins have different one-dimensional polypeptide maps. Synexin-mediated chromaffin granule aggregation and self-aggregation properties have raised the speculation that synexin may have a specific physiological role in the exocytotic release of catecolamines from the medullary cells (9, 10). The significance of the findings reported here is discussed in relation to this proposal.

METHODS

Preparation of Synexins: Synexin fractions were prepared from bovine adrenal medullary or liver homogenates, as previously described (1,2,6), with the following modifications to the gel filtration on columns of Ultragel AcA 34 (LKB, Uppsala, Sweden): (1) addition of 2.5 mM EGTA to the 40.0 mM MES pH 6.02 column buffer and (2) increasing the column dimensions from 1.5 x 60 cm to 1.5 x 75 cm. Two ml fractions were collected at a flow rate of 0.8 ml/min. All procedures were carried out at 0 - 4°C.

Protein concentrations for soluble protein fractions were determined by the Bradford coomassie blue binding assay (11) using bovine serum albumin as the standard. The concentration of lysed chromaffin granule ghosts was determined from the relationship of protein concentration to turbidity at 320 nm: 1.0 µg/ml suspension gives an E_{320} of 0.700 (12).

Assay of Synexin Activity: Synexin-enhancement of Ca^{2+}-induced aggregation of chromaffin granule membranes or PS liposomes was assayed according to the method of Morris et al (6). Activity was measured as the increase in extinction at 320 nm (ΔE_{320}) over an 8 min period after the addition of $CaCl_2$ or $MgCl_2$.

Gel Electrophoresis: One-half ml aliquots of column fractions were dialyzed overnight at 4°C against distilled water containing 1.0 mM PMSF, stored at -70°C and concentrated by freeze drying before electrophoresis on 9 percent polyacrylamide gels under denaturing conditions according to Adams et al. (13).

Sensitivity to Proteases: One-half ml aliquots of synexin I and synexin II peak activity fractions (e.g. fractions 56 and 64 of Fig. 1) containing 50.0 µg/ml of protein were incubated with equal volumes of 45 µg/ml Staphlococcus aureus V-8 protease (Sigma Chemical Co., No. P-8143) or 45 µg/ml chymotrypsin (Sigma Chemical Co., No. C-9381) in 10 mM KCl, 10 mM HEPES pH 7.4 at 37°C. Samples were taken at 5, 10, 20, 30 and 60 min and immediately assayed for synexin activity. The decrease in PS liposome aggregating activity of the fractions, due to protease digestion, was graphed as the percent of control activity (in the absence of enzyme) versus time.

One-dimensional peptide maps were prepared essentially by the method of Cleveland et al (14). Aliquots of tubulin (60 µg/ml) or synexin I or synexin II peak fractions (50 µg/ml) were digested 30 min with protease at 37°C. The reaction was stopped by the addition of an equal volume of 2-fold concentrated sample buffer and heating for 3 min at 100°C before loading onto 20 percent gels.

RESULTS AND DISCUSSION: Prior to the changes in the chromatography procedure, one broad synexin activity peak was observed in the final third of the elution volume (1, 6). In an attempt to increase the resolution and specific activity of our Ultragel AcA 34 purified synexin, we incorporated 2.5 mM EGTA in the column buffer to reduce the possibility of synexin self-association in the presence of Ca^{2+} and lengthened the column an additional 15 cm. These modifications resulted in the resolution of two peaks of activity (Fig. 1A). This result was reproducibly obtained from preparations run on two different columns, using both bovine adrenal medulla (7 preparations) or liver (2

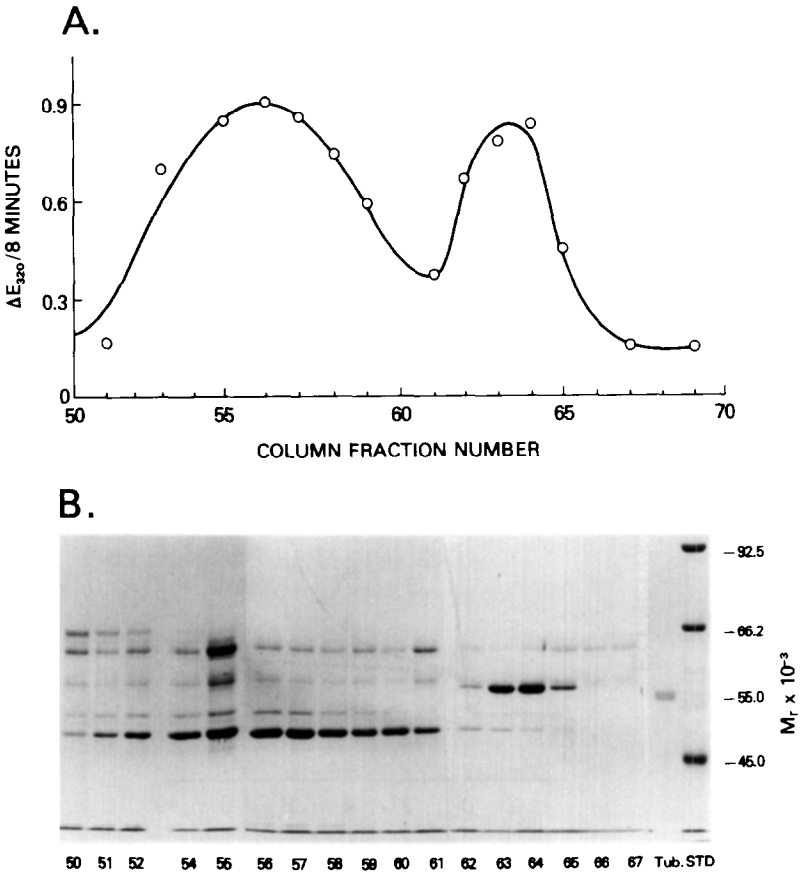

Figure 1: (A) Separation of two peaks of synexin activity. AcA34 column fractions were tested for their ability to enhance Ca^{2+} promoted PS liposome aggregation. Similar results were obtained with chromaffin granule membranes as the substrate. (B) polyacrylamide gel electrophoresis of the same column fractions. Synexin I (M_r ~ 47,000) is the main band of the fractions comprising the first peak of activity with a 62,000 M_r component as the chief contaminant. Synexin II (M_r ~ 56,000) is the main band of peak II with synexin I as the chief contaminant. Molecular weight standards are phosphorylase B, bovine serum albumin, tubulin and ovalbumin in descending order.

preparations) as starting material. It was independent of the absence of HEPES buffer in the 0.3M sucrose homogenization medium (2 preparations), or the presence of 2.5 mM EGTA or 2.5 mM EDTA (2 and 1 preparation respectively). All results presented below are from a single fractionation starting with 210 g of medulla from 60 glands. However, each experiment was performed at least twice on various other preparations.

The first activity peak enhanced Ca^{2+}-induced aggregation of chromaffin granule membranes or PS liposomes as previously reported for 47,000 M_r synexin I (Fig. 2). The addition of Ca^{2+} to synexin I / membrane mixtures resulted in a rapid, pseudo second-order increase in membrane aggregation eventually leading to a pseudo-steady state of aggregation (1, 3, 15). Different kinetics were observed for the second peak of activity. Initially, there was a slow, almost linear increase in membrane aggregation followed by a more rapid rate of change in extinction, leading eventually to a pseudo-steady state. Substitution of $MgCl_2$ (0.5 - 2.0 mM) for $CaCl_2$ had no effect on

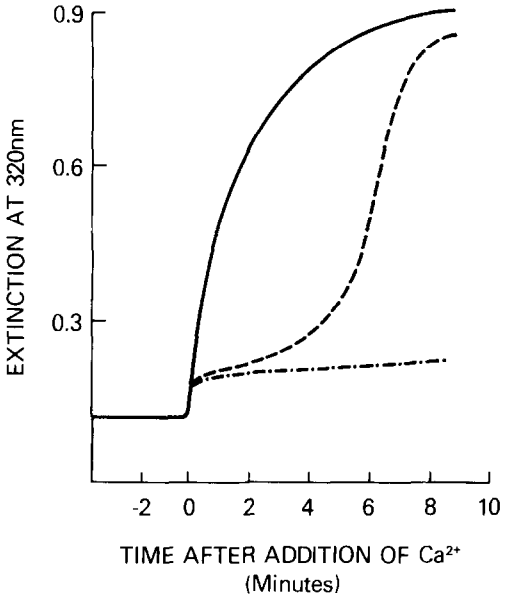

Figure 2: Kinetics of synexin I and synexin II aggregation of chromaffin granule membranes. Assays were performed under the conditions described previously (6). At time 0, $CaCl_2$ (final concentration = 0.80 mM) was added to the chromaffin granule membranes (180 µg protein/ ml; A_{320} = 0.125) in 15 mM KCl, 10 mM HEPES pH 7.4 and the change in A_{320} recorded as a function of time. (-- • --) Control (no synexin present). (------) Synexin I (fraction 56 of Fig. 1) at 7.5 µg/ml present in the assay mixture. (- - -) Synexin II (fraction 64 of Fig. 1) at 8.0 µg/ml present in the assay mixture.

enhancement of membrane aggregation activity by either peak I or peak II fractions. A stopped-flow study of the kinetics of synexin I and II-promoted membrane aggregation is currently under way.

Gel electrophoresis patterns of synexin I and II fractions are displayed in Fig. 1B. The relative increase and decrease in concentration of the 47,000 M_r protein in fractions 50 through 60 corresponds with the activity observed in these fractions, while the increase and decrease of a 56,000 M_r protein, present in the gel patterns of fractions 61 through 67, corresponds to the rise and fall of the second activity peak. The presence of the 56,000 M_r protein in the later fractions suggests that either it has a more globular structure or possesses greater affinity for the Ultragel matrix when compared to the 47,000 M_r synexin I, or that synexin I may chromatograph as a dimer but electrophoresis as a monomer. From the results presented below, it is unlikely that Synexin II represents a monomeric form of synexin I.

The effects of Staphlococcus aureus V-8 and chymotrypsin proteases on the aggregation activity of synexin I and II peak fractions (e.g. Fig. 1, fractions 56 and 64 respectively) are illustrated in figure 3. Both peak I

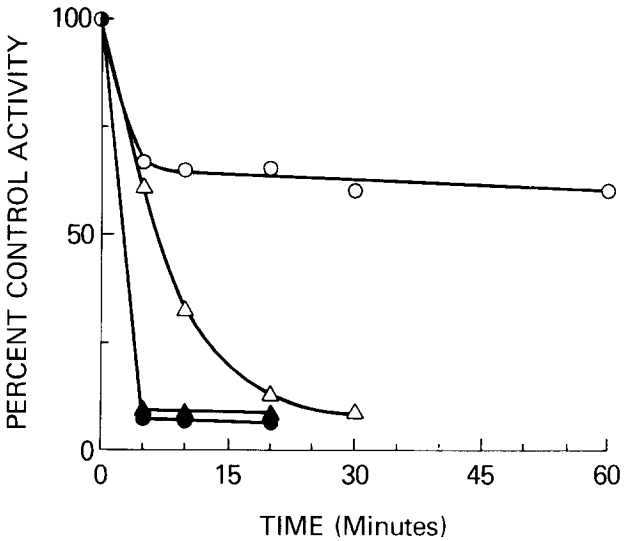

Figure 3: Inactivation of synexin activity by protease digestion. After varying times of digestion, aliquots were taken and tested for PS liposome aggregation activity as described in experimental procedures. V-8 protease-treated synexin I (Δ) and synexin II (o). Chymotrypsin-treated synexin I (▲) and synexin II (●).

and II activities were abolished by chymotrypsin. In sharp contrast, V-8 protease rapidly inactivates synexin I, while the bulk of Synexin II activity remains intact, indicating that the two fractions contain different proteins. The initial rate of peak II inactivation by V-8 protease parallels that of peak I inactivation, suggesting that part of the total peak II activity is made up of the V-8 protease sensitive 47,000 M_r synexin I (cf Fig. 1B). However, the shape of the aggregation curve for V-8 protease did not change appreciably from that of untreated synexin II (Fig. 2). Furthermore, one-dimensional peptide maps of both V-8 protease and chymotrypsin digests of fractions 56 and 64 have different patterns (Fig. 4) which argues that synexin I and II are entirely different proteins.

Synexin II and tubulin have quite similar molecular weights (Figure 1B). Recently, it has been demonstrated that tubulin can be intercalated into sonicated dipalmatyl PC vesicles and that these vesicles will aggregate and fuse in the presence of calcium (16). One dimensional peptide maps of V-8 and chymotrypsin-digested tubulin showed no significant overlaps with either synexin I or II (Fig. 4) and tubulin had no effect on the aggregation of PS vesicles and chromaffin granule membranes (data not shown).

The 62,000 M_r protein, seen as the principal contaminant of the synexin I peak (Fig. 1B), almost certainly does not inhibit synexin I activity, as previously speculated (6). This component peaks in fraction 55 and is greatly reduced in fractions 56-59, whereas synexin I activity peaks in fraction 56 and is very high in fractions 56-59. We found inhibitory activity in a series of column fractions eluting earlier than synexin I which lacked the 62,000 M_r band. When added to the aggregation assay, these fractions produced inhibition of both synexins. However, this peak of inhibition activity contained several proteins of M_r > 65,000, precluding a positive identification of the inhibitor (data not shown).

It has been suggested that synexin I plays a definite role in the exocytotic release of chromaffin granule contents from the adrenal medulla, by attaching the granule to the cell membrane in response to the influx of

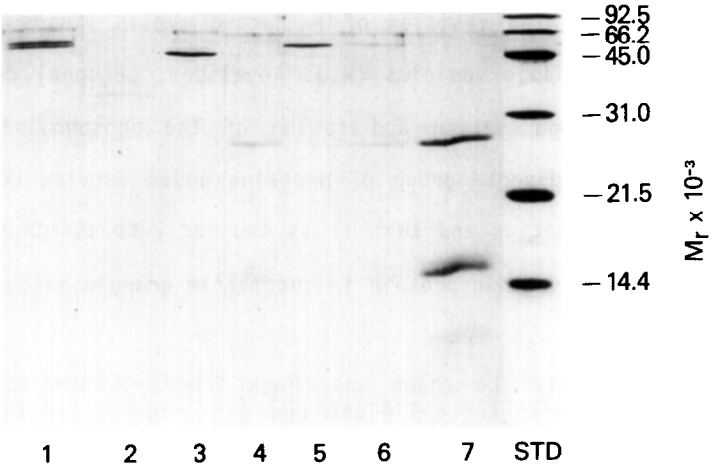

Figure 4: One-dimensional peptide maps of chymotrypsin-treated protein samples as described in the methods section. Lane 1, 1.5 μg tubulin; lane 2, 1.5 μg tubulin plus 0.6 μg chymotrypsin; lane 3, 2.0 μg synexin I peak fraction; lane 4, 2.0 μg synexin I peak plus 1.8 μg chymotrypsin; lane 5, 2.0 μg Synexin II peak fraction; lane 6, 2.0 μg synexin II peak plus 1.8 μg chymotrypsin; lane 7, 3.6 μg chymotrypsin; lane 8, molecular weight standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soy bean trypsin inhibitor and lysozyme, in descending order). The chymotrypsin sample contained four peptides of $M_r \cong 26,900$, 14,900, 14,300 and 10,600, which are visible in lanes 4 and 6 but not in lane 2. Digestion of tubulin produced a large number of peptides of which five ($M_r \cong 33,900$, 33,100, 32,750, 27,300 and 18,200) are still resolvable after 30 minutes. Synexin II produced one peptide ($M_r \cong 38,000$). Synexin I produced no peptides of $M_r > 10,000$.

calcium from activated late calcium channels (9, 10). Although it is quite possible that both of the synexin molecules described here have the same or synergistic functions, it may well be that the aggregation of chromaffin granule or other negatively charged membranes is only a convenient method for the identification of a large number of calcium binding proteins which have entirely different physiological roles. This idea is strengthened by several recent reports. A 32,000 M_r protein isolated from the electric organ of Torpedo marmorata has a Ca^{2+}-specific ability to improve the aggregation of chromaffin granules as well as Torpedo synaptic vesicles and extracted synaptic vesicle lipids but inhibits aggregation of PS liposomes (17). Tubulin can be intercalated into PC vesicles. These vesicles will then fuse if calcium is added (16). Clatharin has a similar effect on PC vesicles when

the pH of the protein/vesicle mixture is lowered (18). The addition of myelin basic protein to sonicated vesicles of extracted myelin lipids (19) or to a variety of artificial lipid vesicles (K.J. Angelides, personal communication) also promotes rapid aggregation and fusion of the phospholipid membranes. Thus, it seems that a diverse group of proteins under varying conditions can promote membrane aggregation and that it is too early to assign synexin I the role of the specific fusogenic protein in chromaffin granule exocytosis.

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