Synexin binds in a calcium-dependent fashion to oriented chromaffin cell plasma membranes

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Oriented plasma membrane fragments from chromaffin cells, isolated on polylysine-coated polyacrylamide beads, bind synexin in a calcium dependent manner Synexin binding was also detected on beads coated with chromaffin granule membranes, but not to beads coated with erythrocyte membranes or to uncoated beads Synexin binding to plasma membrane coated beads showed a specific requirement for calcium ($K_{1/2}$ = 200 μM) and was insensitive to other divalent cations such as magnesium, strontium and barium Synexin binding to either plasma membrane or granule membrane coated beads was saturable, was partially reversible by EGTA and was directly observed by SDS-polyacrylamide gel electrophoresis

Synexin *Plasma membrane* *Chromaffin granule* *Calcium*

1. INTRODUCTION

Exocytosis of catecholamines from adrenal medullary chromaffin cells depends on calcium and involves fusion of chromaffin granules with both plasma membrane and membranes of previously fused granules (compound exocytosis). The exact biochemical mechanism of calcium action is still unknown. However, one candidate for involvement in this process is synexin, a calcium-binding protein from adrenal medulla and other tissues, that aggregates chromaffin granules in the presence of calcium [1,2]. Addition of *cis*-unsaturated fatty acids, such as arachidonic acid, also causes the aggregated granules to fuse [2], and we have thus proposed that synexin might be a mediating factor in calcium-dependent exocytosis [4].

Thus far, synexin has only been demonstrated to interact with chromaffin granules. We have now tested the ability of synexin to bind directly to the cytoplasmic side of plasma membrane fragments of chromaffin cells and find that synexin indeed binds selectively to these plasma membrane fragments in a calcium-dependent manner. These new data further support the hypothesis that synexin may mediate interactions between secretory granules and plasma membranes during exocytosis.

2. MATERIALS AND METHODS

2.1. *Preparation of chromaffin granules and chromaffin cells*

Chromaffin granules were prepared from bovine adrenal medullary tissue by a modification of the differential centrifugation procedure of Taugner [5] as described by Pollard et al. [6]. Chromaffin cells were isolated by collagenase digestion, using a modification of the method of Schneider et al. [7] as described by Pollard et al. [8]. Briefly, adrenal glands were perfused four times with balanced salt buffer, and a further four times with a solution of 0.1% collagenase (Worthington, Type II) in the balanced salt solution at 37°C. Subsequently, the medullary tissue was separated

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from the cortex, minced, washed, and incubated with fresh collagenase solution for 30 min at 37°C. Dispersed cells were then isolated by filtration and washed 3 times with salt solution containing 1% bovine serum albumin (CalBiochem).

2.2. *Purification and assay of synexin*

Synexin was purified from bovine adrenal medullary tissue or liver by ammonium sulfate precipitation and gel filtration as described by Creutz et al. [1]. The pooled synexin peak from the Ultragel AcA34 column (80% pure) was employed in these experiments. Since liver and adrenal medullary synexin were virtually indistinguishable on the basis of chemical and physical properties [9], and since large amounts of liver synexin were more easily obtained, much of this work was done with the liver synexin.

Synexin was assayed by following the aggregation of chromaffin granules at 540 nm as described by Creutz [3]. Essentially, synexin and chromaffin granules were mixed in the presence of 240 nM sucrose, 28 mM histidine (pH 6.0), 30 mM KCl and the appropriate calcium/EGTA buffer (p*Ca* 3.4, high calcium; p*Ca* 5.4, low calcium). The initial absorbance was 0.3, and one unit of synexin was defined as a 1% increase in absorbance in 10 min at 37°C. Assays were corrected for the absorbance change in a baseline blank in the absence of synexin. We found it unnecessary to correct for absorbance changes due to synexin association in the presence of calcium as proposed by Dabrow et al. [10].

2.3. *Preparation of membrane-coated beads*

Membrane-coated beads were prepared as described by Jacobson [11] with the following modifications for chromaffin cells. Polylysine-coated polyacrylamide beads were prepared as described by Jacobson and Branton [12] (50 mg polylysine (M_r 80000, Miles Laboratory)/g polyacrylamide beads). The beads and cells were each washed 3 times in 0.3 M sucrose containing 10 mM Na acetate, pH 5.0. A 50% suspension of beads was added dropwise to an equal volume of freshly dissociated cells (400 million cells/ml of packed beads) and gently swirled. After 10 min on ice, the cell-coated beads were washed 3 times with sucrose acetate buffer and the attached cells were lysed by the addition of 5 vols of 10 mM Tris (pH 7.5) and 2.5 mM EGTA (Tris/EGTA buffer). The beads solution was vortexed at high speed for 30 s, washed twice with Tris/EGTA buffer, sonicated at 50 W for 5 s, then washed 3 times with sucrose histidine buffer (0.3 M sucrose, 40 mM histidine HCl, pH 6.0). The membrane-coated beads were stored in sucrose histidine buffer as a 50% solution at 4°C. Granule membrane-coated beads were prepared in the same way, except that the granules were centrifuged once (10000 × *g*, 20 min) and resuspended in sucrose acetate buffer Beads were added to a granules suspension containing 150 A_{540} of granules/ml packed beads.

Because chromaffin cells did not coat the beads as efficiently as red blood cells, bare spaces were present which could potentially have bound chromaffin granules or intracellular protein after cell lysis. Granule membrane contamination was minimized by using a large excess of cells. The use of polyglutamate to neutralize these uncoated portions of the beads as suggested by Jacobson [13] resulted in a 70–80% reduction in protein bound to the beads without either an increase in acetylcholinesterase specific activity or a decrease in dopamine-β-hydroxylase specific activity and we therefore did not incorporate this approach into our procedure.

2.4. *Assay of synexin binding to membrane-coated beads and release by EGTA*

Synexin binding to membrane-coated beads was measured as the loss of synexin activity from the supernatant solution after exposure to membrane-coated beads. The binding conditions were comparable to conditions used for assaying synexin by granule aggregation. Each assay tube contained 30 mM KCl, 240 mM sucrose, 28 mM histidine-HCl (pH 6.0), and EGTA/calcium buffer with a p*Ca* of either 5.4 or 3.4, membrane-coated beads (usually 100 μl packed beads) and synexin (50–400 units) in a total volume of 0.5 or 1 ml. The mixture was incubated at room temperature for 15 min, with periodic shaking to resuspend the beads. The supernatant solution was removed from the settled beads and samples were assayed immediately for synexin activity. The residual activity was compared to a control sample without beads to determine the amount of synexin bound.

Synexin was released from membrane-coated beads by incubation with EGTA, as follows. Beads

to which synexin had been bound were washed twice in sucrose histidine buffer containing the same amount of Ca^{2+} as for binding, then resuspended in sucrose histidine buffer containing 2.5 mM EGTA. The bead suspensions were shaken at room temperature for 1 h, the beads allowed to settle and samples of the supernatant solution assayed immediately for synexin activity in the presence of 2 mM calcium to compensate for the EGTA present in the extraction medium.

2.5. *SDS–polyacrylamide gel electrophoresis (SDS-PAGE)*

Samples were electrophoresed on 10% polyacrylamide gels as described by Laemmli [14] and stained with Coomassie blue Synexin was bound to beads and released by EGTA as described above, except 200 μl of packed beads were used in a total volume of 1 ml and high calcium was 1 mM rather than 400 μM. Samples of both supernatant solutions and membrane bead pellets were saved after both binding and release. The protein in the supernatant samples (0.8 ml) was precipitated with 1.6 ml 7% trichloroacetic acid and resuspended in 100 μl of sample buffer, boiled and applied to the gel.

2.6. *Enzyme assays and protein determination*

Dopamine-β-hydroxylase was assayed as described by Nagatsu and Undenfriend [15]. A unit of dopamine-β-hydroxylase produces 1 nmol octopamine/min. Acetylcholinesterase was assayed radiochemically as described by Potter [16]. One unit of acetylcholinesterase produces 1 nmol acetate per min. [^{3}H]Acetylcholine was obtained from New England Nuclear. Protein was measured by the Bradford dye-binding technique [17] using bovine gamma globulin as a standard. For bead-bound membrane protein, samples (100 μl) were incubated in 300 μl of 1 M NaCl for approx. 2 h at room temperature on a shaker to release membrane from the beads. The beads were allowed to settle and 200 μl samples of the supernatant solution were assayed for protein.

3. RESULTS AND DISCUSSION

3.1. *Preparation of membrane-coated beads*

Oriented fragments of plasma membrane from chromaffin cells were prepared on polylysine-coated beads, as described in section 2.3. As shown in table 1 (line 1), plasma membrane, as measured by acetylcholinesterase activity, was clearly bound to the beads. However, a small but measurable amount of dopamine-β-hydroxylase activity, a dependable chromaffin granule marker, was also present on the plasma membrane bead preparation. Since synexin had already been shown to bind to chromaffin granules, it was important to assess the degree of granule membrane contamination of the plasma membrane preparation which could occur during the lysis of the attached cells. We proceeded to prepare chromaffin granule membrane-coated beads and as anticipated, the granule membrane beads had a much higher level of dopamine-β-hydroxylase activity than the plasma membrane beads (table 1, line 2). The granule membrane beads also displayed a significant amount of acetylcholinesterase activity, which may actually be intrinsic to the chromaffin granules as reported recently by Gratzl et al. [18]. It was clear, however, that the beads prepared using freshly dissociated

Table 1

Membrane bead analysis

	Protein (mg/ml)	Plasma membrane marker acetylcholinesterase (units/mg protein)	Granule membrane marker dopamine-β-hydroxylase (units/mg protein)
Plasma membrane-coated beads	0.97 ± 0.36	15.6 ± 6 6	21.4 ± 12.0
Granule membrane-coated beads	0.79 ± 0 33	4 4 ± 1.8	141 9 ± 70.4

These are average values of 16 plasma membrane and 10 granule membrane preparations, ± SD

chromaffin cells were enriched in membrane that was clearly not derived from chromaffin granules and were most probably plasma membrane.

This conclusion was also substantiated by an examination of SDS–polyacrylamide gels of membranes eluted from both plasma membrane and granule membrane-coated beads. As shown in fig.3, bands characteristic of granule membranes (lane A2, plasma membrane, lane B2, granule membrane), were essentially undetectable in the plasma membrane lane.

3.2. *Synexin binding to membrane-coated beads*

Synexin was mixed with membrane-coated beads in the presence or absence of calcium and incubated for 15 min at room temperature as described in section 2. The data, shown in table 2, indicated that both plasma and granule membrane preparations could bind synexin in a calcium-dependent manner, since virtually all the synexin was bound at 400 μM calcium (pCa = 3.4), but not at 4 μM calcium (pCa = 5.4). Beads alone did not bind synexin in the presence or absence of calcium. As indicated in table 2, we also tested red blood cell membrane-coated beads for synexin binding and found them to be inactive. The fact that red blood cell membrane beads did not bind synexin in high or low calcium conditions indicated to us that some degree of membrane specificity existed in the interaction.

We were concerned that the binding of synexin to the plasma membrane could be due to the small amount of chromaffin granule membrane contamination. To check this possibility, we assayed granule membrane-coated beads with comparable dopamine-β-hydroxylase activity (~15% of the amount of granule membrane beads used in the standard assay) for synexin binding. We were unable to measure any binding of synexin, presumably because not enough binding sites were available to give a detectable signal. We also tested for potential problems due to contamination by other cytoplasmic components by mixing the soluble fraction of a cell lysate prepared by hypotonic lysis of cells and centrifugation at 20000 rpm for 30 min with beads. Here again, no binding of synexin was detected. We concluded that synexin binding to plasma membrane-coated beads could not be attributed primarily to contaminating granule membranes or cytosolic components.

Table 2

Synexin binding to membrane-coated beads

	% synexin bound	
	400 μM calcium	4 μM calcium
Plasma membrane-coated beads	90–100	0
Granule membrane-coated beads	90–100	0
Red blood cell-coated beads	0	0
Beads alone	0	0

This is a summary of 10 experiments. Binding assays contained 90 μg protein and 50–100 units of synexin

Convinced now that the association of synexin with plasma membrane-coated beads was indeed to plasma membranes, we proceeded to characterize the binding in more detail. Synexin binding to plasma membrane-coated beads was specifically dependent on calcium. Inclusion of millimolar concentrations of divalent cations resulted in 100% binding of synexin with calcium, but only 6% with magnesium or barium and none at all with strontium. A more detailed analysis of calcium activation of binding revealed that half maximal binding occurred at a calcium concentration of approximately 200 μM (fig.1). This calcium titration curve was virtually indistinguishable from the titration curves for granule aggregation [1] and synexin self-association [2].

The interaction of synexin with both plasma membranes and granule membranes appeared to be saturable processes, with maximum binding of about 1300 and 3000 units synexin/mg membrane protein, respectively (fig.2, data for granule membrane beads not shown). Synexin binding was at least partially reversible since incubation of beads to which synexin had been bound with EGTA caused about 40% of the bound synexin activity to be released (fig.2).

Synexin binding to plasma membrane-coated beads was independent of temperature. Similar amounts of synexin binding were seen at 2, 20 and 37°C. This result agreed with the temperature dependence of synexin self-association but contrasted with the strong temperature dependence of

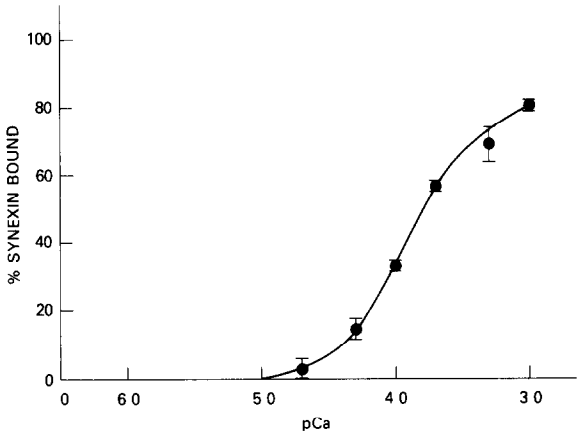

Fig.1 The effect of calcium concentration on synexin binding to plasma membrane-coated beads Synexin was bound to plasma membrane-coated beads (assay volume ± 0.5 ml) in the presence of calcium/EGTA buffers to provide the stated calcium concentrations. Samples of the supernatant solution were assayed for unbound synexin activity The percent of bound synexin was calculated using the activity of a control without beads as equivalent to 0% bound. The values are the average of two separate experiments, one using 50 μg membrane protein and 165 units of synexin, the other 35 μg membrane protein and 150 units of synexin.

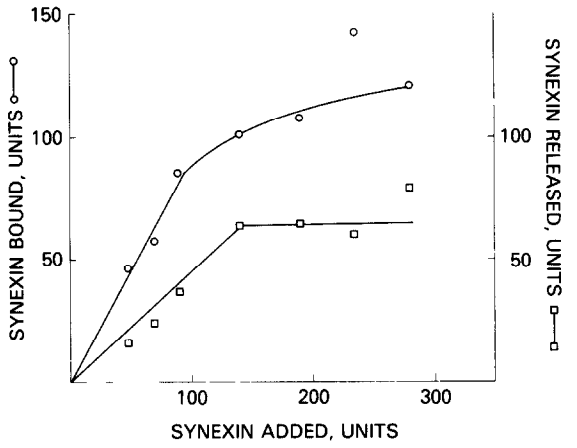

Fig.2. Effect of synexin concentration on binding to plasma membrane and release of synexin by incubation with EGTA Plasma membrane-coated beads (110 μg membrane protein) were incubated in standard 1 ml binding assay conditions (p*Ca* = 3.4) with synexin concentration varied as shown. Synexin binding (○) and release (□) were measured as described in section 2

the synexin induced granule aggregation.

Synexin binding to membrane-coated beads could also be demonstrated directly using PAGE, and the results, shown in fig.3, paralleled those obtained by the standard synexin binding assay. Synexin was sedimented with granule or plasma membrane-coated beads only in the presence of high calcium (lanes 3A and 3B vs lanes 7A and 7B, respectively). The amount of synexin bound to the membrane-coated beads in the presence of calcium appeared as a major component compared to the membrane proteins (compare lanes 4A and 4B to 2A and 2B). A band with similar mobility to synexin was not seen in the membrane controls. EGTA removed much of the bound synexin (lanes 5A and 5B), but some of the synexin remained with the membranes (lanes 6A and 6B).

Our main conclusion is that synexin binds to oriented plasma membrane fragments of chromaffin cells. The isolation of plasma membrane on polylysine-coated beads is reported to expose only the cytoplasmic surface of the plasma membrane of a variety of cell types to the medium [19,20]. We assume that this is probably also the case in our system. However, the only direct evidence for this assumption is that calcium-dependent binding of synexin is essentially undetectable if the cells attached to the beads are not first broken. Therefore, we are content to identify our membrane fragments as oriented in a manner which at least exposes the inner surface of the plasma membrane. The outer surface of the plasma membrane may also be accessible to the synexin but does not provide a binding site. These data thus support the concept that simple exocytosis, making contact and fusion between chromaffin granules and plasma membranes, can occur in a synexin-regulated manner.

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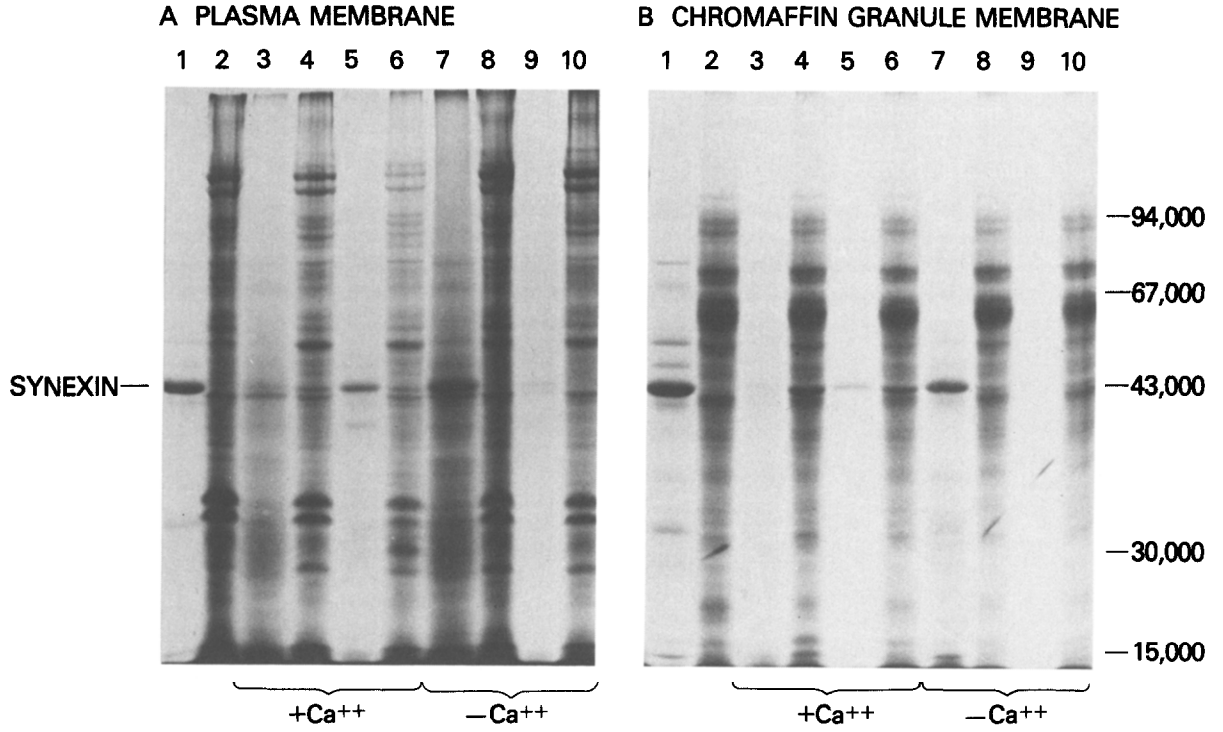

Fig.3. SDS–PAGE of synexin binding and release to plasma membrane- and granule membrane-coated beads A1 and B1 are samples of synexin. A2 and B2 are plasma membrane-coated and granule membrane-coated beads, respectively. Upon addition of synexin and calcium (pCa = 3.4) to plasma membrane-coated beads (panel A), synexin is removed from the supernatant (A3) but appears in the plasma membrane fraction (A4). Treatment of synexin–plasma membrane complex with EGTA releases the bound synexin into the supernatant (A5), leaving plasma membrane depleted of synexin (A6). The same experiments shown in A3–6 are repeated in A7–10, except that pCa^{2+} = 5.4. In this case synexin is not removed from the supernatant (A7), is therefore not bound to the plasma membrane (A8), is thus not released from the membrane by EGTA (A9) and of course, none is left on the plasma membrane (A10) Parallel experiments are done with the chromaffin granule beads in panel B, with similar results.

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