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DIFFERENTIAL RECOGNITION OF SECRETORY VESICLES BY ANNEXINS

Carl E. Creutz[1], Stephen Moss, J. Michael Edwardson[2], Izumi Hide, Bastien Gomperts, and Students[3] taking the European Molecular Biology Organization Course "Advanced Techniques for Studying Secretion"

Department of Physiology, University College London
Gower Street, London WC1E 6BT, U.K.

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The interactions of synexin, calpactin, and p68 (annexins VII, II, and VI) with zymogen granules and adrenal medullary chromaffin granules were compared. Synexin promoted the aggregation and fusion of both types of organelles. Calpactin inhibited the action of synexin on zymogen granules but would aggregate only chromaffin granules. p68 inhibited aggregation of chromaffin granules by calpactin and fusion of zymogen granules by synexin. The results demonstrate that these annexins, all of which bind acidic phospholipids, interact differently with different biological membranes and therefore may play specific and non-overlapping roles in cells where they are co-expressed.

The annexins are a family of structurally related calcium-dependent membrane-binding proteins [1]. Because most members of the family are capable of promoting the calcium-dependent aggregation of secretory vesicles it has been proposed that these proteins may serve as mediators of membrane fusion in exocytosis. In particular, the ability of synexin (annexin VII) and calpactin

[1]Corresponding author: Permanent address: Department of Pharmacology, University of Virginia, Charlottesville, VA 22908. FAX: (804) 982-3878.

[2]Permanent address: University of Cambridge, Department of Pharmacology, Tennis Court Road, Cambridge CB21QJ, U.K.

[3]Students participating in these experiments were:
Giampietro Schiavo, Istituto di Patologia Generale, Padova, Italy; Leena Nevalainen, Institute of Biotechnology, Helsinki, Finland; Ludolf von Ruden, Max Planck Institut fur biophysikalische Chemie, Gottingen, Germany; Lynda Fletcher, Department of Physiological Sciences, Manchester University, U.K.; Frank Bartels, Medizinische Hochschule Hannover, Germany; Ingo Waschulewski, Department of Anatomy and Cell Biology, University of Marburg, Germany; Roberto Buccione, Consorzio Mario Negri Sud, Centro di Ricerche Farmacologiche e Biomediche, Italy; Jef Pinxteren, Department Neurofarmacologie, UIA, Belgium; Christopher Magnus, Department of Pharmacology, School of Pharmacy, London, U.K.2222.

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(annexin II) to promote chromaffin granule aggregation and fatty-acid dependent fusion has been documented [2,3]. Since the annexins can bind to acidic phospholipids it has not been obvious they should exhibit a high degree of specificity for binding or aggregating particular types of biological membranes. In this study the ability of synexin and the calpactin tetramer ($p36_2p10_2$) to aggregate and fuse pancreatic zymogen vesicles and chromaffin granules was compared. The inhibitory effects of p68 (annexin VI) on the aggregation of both organelles was also examined. Surprisingly, the calpactin tetramer was found to have a specific action on chromaffin granules while synexin and p68 were active with both organelles.

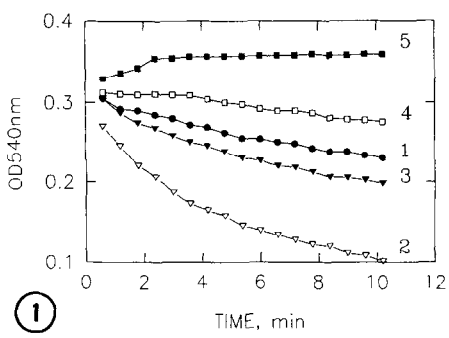
MATERIALS AND METHODS

Bovine lung calpactin tetramer [3], recombinant human synexin [4], recombinant murine p68 [4], bovine chromaffin granules [2], and rat pancreatic zymogen granules [5] were isolated as previously described. Turbidity measurements were performed on an LKB Ultrospec 4050 recording spectrophotometer in 240 mM sucrose, 30 mM KCl, 25 mM MES-NaOH, pH 6.0 at 21°C, with other conditions as described [2]. Granule preparations were diluted to an OD540nm of 0.3 (approximately 50ug protein/ml for zymogen granules and 100ug/ml for chromaffin granules). Fusion of zymogen granules was determined by monitoring the dequenching of octadecyl rhodamine incorporated in one population of granules incubated with an equal concentration of unlabelled granules. Fluorescence dequenching was measured in a Perkin-Elmer LS-5 fluorescence spectrometer at 37°C in the above buffer with other conditions as described [5]. Due to the ample manpower available for this study, experiments were performed at least twice by independent investigative teams. Representative results are shown.

RESULTS AND DISCUSSION

Aggregation of chromaffin granules by recombinant human synexin and bovine lung calpactin. The synexin used in this study was expressed in Saccharomyces cerevisiae using a cDNA isolated from a human myoblast library [4]. Accordingly it was important to establish that this protein preparation exhibited activity similar to that of the native bovine protein previously characterized [2]. The time course, extent, protein concentration dependence, and calcium sensitivity of chromaffin granule aggregation by the recombinant human protein were found to be indistinguishable from the characteristics of the native bovine protein. In particular, maximal granule aggregation monitored by turbidity measurements on granule suspensions was promoted by 4ug/ml of human synexin, and half-maximal aggregation was promoted by 200uM free calcium.

Since the bovine lung calpactin was found to be unable to promote aggregation or fusion of rat pancreatic zymogen granules (see below) it was important to determine if the calpactin preparation aggregated chromaffin granules as previously described. Indeed, the preparations used in this study behaved characteristically [3] with chromaffin granules as substrate (eg., see fig. 5 and 6).



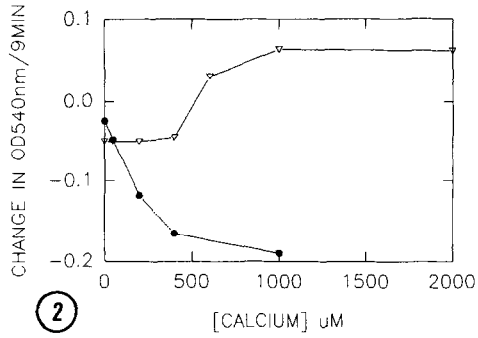


Figure 1. Time course of turbidity change of zymogen granule suspensions incubated with increasing amounts of synexin. 1mM Ca^{2+} was added at time 0. Synexin concentrations: 1, 0μg/ml; 2, 10.7 μg/ml; 3, 21.4 μg/ml; 4, 42.8 μg/ml; 5, 85.6 μg/ml.

Figure 2. Calcium dependence of the change in turbidity of zymogen granule suspensions incubated with 4.3 μg/ml (circles) or 85.6 μg/ml (triangles) synexin.

Turbidity measurements of zymogen granule suspensions incubated with synexin. Figure 1 illustrates the time course of turbidity changes when zymogen granules were incubated with increasing concentrations of synexin and 1mM free Ca^{2+}. In the absence of synexin the turbidity of the suspension slowly declines (fig.1, trace 1); this may be due to spontaneous granule lysis. Low concentrations of synexin enhance the rate of this decline (fig.1, traces 2,3); this may be due to enhanced lysis or to aggregation and subsequent fusion of granules [6]. High concentrations of synexin stabilize or increase the turbidity of the suspension (fig.1, traces 4,5); this was confirmed to be due to granule aggregation by direct observation in a phase microscope.

The decrease in turbidity of zymogen granule suspensions incubated with low concentrations of synexin exhibited a calcium sensitivity characteristic of synexin-induced chromaffin granule aggregation [2], with half-maximal response at approximately 200uM Ca^{2+} (fig. 2). The increase in turbidity induced by higher concentrations of synexin appeared to require higher concentrations of calcium (fig. 2).

Fusion of zymogen granules promoted by synexin. A fluorescence assay of lipid mixing, based on the dequenching of octadecyl rhodamine [5], was used to monitor fusion of labelled with unlabelled zymogen granules in the presence of synexin. Low concentrations of synexin, which caused a decline in the granule suspension turbidity, promoted granule fusion (fig. 3, traces 2,3)). This suggests that at least part of the synexin-dependent turbidity decline (fig.1) was due to granule fusion, as is characteristic of synexin- and fatty acid-dependent chromaffin granule fusion [6]. Further increases in synexin concentration reduced the extent of zymogen granule fusion (fig.3, trace 4)). This behavior has also been described for the fusion of chromaffin granules by

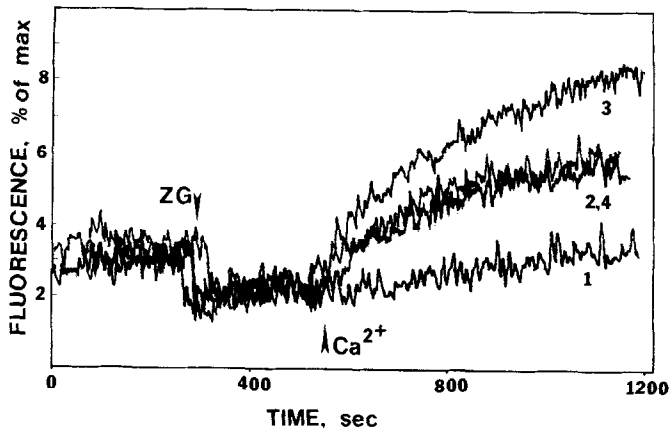


Figure 3. Fusion of zymogen granules incubated with synexin. Fluorescence of octadecyl rhodamine (normalized to maximum intensity in the presence of 0.2% Triton X-100) is recorded as a function of time. Labelled zymogen granules are present in the cuvette at the beginning of the experiment. An equal concentration of unlabelled granules is introduced at "ZG", 1 mM Ca^{2+} is introduced at "Ca^{2+}", and synexin is present from time 0. Trace 1, 0μg/ml; 2, 8 μg/ml; 3, 16 μg/ml; 4, 40 μg/ml synexin.

synexin [7], and may be due to extensive coating of the granule membrane with synexin which continues to promote membrane aggregation but which prohibits the close approach of the membranes required for fusion.

Interaction of calpactin and p68 (annexin VI) with chromaffin and zymogen granules. Calpactin, at concentrations up to 85 ug/ml, did not alter the turbidity of zymogen granule suspensions, although this is 9 times the calpactin concentration which gave maximal aggregation of chromaffin granules and 7 times the synexin concentration that gave maximal fusion of zymogen granules. Simultaneous membrane fusion might have masked any increase in turbidity associated with aggregation. However, at similar concentrations, calpactin caused no change in the rhodamine fluorescence indicative of membrane fusion. However, calpactin did appear to bind to zymogen granules as it inhibited the effects of synexin on the turbidity of zymogen granule suspensions (fig.4).

The ability of calpactin to aggregate membranes was not destroyed by incubation of the protein with the zymogen granules. As seen in figure 5, when chromaffin granules were added to calpactin preincubated with zymogen granules under conditions of the turbidity assay, the chromaffin granules were still aggregated by the calpactin.

p68 has been described as a synexin inhibitor ("synhibin", [8]) because it blocks the aggregation of chromaffin granules by synexin. This inhibition appears to be dependent upon the binding of the p68 to the granule membrane [9]. Recombinant murine p68 produced in yeast [4] was tested in this study

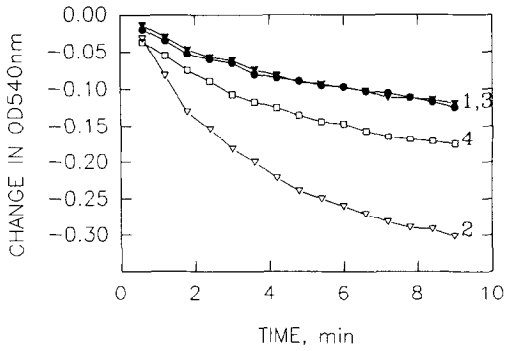
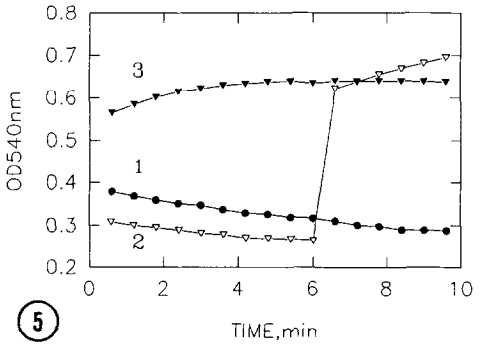


Figure 4. Effect of calpactin on turbidity changes in zymogen granule suspensions induced by synexin. 1, no annexin; 2, 2.2 μg/ml synexin; 3, 4.3 μg/ml calpactin; 4, 2.2 μg/ml synexin plus 4.3 μg/ml calpactin. 1 mM Ca^{2+} present in all cases.

and found to be comparably effective in blocking synexin action on chromaffin granules. Interestingly, the p68 was also found to be an effective inhibitor of the aggregation of chromaffin granules by calpactin (fig. 6).

p68 was also found in this study, at 15 ug/ml, to block the fusion of zymogen granules by 16 ug/ml synexin, suggesting that p68 can bind to both types of secretory organelle.



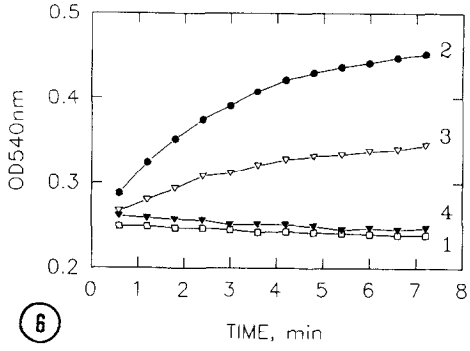


Figure 5. Pre-incubation of calpactin with zymogen granules does not alter its ability to aggregate chromaffin granules. Trace 1: Zymogen granules and 1mM Ca^{2+} present from time 0, no annexin. Trace 2: Zymogen granules, calcium, and 8.5μg/ml calpactin present from time 0, chromaffin granules added at 6min. After turbidity jumps to 0.6, the subsequent increase represents aggregation. Trace 3: Zymogen granules, chromaffin granules, calcium, and calpactin present from time 0.

Figure 6. Effect of p68 on the aggregation of chromaffin granules by calpactin. Granules were incubated with 1 mM Ca^{2+} and 0μg/ml (1) or 8.5 μg/ml calpactin (2,3,4) and 0μg/ml (1,2), 10μg/ml (3), or 20μg/ml (4) p68.

CONCLUSION

These results suggest that the calpactin tetramer can distinguish between particular biological membranes, while synexin and p68 may be more promiscuous. The specificity of the calpactin tetramer may arise from the ability to distinguish subtle differences in membrane lipid composition through coordinated action of the four homologous but distinct lipid binding domains present in each heavy chain. Alternatively, the associated 10kDa light chains may contribute to this unique behavior. Multiple annexins have been identified in single cell or tissue types. The differential specificities documented here suggest that the annexins may play distinct roles in a single cell by interacting with different membranes. Because the calpactin tetramer appears specific for chromaffin granules, this protein may play a unique role in neuroendocrine secretion.

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