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CALCIUM DEPENDENCE OF THE BINDING OF SYNEXIN TO ISOLATED CHROMAFFIN GRANULES

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The calcium dependence of the binding of synexin to isolated chromaffin granules has been investigated. The calcium dependence was found to be pH sensitive, binding occurring at higher Ca^{2+} concentrations at lower values of pH. At pH 7.2 half-maximal binding occurred at 4 μM Ca^{2+}. This is a lower Ca^{2+} concentration than the 200 μM that is required to give half-maximal self-association of synexin or membrane aggregation by synexin. The data therefore suggest that in the chromaffin cell stimulated to release catecholamines and proteins by exocytosis synexin first binds to membranes and then associates with itself to draw membranes together in preparation for fusion.

Synexin is a widely distributed , soluble protein of mass 47,000 daltons that in the presence of Ca^{2+} undergoes self-association (1), binds to biological membranes (2) and liposomes (3,4), and draws secretory vesicle membranes into "pentalaminar" complexes (2) that are subject to fusion (5). Because of these properties synexin has been hypothesized to be an intracellular receptor for Ca^{2+} in the process of exocytosis that functions to attach secretory vesicle membranes to the plasma membrane, or vesicle membranes to one another in compound exocytosis. The Ca^{2+}-dependencies of self-association of synexin and of the aggregation of chromaffin granules by synexin have been examined in detail and found to be virtually identical (1). Both processes appear to be activated by a Ca^{2+} binding site with dissociation constant 200 μM, the activations are positively cooperative with a Hill coefficient of 2.3, and the processes are

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highly specific for Ca^{2+}. It has also been demonstrated that at pH 6.0 synexin binds to chromaffin granules in the presence of 400 μM Ca^{2+}, but not 4 μM Ca^{2+} (2). However, a complete Ca^{2+} titration curve of this binding process has not previously been investigated and therefore the relationship between the membrane binding event and the self-association and inter-membrane attachment events has not been clear.

In recent studies on the isolation by affinity chromatography of proteins that bind to chromaffin granule membranes in the presence of Ca^{2+} (6,7) we have found that synexin binds to membranes at pH 7.3 at concentrations of Ca^{2+} (40 μM) that are lower than might be predicted on the basis of the Ca^{2+} dependence of chromaffin granule aggregation by synexin. This observation stimulated a more detailed analysis of the Ca^{2+} dependence of synexin binding to chromaffin granules. We report here that Ca^{2+} apparently interacts with a site to cause binding of synexin to membranes that is distinguishable from the Ca^{2+} binding site that activates self-association or the formation of inter-membrane contacts. This new site is highly pH sensitive and exhibits a higher affinity for Ca^{2+} than the other site(s).

MATERIALS AND METHODS

Materials. Synexin was prepared from bovine liver tissue by ammonium sulfate induced precipitation from a post-microsomal supernatant and gel filtration on LKB Ultrogel AcA34 (2,8). Chromaffin granules were prepared from bovine adrenal medullary tissue by differential centrifugation in 0.3M sucrose (9).

Binding experiments.

The binding of synexin to chromaffin granule membranes was determined by assaying the unbound synexin present in the supernatant of a reaction mixture after sedimenting whole chromaffin granules with bound synexin. Four hundred microliter samples of synexin ($\sim$75 μg/ml) in the gel filtration buffer (0.3M sucrose, 40 mM Mes-NaOH pH 6.0, for experiments conducted near pH 6) or in the gel filtration buffer plus 40 mM Hepes-NaOH, pH 7.3 (for experiments conducted near pH 7.3) was combined with 400 μl of 0.3M sucrose, 40 mM MES -NaOH pH 6.0 or 400 μl of 0.3M sucrose, 40 mM Hepes-NaOH pH 7.3, 100 μl of a chromaffin granule suspension in 0.3M sucrose with A_{540} = 3.0 ($\sim$1.3 mg/ml), and 20

μl of a calcium-EGTA buffer containing 125 mM EGTA. Therefore, final concentrations in the binding assay were 32 ug/ml synexin, 141 ug/ml chromaffin granule protein, 0.294 M sucrose, 35 mM MES-NaOH or 17 mM MES-NaOH and 35 mM Hepes-NaOH, 2.7 mM EGTA and various concentrations of $CaCl_2$. The additional presence of 30 mM KCl in one experiment conducted at pH 7.2 was found not to alter the calcium sensitivity of binding. The granules and synexin were incubated for 10 min at 37°C then samples were placed in a Sorvall SM-24 rotor that had been warmed to 37°C, then centrifuged for 30 min at 15,000 rpm (25,000 g) at room temperature. The supernatants were transfered to seperate tubes and stored at -20°C overnight.

The free Ca^{2+} concentrations of the binding reaction mixtures were calculated from the Ca^{2+} and EGTA concentrations and the measured pH's of the binding supernatants. Specifically, two sets of "calcium buffers" were prepared to cover the range pCa 7.0 to 3.0, one set to be used for experiments near pH 6.0, and the other to be used near pH 7.3. At the completion of each experiment, the pH of the reaction mixtures was checked at 37°C. The measured value of the pH was then used to recalculate the free Ca^{2+} concentrations, using the known Ca/EGTA ratios of the Ca^{2+} buffers. All calculations were carried out according to Caldwell (10), using a programmable calculator (Hewlett-Packard HP41CV).

Analytical Procedures.

The supernatants from the binding experiments were assayed for Ca^{2+}-dependent chromaffin granule aggregating activity characteristic of synexin (2). Four hundred μl of supernatant was added to 200 μl of 0.15M KCl, 400 μl of 0.3M sucrose containing 130 μg of chromaffin granule protein, and 20 μl of 100 mM $CaCl_2$. Because of the EGTA and variable Ca^{2+} contents of the supernatants this procedure resulted in a range of free Ca^{2+} in the assay mixture of 1.0 to 2.4 mM, a range in which synexin is fully activated to cause granule aggregation. Aggregation of the granules was monitored by continuous measurement of the turbidity (A_{540}) of the granule suspension. The increase in turbidity seen at 10 min was taken as a measure of synexin activity in the supernatants.

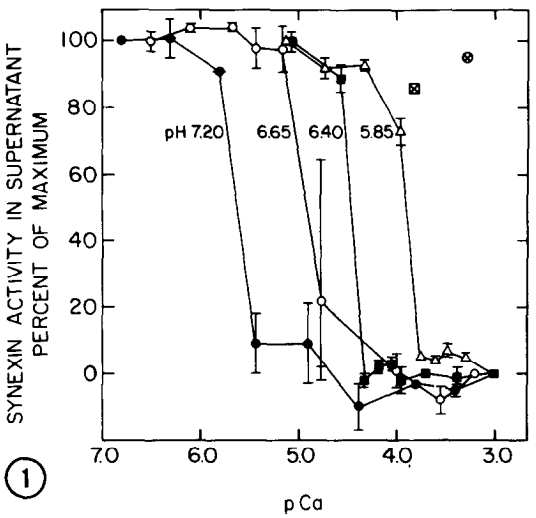
Pellets of chromaffin granules with or without bound synexin were analyzed by 10% polyacrylamide gel electrophoresis in the presence of SDS according to Laemmli (11) and stained with Coomassie Blue.

Protein was assayed by the method of Bradford using bovine serum albumin as standard for chromaffin granule proteins (12), and bovine gamma globulin as standard for the synexin preparations (5). The use of these two standards gives results that agree with the Lowry (14) assay using bovine serum albumin as standard (5).

RESULTS

Figure 1 illustrates the Ca^{2+} titration curves for the binding of synexin to chromaffin granules at 4 values of pH ranging from 5.85 to 7.2. It is seen that binding occurs at lower concentrations of Ca^{2+} at the more neutral pH values.

Figure 2 is a composite of data obtained in repeated titration experiments conducted at different values of pH, and



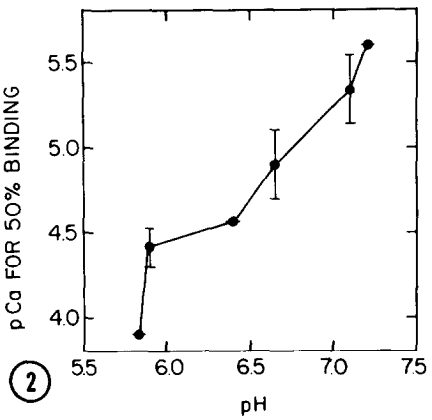


Figure 1. Reduction in synexin activity in supernatants due to binding to chromaffin granules. The activity was measured as the percent increase in turbidity of a granule suspension induced by 400 μl of supernatant. The titration curves are normalized to activity left in the supernatant at the lowest Ca^{2+} concentration (which led to a 50 to 60% increase in turbidity) as 100% and activity at the highest Ca^{2+} concentration (where no aggregating activity was found in the supernatant) as 0%,
⊗, control experiment at pH 5.85 in the absence of chromaffin granules.
⊠, control experiment at pH 7.2 in the absence of chromaffin granules.

Figure 2. pH dependence of the Ca^{2+} concentration leading to binding of 50% of synexin activity to chromaffin granules.

illustrates the relationship between the Ca^{2+} concentration giving half-maximal synexin binding and the pH of the incubation medium. The binding was specific for Ca^{2+} in that 1 mM Mg^{2+} was found not to promote binding.

Since chromaffin granules contain peptidases involved in processing neuropeptides (13), one concern in interpreting the binding data was that the loss of synexin activity in the supernatants might have been due to inactivation of synexin by these peptidases rather than binding of synexin to the granules. We examined this possibility by two approaches. First we analyzed by electrophoresis the granule pellets from the binding assay for the presence of the 47,000 dalton synexin band (Fig. 3). This band appeared in gels of the pellets in good

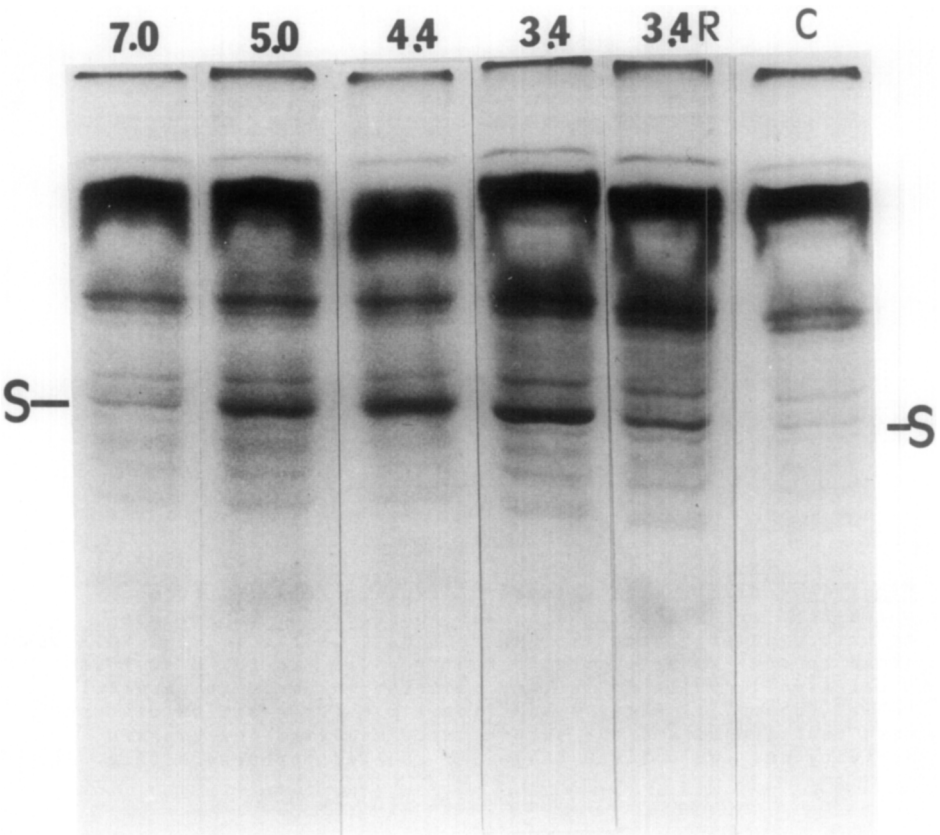


Figure 3. Polyacrylamide gel electrophoresis of granule pellets after a binding experiment. Binding was conducted at pH 7.25 and the loss of synexin activity in the supernatants is illustrated in figure 4. Numbers at the top of the gels indicate the pCa values of the binding experiments. "3.4R" marks a gel of granules pelleted after synexin bound at pCa 3.4 was removed by treatment with EGTA (see legend to fig. 4). "C" marks a gel of a control pellet of granules incubated without synexin at pCa 4.4. "S" marks the position of the synexin band, interpolated as 47,000 daltons in comparison with ovalbumin and bovine serum albumin standards (not shown).

correlation with the disappearance of the activity from the supernatants (Fig. 4). Furthermore, no other bands in the gels were modified, suggesting that proteolytic fragments of synexin were not bound to the granules.

As a second demonstration that synexin was not being inactivat ed by proteolysis, we tested the reversibility of the binding reaction by adding EGTA to the binding reaction mixtures to chelete the Ca^{2+} and promote the release of the bound synexin.

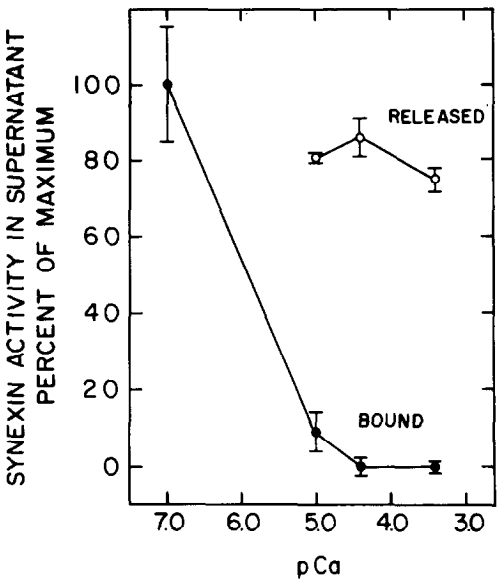


Figure 4. Binding and release of synexin activity from chromaffin granules. Binding was conducted at pH 7.25 as described under "Materials and Methods". After 10 min, EGTA was added to some tubes to reduce the free Ca^{2+} to 10^{-7} M or less ("released"), while no further addition was made to parallel tubes ("bound"). After 5 additional min the reaction mixtures were centrifuged and the supernatants analyzed for synexin activity and the pellets subjected to electrophoresis (fig. 3).

As seen in figure 4, most of the binding activity was recovered by this procedure. The gel in figure 3 also shows that most of the 47,000 dalton band was released by this procedure, although some synexin does appear to be irreversibly bound. Synexin induced aggregates of chromaffin granules cannot be completely dissociated by EGTA (2), and the synexin that remains bound to the granules may be involved in irreversible linkages between granules.

It was previously reported that the Ca^{2+} dependence of chromaffin granule aggregation by synexin was unaffected by pH in the range 6.0 to 7.3 (2). We re-examined the Ca^{2+} dependence of granule aggregation at these two extreme values of pH. We found that the magnitude of the light scattering change seen at any given Ca^{2+} concentration is slightly greater at pH 6.0 than 7.3, probably reflecting more extensive aggregation at the lower pH.

However, the calcium dependence of the turbidity change was indeed similar at the two values of pH. Similarly, we investigated the pH dependence of the synexin self association reaction, since previously reported data was obtained at pH 6.0 (1). Again, we found that the Ca^{2+} dependence of the self-association reaction was not significantly altered at pH 7.3.

DISCUSSION

The report that synexin self-association has the same Ca^{2+}-dependence as membrane aggregation by synexin (1) led to the suggestion that the self-association of synexin played some mechanistic role in the process of membrane aggregation. At least two mechanisms may be envisioned, although additional alternatives are possible. In one mechanism, the self-association of synexin monomers leads to the formation of small polymers shaped like 50 by 150 A^{o} rods (1). These rods may have different solubility properties than the synexin monomers and therefore stick to membranes. By sticking to more than one membrane, one rod or a group of rods could bind two membranes together. This final step in the process might even be Ca^{2+} independent. In the alternative mechanism, synexin monomers would bind to membranes in a Ca^{2+} dependent step and then by a process of Ca^{2+} dependent self-association draw membranes together.

We believe our present data support the second mechanism of synexin action, particularly as events may occur in the cell (fig. 5). As the cytoplasmic free calcium concentration increases in a stimulated cell into the range 1 to 10 μM synexin would bind to membranes but not undergo significant self-association. If in certain areas of the cell, such as near the plasma membrane Ca^{2+} channels, the Ca^{2+} concentration rises

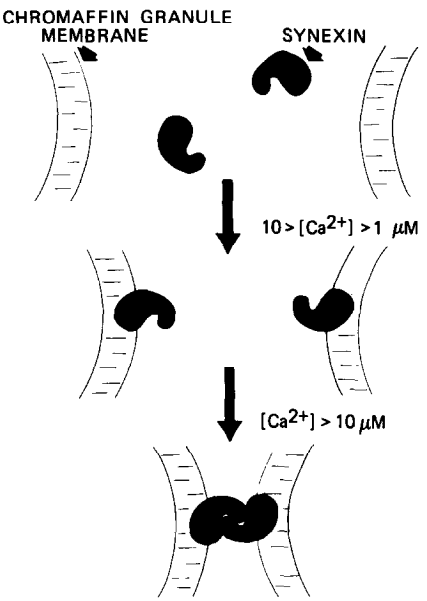


Figure 5. An hypothesis for the sequence of events that may lead to the formation of synexin induced inter-membrane contacts during exocytosis in the chromaffin cell. In the resting cell (top), the Ca^{2+} concentration is less than 1 μM and synexin is freely soluble. In the stimulated cell (middle), as the Ca^{2+} concentration rises to between 1 and 10 μM, synexin attaches to membranes. When the Ca^{2+} concentration further increases above 10 μM Ca^{2+} (bottom), synexin molecules on different membranes self-associate to bring the membranes together. Although isolated synexin in the presence of ca^{2+} self-associates to form 50 by 150 A^{o} rods similar to the structure illustrated at the bottom of the figure, the actual number of synexin molecules that associate to form this structure, the orientation of this structure relative to the membrane, and the number of such structures involved in creating an inter-membrane contact are not known.

above the 10 μM range, self association of membrane bound synexin will occur, drawing membranes together to form pentalaminar complexes. (The threshold Ca^{2+} concentration necessary to initiate inter-membrane contact is more difficult to determine than the concentration of Ca^{2+} (200 μM) required to give the half-maximal effect in a complete titration curve. The threshold has been variously estimated as 6 μM on the basis of electron microscopy (2), 10 μM on the basis of monitoring fusion of the aqueous compartments of liposomes (15), or as high as 40 μM if using simply light scattering from granule suspensions (2).) Membranes attached to one another by synexin could subsequently

fuse as a result of osmotic pressure increases in the secretory vesicle and/or a weakening of the pentalaminar structure by free arachidonic acid mobilized by Ca^{2+} activated phospholipase A_2 (16,17,5).

It is possible that a Ca^{2+} binding site on the chromaffin granule membrane regulates the binding of synexin to this membrane. However, we have found that synexin also binds to a phosphatidyl serine affinity column at a low Ca^{2+} concentration, eluting at between 40 and 0.1 μM Ca^{2+} at pH 7.3. Therefore, we suggest that synexin may have a high-affinity Ca^{2+} binding site that regulates interaction of this protein with membranes, as well as a lower affinity site that regulates self-association. Direct studies of Ca^{2+} binding to purified synexin are clearly needed to sort out the number and affinity of binding sites on this Ca^{2+} receptor.

Our data also suggest that even in regions of a stimulated secretory cell where the free Ca^{2+} concentration does not rise above 10 μM permitting membrane contacts to be initiated by synexin, the reversible association of synexin monomers with secretory vesicle membranes occurring at lower Ca^{2+} concentrations (1 to 10 μM) could possibly regulate other important steps in the secretory pathway.

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