Biochimica et Biophysica Acta, 1029 (1990) 149–160
Elsevier

BBAMEM 75042

Annexin-chromaffin granule membrane interactions: a comparative study of synexin, p32 and p67

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(Received 10 July 1990)

Key words: Annexin; Synexin; Calelectrin; Chromaffin granule; Exocytosis; Calcium ion binding protein

The chromaffin granule membrane binding and aggregating properties of three annexins, synexin, p32 and p67, have been studied and compared. Each protein was activated to bind and aggregate membranes with a biphasic Ca^{2+} dependence, with one phase titrating between pCa 5.0–3.5 and the second at higher levels of calcium (pCa < 3.5). *cis*-Unsaturated free fatty acids lowered these Ca^{2+} requirements by approximately one log unit. Barium and strontium were able to partially substitute for calcium, with the order of sensitivity $Ca^{2+} > Sr^{2+} > Ba^{2+}$. The proteins appeared to bind to distinct but overlapping populations of receptor sites, and did so in a manner displaying positive cooperativity at the higher Ca^{2+} levels. The maximal efficacy of the proteins as membrane aggregators differed with synexin being 1–2-fold more efficacious than p32, which in turn was 7-fold more efficacious than p67. In combination, p67 was an effective inhibitor of granule aggregation induced by synexin or p32, while p32 was able to both promote and inhibit synexin-induced granule aggregation in a manner which varied with synexin concentration. The complexity of these annexin–membrane interactions may be a reflection of the multidomain structure of the annexins and may have implications for the differential functions of these proteins in cells.

Introduction

The annexins are a new family of Ca^{2+}-binding proteins which bind to acidic phospholipid and biological membranes in the presence of calcium [1]. At least eight distinct members are known and have been identified by several names reflecting the different contexts in which they have been studied. Included in this family are p32 (endonexin [2], chromobindin 4 [3], protein II [4], lipocortin IV [58]), endonexin II [5] (lipocortin V [58]), calpactin I and II [6] (lipocortin II and I [7], chromobindin 8 and 9 [3], p36 and p35 [8]), synexin [9], lipocortin III [58], anchorin CII [59], and p67 [12] (lipocortin VI [58], chromobindin 20 [3], protein III [4], 67 kDa calelectrin [13]). Although the physiological function of these proteins has not been determined, in vitro studies indicate that some of these proteins self-associate [14–16], bind F-actin [5,17–19] and spectrin [17], are substrates for phosphorylation by C-kinase [20,21], EGF-receptor kinase or src-kinase [8], inhibit phospholipases [8,22–24] or clotting factor action [25], and potentiate the aggregation of membranes [9,13,26]. A comparison of the amino acid sequences [20,25,28], now known for several of these proteins, reveals that each protein has two regions, a short amino-terminal region lacking homology with the other members of the family, and a core region consisting of 4 or 8 repeats of a 70 amino acid segment sharing 40–60% homology between family members. Modeling studies [29] predict that the 70 amino acid segment may resemble the intestinal Ca^{2+}-binding protein with truncated Ca^{2+}-binding loops. It is proposed that these loops bind Ca^{2+} and phospholipid in a ternary complex. In fact, four Ca^{2+}-binding sites in the presence of acidic phospholipid have been detected on some members of this family, in agreement with this model [18,52]. The existance of multiple putative Ca^{2+}/phospholipid binding domains in each protein has been suggested to underlie the ability of these proteins to aggregate phospholipid vesicles and secretory granule membranes [1].

It has been proposed that these proteins may play a role in intracellular signal transduction by binding to intracellular membranes in the presence of Ca^{2+}. Such

Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; ADA, *N*-(2-acetamido)iminodiacetic acid; BSA, bovine serum albumin;

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an event may lead to inhibition of phospholipase A_2 or to promotion of intermembrane contacts such as those occurring in exocytosis. Despite the fact that the ability of annexins to bind to and aggregate membranes is well known, little is known about how these proteins perform these functions, whether these functions are regulated, and whether there are differences between individual family members.

In this paper we examine the interaction of three annexins, synexin, p32 and p67 with a natural membrane 'substrate'. Their interactions are found to be qualitatively similar but complex and quantitatively distinct. Each protein shows complex, biphasic activation by Ca^{2+} but with slightly different Ca^{2+} affinities. The complexity of their calcium activation curves may be a reflection of the multi-domain core structure shared by all. Each protein binds to a slightly different population of sites on the chromaffin granule membrane, and although they all appear to self-associate on the membrane surface to promote intermembrane contacts, they differ in their effectiveness as membrane aggregators and function as partial agonists in combination. As these proteins are simultaneously present in a number of cells, these annexin–annexin interactions may be important in regulating their cellular functions.

Materials and Methods

Fatty acids and their derivatives, egg yolk PS and PC were obtained from Sigma Chemical Co. and used as obtained without further analysis or purification. They were maintained as 20 mg/ml stock solutions in ethanol. ^{125}I was supplied by Amersham Corporation, and IODO-GEN was from Pierce Chemical Co.

Preparation of chromaffin granules. For turbidity assays of chromaffin granule aggregation, granules were prepared by differential centrifugation in 0.3 M sucrose as described [32]. For preparation of chromaffin granule membranes, chromaffin granules were prepared from the large granule fraction by sedimentation through a 1.6 M sucrose shelf [33]. The granule pellet was resuspended in 100 ml of deionized water and lysed with several strokes of a tight fitting Dounce homogenizer. The membranes were harvested by centrifugation at $35000 \times g$ for 30 min, resuspended in deionized water at a concentration of approximately 2 mg/ml, and stored at −80°C until use. These preparations have been extensively characterized [27,30]. Typically granules prepared by differential centrifugation may contain 5 to 10% of the total cellular mitochondrial and lysosomal enzyme activities, while the membrane preparation has 2 to 5% contamination. However, since the chromaffin granule is by far the most prominent organelle in the cell, the amount of contaminating membrane 'substrate' in the binding assays should be several fold less than these figures. The isolated chromaffin granules were stable for several days in isotonic sucrose at 4°C, but were generally used within 2–3 days of preparation. Multilamellar liposomes were prepared as follows. A chloroform solution of PS/PC (1:1, w/w) was dried under nitrogen and resuspended at 1 mg/ml by vortex mixing into buffer containing 0.24 M sucrose, 30 mM KCl, 40 mM Hepes (pH 7.0).

Annexin preparation. The mammalian calelectrins (p32 and p67) were prepared from bovine liver as described [3]. Using these purification methods 1.0–1.5 mg of p32 and p67 were routinely prepared from 150 g of liver, in greater than 95% purity as assessed by SDS-PAGE.

Bovine liver synexin was prepared as follows. 250 g of chilled bovine liver was minced and homogenized in 500 ml ice-cold 0.3 M sucrose, 2 mM EGTA, 25 mM Mes (pH 6.0) buffer by two 30-s pulses with a Waring blender followed by one complete pass at 2500 rpm with a Potter-Elvejhem tissue grinder. The homogenate was centrifuged at $23000 \times g$ for 30 min at 4°C and the supernatant was isolated and recentrifugated at $100000 \times g$ for 60 min at 4°C. The supernatant from this high speed spin (cytosol) was filtered through two layers of cheesecloth and brought to 20% saturation in ammonium sulfate. After 15 min, the precipitated proteins were pelleted by centrifugation at $100000 \times g$ for 60 min at 4°C and resuspended into 20 ml of ice-cold 0.3 M sucrose, 25 mM Mes (pH 6.0) with the aid of a Dounce homogenizer. The solution was clarified by centrifugation at $100000 \times g$ for 40 min at 4°C and the supernatant was applied to a column (2.5 × 12 cm) of DEAE-Sephacel equilibrated and then eluted with 0.3 M sucrose, 25 mM Mes (pH 6.0) at 4°C. The eluant fractions containing chromaffin granule aggregating activity were pooled, fortified with 1 mM $CaCl_2$, and applied to a Mono S column connected to a Pharmacia FPLC system containing buffer A (0.3 M sucrose, 1 mM $CaCl_2$, 25 mM Mes (pH 6.0)) and buffer B (0.3 M sucrose, 1 M KCl, 25 mM Mes (pH 6.0)). The column was washed with buffer A and synexin was eluted with a linear gradient of buffer A and buffer B. This chromatography step was done at room temperature. Fractions containing chromaffin granule aggregating activity, eluting between 0.5–0.6 M KCl, were pooled. On SDS-PAGE three bands were seen in these fractions, synexin (80%) at 47 kDa, μ-synexin (5–10%, the predominant skeletal muscle isotype; Ref. 31) at 51 kDa and a 56 kDa contaminant (10–15%) which was removed by a repeat 20% ammonium sulfate precipitation step when preparing synexin for ^{125}I-synexin binding experiments.

Purified synexin, p32 and p67 were iodinated by the IODO-GEN method as described [3].

Binding assays. The binding of ^{125}I-labelled protein to membranes was measured by a modification of the method of Burgoyne and Geisow [34]. Intact chromaffin granules, chromaffin granule membranes, or multi-

lamellar PS/PC liposomes were preincubated for 15 min at 22°C in buffer containing 0.24 M sucrose, 30 mM KCl, 40 mM Hepes-NaOH (pH 7.0) and varying concentrations of free fatty acids. An aliquot of this mixture was then added to a 1.5 ml microfuge tube so as to contain (in final concentration) 0.24 M sucrose, 30 mM KCl, 40 mM Hepes-NaOH (pH 7.0), 52 μg/ml chromaffin granule membrane protein (or 50 μg/ml total chromaffin granule protein), 2.5 mM chelator (EGTA,HEDTA, or ADA) and varying concentrations of $CaCl_2$, fatty acids, ^{125}I-labelled protein, and nonlabelled protein. The reactions were started by addition of ^{125}I-labelled protein (in competition binding experiments, nonlabelled protein was first allowed to react with the membranes for 15 min at 22°C prior to addition of labelled protein), allowed to incubate for 15 min at 22°C, then centrifuged at 15 600 × *g* for 15 min in an Eppendorf microcentrifuge. The supernatants were aspirated and the pellet and tube wall were washed twice with binding buffer containing the same Ca^{2+} concentration as used in the binding step. The radioactivity was measured in both the pellets and the supernatants with a Beckman Model 300 gamma counter. Specific binding was defined as the difference in membrane-bound radioactivity in the absence and presence of excess EGTA or excess nonlabelled protein. Both methods of calculation gave identical results and nonspecific binding was generally defined as binding in the presence of 2.5 mM EGTA (unless otherwise noted). In the absence of granule membranes or carrier proteins (1 mg/ml ovalbumin or bovine serum albumin), ^{125}I-labelled proteins bound to the polypropylene microfuge tube in both a Ca^{2+}-independent and Ca^{2+}-dependent manner. Hence, experiments involving binding to liposomes were done in the presence of 1 mg/ml BSA. The presence of carrier protein had no effect on the Ca^{2+} dependence of protein binding to chromaffin granule membranes and was often omitted from these studies. Ovalbumin was included in all studies involving free fatty acids to protect the labelled protein from detergent effects of the non-membrane-bound lipid.

Chromaffin granule aggregation was assayed by measuring the change in turbidity (absorbance at 540 nm) induced in a dilute suspension of chromaffin granules by addition of calcium. Absorbance measurements were made on a Gilford System 2600 microprocessor controlled spectrophotometer equipped with an automatic cuvette positioner which allowed intermittant monitoring of four simultaneous reactions. The standard aggregation assay was conducted at 22°C on a 1 ml granule suspension having an initial absorbance at 540 nm of 0.3 (50–70 μg/ml granule protein) in a buffer containing 0.24 M sucrose, 30 mM KCl, 40 mM Hepes-NaOH (pH 7.0) and variable amounts of synexin or calelectrin. After establishing a baseline absorbance for 1 min, 20 μl of Ca^{2+}-buffer was added with hand mixing to give a final concentration of 2.5 mM chelator and 0–15 mM $CaCl_2$, and absorbance was recorded for an additional 8 min. Controls were examined in which the change in absorbance due to granules alone or protein alone were monitored. Granule aggregation was expressed as:

$$\frac{A_{540}^{6\text{min}} - (A_{540}^{6\text{min}(-\text{protein})} - A_{540}^{\text{baseline}})}{A_{540}^{\text{baseline}}} \times 100\%$$

where $A_{540}^{\text{baseline}}$ and $A_{540}^{6\text{min}}$ represent the absorbance values at 540 nm at zero time and 6 min after Ca^{2+} addition, respectively. Dabrow et al. observed that synexin exhibited a significant increase in absorbance in the absence of granules and subtracted this component from their expression for granule aggregation [60]. However, we failed to observe a Ca^{2+}-dependent increase in the absorbance of a solution of either synexin, p32 or p67 and hence found such a correction unnecessary.

Protein was measured by the method of Bradford [35] using BSA as the standard for p32, p67 and chromaffin granules, and bovine gamma globulin as the standard for synexin. Using these standards, this assay gives protein concentrations which agree well with those determined by the Lowry assay [36]. Lipid phosphorus was determined according to Bartlett [37].

Ca^{2+} measurements.Ca^{2+} buffers were employed to maintain medium Ca^{2+} concentrations at precisely defined levels. To cover a wide range of Ca^{2+} concentrations, a series of chelators with different buffering ranges was employed as described by Durham [38]. A series of Ca^{2+} buffers (prepared as 50-fold concentrates) ranging from pCa 7 to pCa 3 were made using the stability constants measured by Durham in 100 mM NaCl at 37°C. The pCa of each buffer in 0.3 M sucrose, 30 mM KCl, 40 mM Hepes-NaOH (pH 7.0) at 22°C was then checked with a Ca^{2+}-selective electrode (Radiometer) standardized with solutions of known Ca^{2+} activity (activity coefficients for dilute aqueous solutions of $CaCl_2$ were calculated from the Debye-Huckel limiting law). The values of pCa reported here, therefore, reflect Ca^{2+} activities rather than Ca^{2+} concentrations. To compare these values with pCa values reported in concentration units, all values of pCa given in this paper should be decreased by approximately 0.26 units (i.e., pCa 6.0 (activity) becomes pCa 5.74 (concentration)).

Results

Ca^{2+} dependence of annexin action

The Ca^{2+} concentrations required for binding synexin, p32 and p67 to membranes as well as for membrane aggregation induced by these proteins have previously been studied by different laboratories under nonuniform conditions using an inconsistent variety of artificial or natural membrane substrates. In order to critically compare the Ca^{2+} requirements for activation

of these three annexins, we measured under identical conditions the Ca^{2+} concentrations required for binding synexin, p32, and p67 to the chromaffin granule membrane using a sensitive radioligand binding assay (Fig. 1), and for inducing membrane aggregation by these proteins (Fig. 2). As shown in these figures both membrane binding and induction of membrane aggregation have a multiphasic Ca^{2+} dependence. For both p32 and p67, a significant extent of membrane binding appears to occur at lower Ca^{2+} levels than required to promote appreciable membrane aggregation. However, since turbidity changes may not be linearly related to the extent of granule aggregation, a direct comparison of the Ca^{2+} dependencies of these two events may be misleading. Both membrane binding and granule aggregation have a similar biphasic Ca^{2+} dependence with one phase titrating between pCa 5.0 and 3.5 and the second phase between pCa 3.5 and 2.0. For all three annexins, a dip is observed in the Ca^{2+} titration curve for membrane binding. This is most pronounced in the case of p67 (Fig. 1c) and is most unexpected for a simple binding reaction. However, the binding data presented in Fig. 1 were obtained at lower protein concentrations than the aggregation data. At higher annexin concentrations, such as those used in Fig. 2 for granule aggregation, this dip is not apparent (not shown), and the first 'phase' of the Ca^{2+} titration reaches a stable plateau. The reason for this difference at low and high protein concentration relates to the Ca^{2+}-dependent changes in membrane binding affinity and capacity discussed below. One apparent difference between these three proteins is the slightly lower Ca^{2+} requirements of p32 and p67 for membrane binding when compared to synexin.

The Ca^{2+} requirements for membrane binding also appear to vary depending on the membrane to which the annexins bind. We observed that the Ca^{2+} concentration at half-maximal for the first phase of binding of p32 to chromaffin granule membranes, PS/PC (1:1, w/w) vesicles, and mitochondrial membranes was pCa 5.0, 5.2 and 4.6, respectively.

Not only are the three annexins activated by Ca^{2+} but they are also substrates for activation by Ba^{2+} and Sr^{2+} to varying degrees as shown in Table I. Differences of 5% or more we found to be highly significant and reproducible in this assay. In general, the order of sensitivity to cations appears to be $Ca^{2+} > Sr^{2+} > Ba^{2+}$. For example, this is readily apparent with synexin at 1 mM cation concentration. However, with p32, both 1 mM and 10 mM cation concentrations must be considered. At 1 mM cation concentration, Sr^{2+} is equivalent to Ba^{2+}, and both are less effective than Ca^{2+}, while at 10 mM, Sr^{2+} produces similar aggregation to Ca^{2+} while both produce far more aggregation than Ba^{2+}.

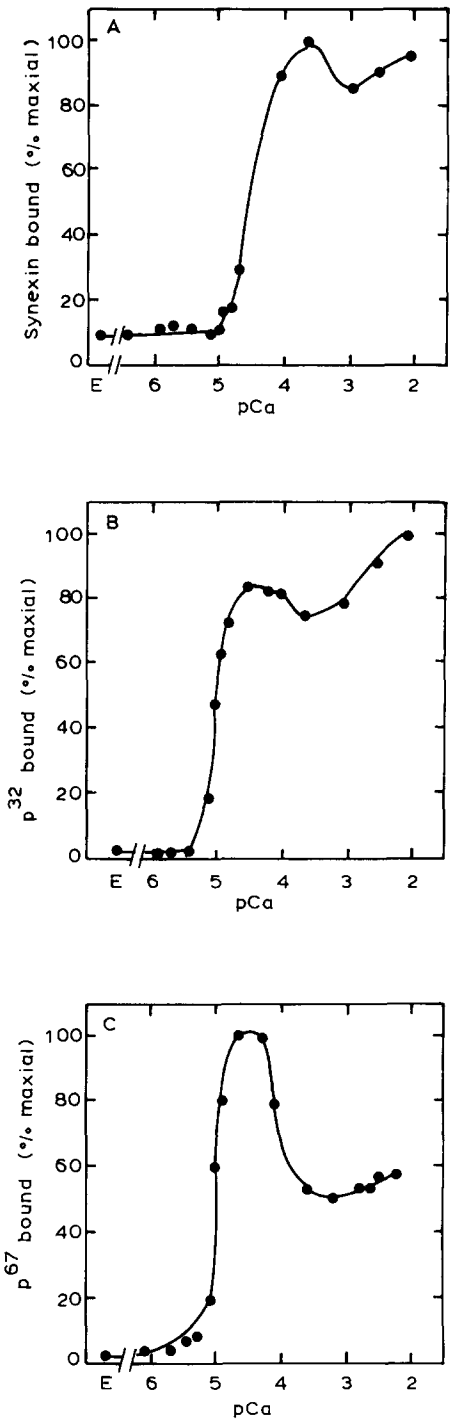


Fig. 1. Ca^{2+}-dependence of synexin, p32 and p67 binding to chromaffin granule membranes. Chromaffin granule membranes were incubated with 20 nM ^{125}I-synexin (4.6×10^4 cpm/pmol) (A), 20 nM ^{125}I-p32 (1.3×10^4 cpm/pmol) (B), or 5 nM ^{125}I-p67 (1.7×10^3 cpm/pmol) (C) for 15min in the presence of various concentrations of free calcium as described under Materials and Methods. Total membrane associated radioactivity, expressed as a percentage of the maximal amount bound, is plotted as a function of the free Ca^{2+} concentration. E = buffer containing 2.5 mM EGTA with no added $CaCl_2$. The binding analyses were performed at least twice with different preparations of granules with essentially the same results. Results from representative experiments are shown.

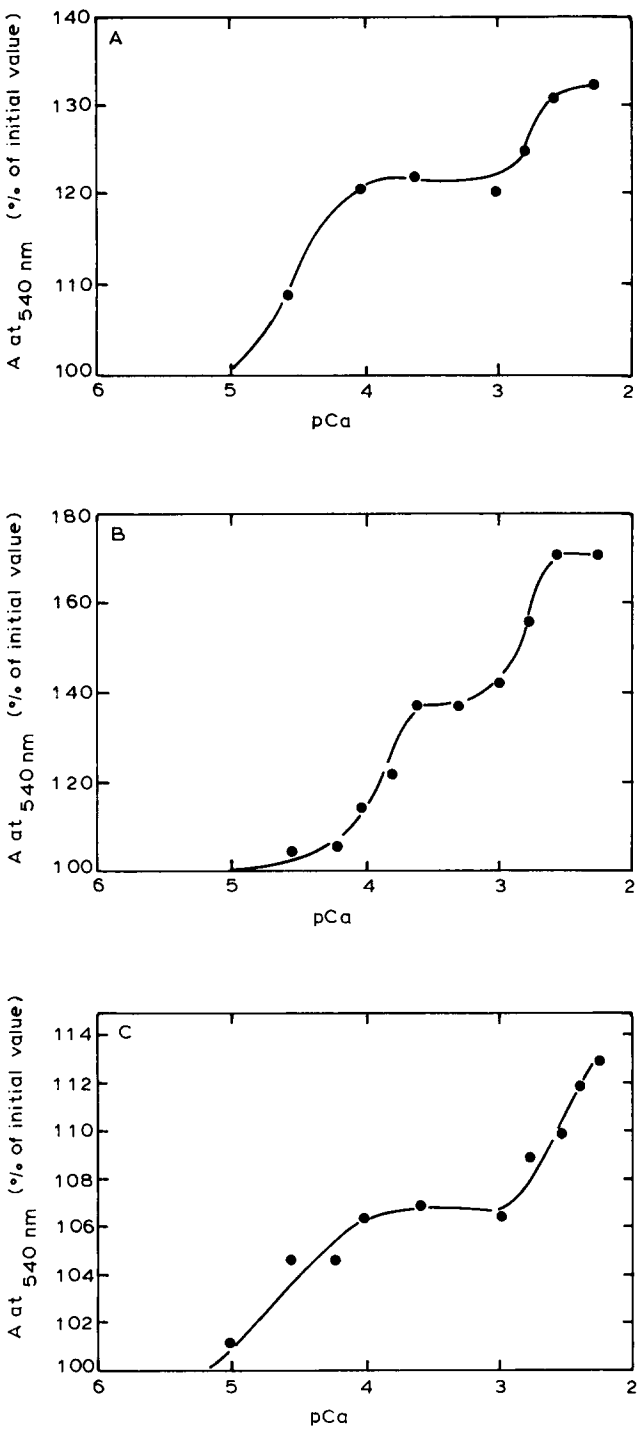


Fig. 2. Ca^{2+}-dependence of annexin-induced chromaffin granule aggregation. Chromaffin granule aggregation in the presence of 10.5 μg/ml synexin (A), 10 μg/ml p32 (B), 20 μg/ml p67 (C) was measured as described under Materials and Methods. The degree of granule aggregation is plotted as a function of free Ca^{2+} concentration. In each case the data are from a representative experiment which has been repeated at least once on a separate preparation of chromaffin granules.

Free fatty acids lower annexin Ca^{2+} requirements

Oleic acid (or arachidonic acid) had previously been shown to increase the Ca^{2+} sensitivity of self-association

TABLE I

Chromaffin granule aggregation induced by synexin and the calelectrins in the presence of Ca^{2+}, Ba^{2+} and Sr^{2+}

Chromaffin granule aggregation was measured in the absence and presence of synexin (19.8 μg/ml), p32 (11.8 μg/ml) or p67 (20.8 μg/ml) as described under Materials and Methods. The reaction was initiated by the addition of $CaCl_2$, $BaCl_2$ or $SrCl_2$ so as to make the final cation concentration 1 or 10 mM when uncorrected for activity. No cation buffer was employed.

Cation	Concn. (mM)	*A* at 540 nm (% of initial value)			
		Annexin present			
		none	synexin	p32	p67
Ca^{2+}	1	93.6	148.1	140.0	104.8
Ca^{2+}	10	98.1	152.1	159.4	126.8
Ba^{2+}	1	94.2	108.2	100.5	101.5
Ba^{2+}	10	97.6	124.0	108.8	104.7
Sr^{2+}	1	92.4	118.5	102.8	104.2
Sr^{2+}	10	94.1	137.8	154.7	108.5

of synexin in solution [16]. As shown in Fig. 3 oleic acid also apppears to increase the Ca^{2+} sensitivity of membrane binding and membrane aggregation by p32 in a concentration-dependent manner. Essentially identical effects were seen with synexin and p67. Chromaffin granule membranes, instead of intact granules, were used in the aggregation assay to avoid turbidity changes associated with free fatty acid induced chromaffin gran-

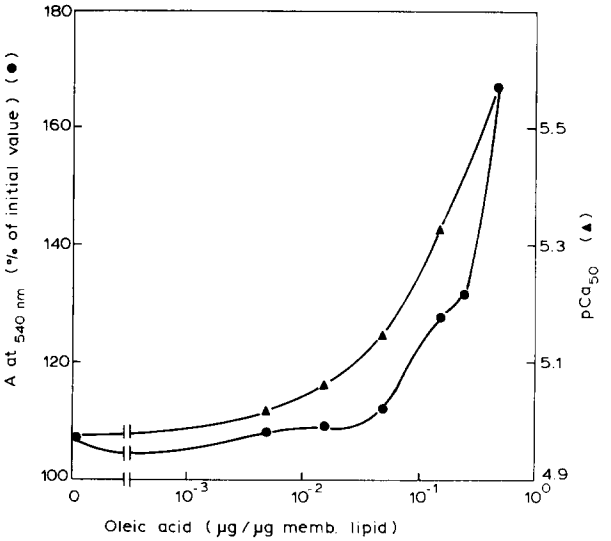


Fig. 3. P32 binding and membrane aggregation as a function of oleic acid concentration. Chromaffin granule membranes (19 μg/ml (●), or 130 μg/ml (▲)) were incubated with varying concentrations of oleic acid for 15 min at 22°C. They were then either diluted 10-fold into standard aggregation buffer containing 27.5 μg/ml p32 and allowed to aggregate at pCa 4.9, or diluted 2.5-fold into a solution containing 12 nM ^{125}I-p32, 1 mg/ml ovalbumin and varying concentrations of free Ca^{2+} as described under Materials and Methods. The extent of membrane aggregation (●) and the Ca^{2+} concentration required for half-maximal binding (▲) are plotted as functions of oleic acid concentration expressed relative to total granule lipid. Data from a single experiment are shown.

TABLE II

Specificity of free fatty acids as modulators of p32 binding and membrane aggregation

Various free fatty acids (at a concentration of 0.5 μg/μg membrane lipid) were substituted for oleic acid in the experiments described in the legend to Fig. 3. The Ca^{2+} concentration required for half-maximal p32 binding (pCa_{50}), and the extent of p32-induced membrane aggregation at pCa 4.9 (A_{540}, % of baseline at 10 min) is tabulated for each free fatty acid. Data are from a single experiment.

Fatty acid	pCa_{50}	A_{540}
None	4.97	
Oleic acid	5.55	167
Linoleic acid	5.50	
Elaidic acid	5.40	
Palmitic acid	5.36	133.7
Myristic acid	5.32	
Methyl oleate	5.00	105.5

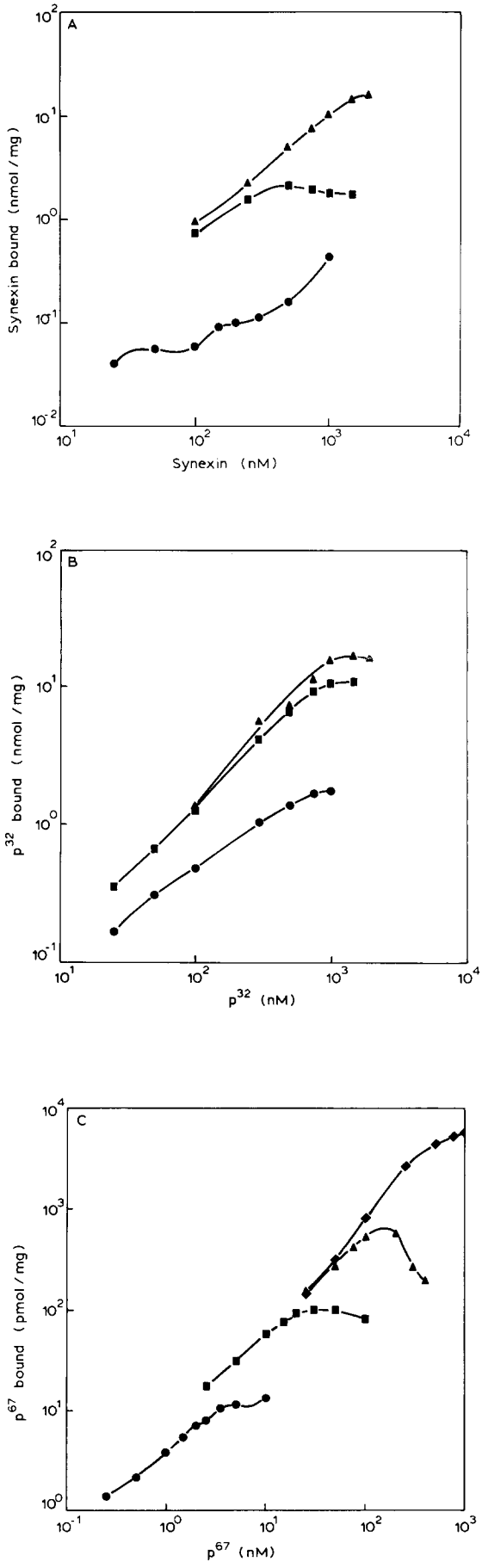
ule fusion [46], since osmotically lysed chromaffin granules do not fuse [39]. Effective concentrations of oleic acid, e.g., greater than 0.05–0.1 μg/μg membrane lipid, are similar to those that promote fusion of chromaffin granules aggregated by synexin [46] and increase the affinity of C-kinase for Ca^{2+} (1.5 μg/μg lipid) [44]. Although these free fatty acid concentrations appear quite high, not all of the added fatty acid paritions into the membrane. Although the membrane-aqueous partition coefficient for oleic acid was not measured in this system, literature values range from 10^3 to 10^5 [47], so it is possible that as little as 10% or as much as 90% of the added lipid is actually membrane bound. The data therefore represent the behavior of the complete system, including Ca^{2+}, the annexin, and the membrane, as well as the aqueous environment. Not all free fatty acids were equally effective at lowering the Ca^{2+} requirements for p32 binding as shown in Table II. *cis*-Unsaturated free fatty acids such as oleic acid or linoleic acid were more effective than trans-unsaturated free fatty acids such as elaidic acid or saturated free fatty acids such as myristic or palmitic acid. Esterified free fatty acids such as methyl oleate were ineffective. The mechanism of these differential fatty acid effects may include differences in degrees of partitioning into the membrane.

Protein concentration dependence of annexin action

Figs. 4a–c and 5a–d show the protein concentration dependence at different Ca^{2+} levels for annexin binding to chromaffin granule membranes, and annexin-induced chromaffin granule membrane aggregation, respectively. Qualitatively, the binding characteristics of the three annexins are very similar. At the low Ca^{2+} concentrations the binding curves well approximate a rectangular hyperbola indicating lack of extensive interprotein interactions, and binding to one or many classes of binding sites of comparable affinity. As the Ca^{2+} level increases, several changes in membrane binding are seen. First of all, there is an apparent decrease in relative binding affinity. Secondly, there is an apparent increase in binding capacity, and thirdly, positive cooperativity is observed. The Hill coefficients for the binding of synexin, p32 and p67 at pCa 3 were 2.0, 5.0, and 1.9, respectively. A curious feature of these titration curves for membrane binding is the decrease in binding observed at high protein concentrations at certain Ca^{2+} levels. This phenomenon is most apparent with p67 (Fig. 4c), but also occurs to a lesser extent with synexin (Fig. 4a), and also possibly with p32 (Fig. 4b). The effect is most dramatic at pCa 4.0 in these figures, but there is variability between protein preparations and in some cases the effect is also seen at both lower and higher Ca^{2+} levels. The biphasic nature of the mass titration of annexin binding is also sensitive to the presence or absence of carrier proteins such as ovalbumin or bovine serum albumin. For example, at pCa 4.8, in the absence of carrier protein, binding of p67 is biphasic (data not shown). Carrier proteins such as BSA also inhibit annexin self-association on membrane surfaces at pCa > 3 (detected by measurement of intermolecular energy transfer using fluorescently labelled annexins; Zaks and Creutz, manuscript in preparation). Therefore, such a self-association event may somehow lead to alterations in the annexin-binding properties of the membrane.

When the protein concentration dependence for binding is compared to that for chromaffin granule aggregation, some common qualitative features are seen. The first such feature is the fact that the maximal response in each situation increases as a function of Ca^{2+} concentration, and secondly, apparent cooperative interactions appear more pronounced at the higher Ca^{2+} levels. The amount of annexin required to generate the maximal (or final) aggregation response correlates with that producing the maximal degree of binding at each Ca^{2+} concentration. However, the maximal membrane aggregating ability of the annexins does not appear to solely depend on the amount of protein that is membrane bound, but rather also on its degree of Ca^{2+} saturation. For example, at saturating protein concentrations, seven times as much synexin is membrane bound at pCa 3 than at pCa 4 while the extent of membrane aggregation is the same at these two levels of calcium. Furthermore different modes of membrane aggregation may occur at different Ca levels: p32-induced membrane aggregation decreases as the protein binding sites on the membrane are saturated, but this is seen only at high and not at low Ca^{2+} levels.

Examination of the data in Fig. 5 reveals that although each protein shares the ability to aggregate chromaffin granules, there are distinct differences between them in this regard. One such difference is the



range of Ca^{2+} concentrations where they are most active. Synexin and p32 are most active from pCa 5 to 3, while p67 is most active from pCa 3 to 2. The second difference is that at physiological Ca^{2+} concentrations, loosely defined as < 1 mM Ca^{2+}, the three proteins differ in efficacy with synexin being approx. 1–2-times more efficacious than p32, which in turn is approx. 7-times more efficacious than p67.

Annexin–annexin interactions affecting membrane binding and aggregation

The membrane binding isotherms generated at low Ca^{2+} levels for synexin, p32 and p67, shown in Fig. 4 indicate that these three proteins bind 140, 2200, and 18 pmol/mg membrane binding sites with apparent affinities of 87, 270, and 2.5 nM. In order to determine if the different annexin proteins compete for available binding sites, we examined the ability of the different annexins to displace each other from the membrane. As shown in Fig. 6, p67 displaces synexin from binding sites on chromaffin granules at pCa 4.8 and pCa 4.4 with a K_i of 7 nM and 26 nM, respectively. These K_i values agree well with the K_d values for p67 binding at these Ca^{2+} levels, being 3 nM and 24 nM, respectively, suggesting that displacement of synexin is a consequence of p67 binding to the membrane and that the binding affinity of p67 is not altered by the presence of bound synexin. Interestingly, the K_i of 15–17 nM for p32-dependent inhibition of synexin or p67 binding is appreciably less than the apparent K_d of 200–400 nM for p32 binding to its receptor. This suggests that only a fraction of p32 binding sites need be occupied to completely displace p67 or synexin from the membrane. However, since the total number of p32 binding sites greatly outnumber those of synexin or p67 at a given Ca^{2+} level, it is conceivable that a direct 1:1 competition for binding sites occurs. If this model is correct, p67 would not be expected to displace p32 from granule membranes, since the number of p32 binding sites greatly exceed the number of p67 binding sites. This prediction appears to be supported experimentally (Fig. 6b). A modest (20%) decrease in p32 binding is seen at high concentrations of p67, but these levels of p67 are in excess of those needed to saturate p67 binding sites on the granule membrane at pCa 4.8, and appear inde-

Fig. 4. Protein concentration dependence of annexin binding to membranes. Chromaffin granule membranes (52 μg/ml) were incubated with varying concentrations of ^{125}I-synexin (A, at pCa 4.55 (●), 4.0 (■), and 3.0 (▲)), ^{125}I-p32 (B, at pCa 5.0 (●), 4.0 (■), and 3.0 (▲)), and ^{125}I-p67 (C, at pCa 4.8 (●), 4.55 (■), 4.0 (▲), and 3.0 (◆)) as described under Materials and Methods. Specific binding (total binding minus binding in the presence of 2.5 mM EGTA alone) is plotted as a function of total annexin present. A log-log plot is required to incorporate the wide range of data on a single graph. Data are from a representative experiment, repeated at least once.

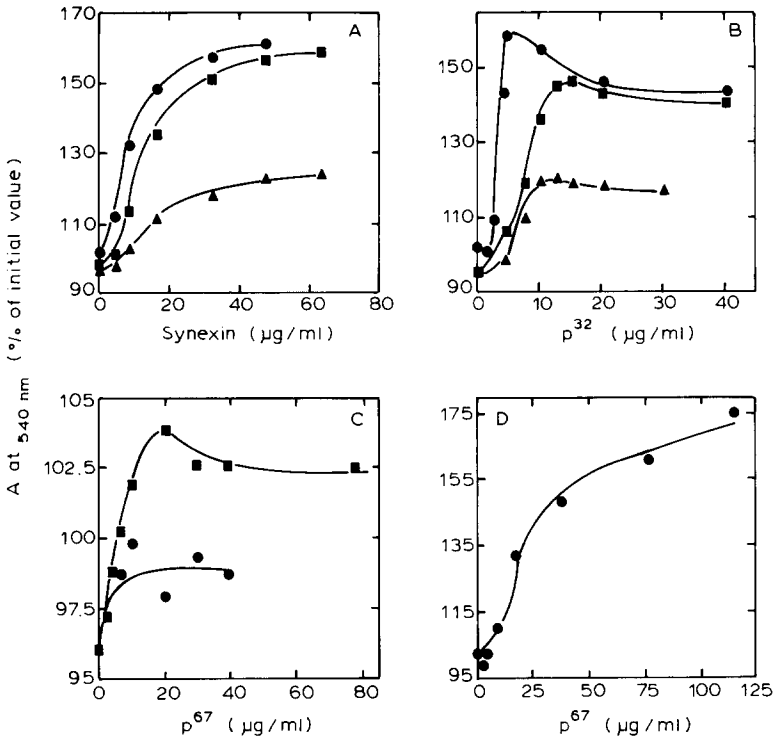


Fig. 5. Annexin concentration dependence of chromaffin granule aggregation. Chromaffin granule aggregation was measured in the presence of varying concentrations of synexin (A, at pCa 2.26 (●), 3.0 (■), and 4.55 (▲)), p32 (B, at pCa 2.26 (●), 3.3 (■), and 4.0 (▲)), and p67 (C, at pCa 4.55 (●) and 3.0 (■)) and (D, at pCa 2.26) as described under Materials and Methods. The data are from a representative experiment, repeated at least once with a separate preparation of chromaffin granules.

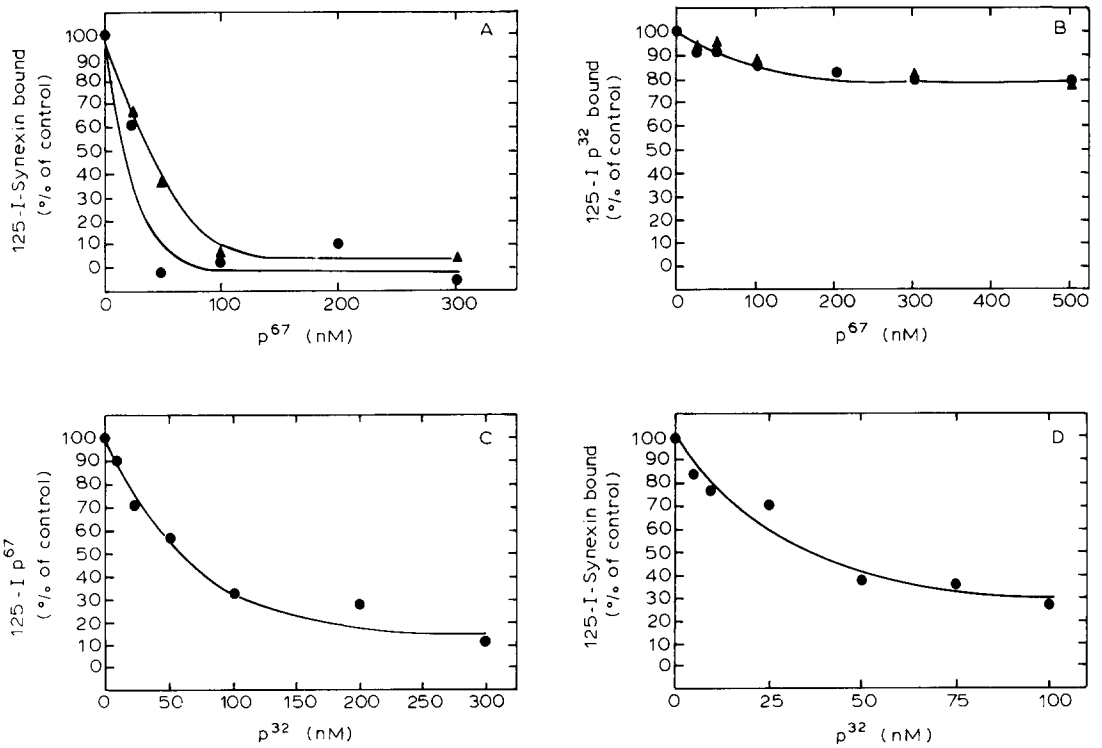


Fig. 6. Competitive granule binding interactions of the annexins at low Ca^{2+} concentrations. Chromaffin granule membranes (52 μg/ml) were preincubated with varying concentrations of nonlabelled p32 or p67 for 15 min at 22°C in the presence of 1 mg/ml ovalbumin at pCa 4.8 (●) and 4.4 (▲) (A), pCa 4.8 (B), pCa 4.9 (C), and pCa 4.55 (D). To these mixtures were added (in final concentration) ^{125}I-synexin (Fig. A, 50 nM ($1x10^4$ cpm/pmol) (●), 20 nM (300 cpm/pmol) (▲); Fig. D. 14 nM (9500 cpm/pmol)) ^{125}I-p32 (Fig. B, 5 nM (7875 cpm/pmol) (●), 50 nM (237 cpm/pmol) (▲)), or ^{125}I-p67 (Fig. C, 10 nM (1.3×10^4 cpm/pmol)), and each was allowed to incubate an additional 15 min as described under Materials and Methods. Iodinated protein binding (expressed as a percentage of binding in the absence of nonlabelled protein) is plotted as a function of total nonlabelled protein concentration. Data are from a single experiment.

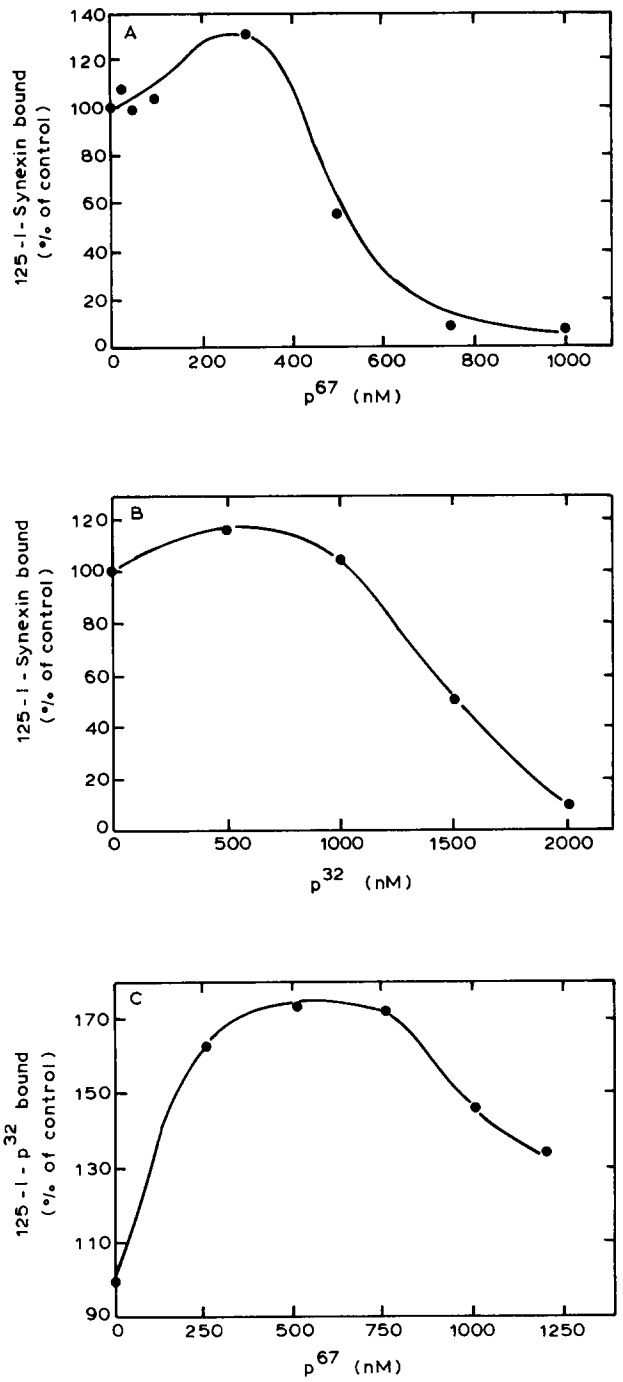


Fig. 7. Competitive granule binding interactions of the annexins at high Ca^{2+} concentrations. Chromaffin granule membranes (52 μg/ml) were preincubated for 15 min at 22°C with varying concentrations of p67 (A,C) or p32 (B) for 15 min at pCa 3.0 in the presence of 1 mg/ml ovalbumin. ^{125}I-synexin (A, 20 nM (3000 cpm/pmol);B, 5 nM (5000 cpm/pmol)) or ^{125}I-p32 (C, 5 nM (1.5×10^4 cpm/pmol)) was then added, and after an additional 15 min incubation ^{125}I-synexin (A,B) and ^{125}I-p32 (C) specific binding was measured as described under Materials and Methods. The results are expressed as a percentage of binding observed in the absence of nonlabelled protein, and plotted as a function of total concentration of nonlabelled protein added. Data from a single experiment are shown.

pendent of the concentration of p32 present, suggesting that this inhibition is by a mechanism other than competition for membrane binding sites. One possible mechanism is p32–p67 interaction in solution which prevents p32 from binding membranes.

At the higher Ca^{2+} levels, where cooperative binding prevails, a different type of annexin interaction is observed. At low concentrations of one radiolabelled annexin, binding of this protein is affected in a biphasic manner by a heterologous annexin protein (Fig. 7). In the absence of synexin, p32 binding occurs mainly at concentrations below 1000 nM (Fig. 4). At these concentrations, p32 appears to enhance ^{125}I-synexin binding. At higher p32 concentrations, inhibition of ^{125}I-synexin binding occurs. The mechanism of this inhibition is uncertain, however, since it occurs over a range of p32 concentrations where little additional p32 is binding to the granule membrane. Binding of ^{125}I-synexin to aggregates of p32 in solution may be occurring, although there is no direct evidence to support this theory. The binding competition data shown in Fig. 7 also reveals that p32 and p67 have nearly identical effects on synexin binding, and that synexin and p32 binding is similarly affected by p67. At higher concentrations (500–1000 nM) of a radiolabelled protein, however, the binding of this protein is not enhanced by the presence of heterologous protein. In the presence of a saturating amount (1000 nM) of ^{125}I-p32, p67 at concentrations as high as 200 nM, produce only a modest 13% decrease in p32 binding (data not shown).

Although p67 has little ability to inhibit p32 binding, we have previously shown that it is a very effective inhibitor of chromaffin granule aggregation induced by p32 or synexin (Fig. 2, Ref. 3), and is effective at p67 concentrations equal to or less than those required for

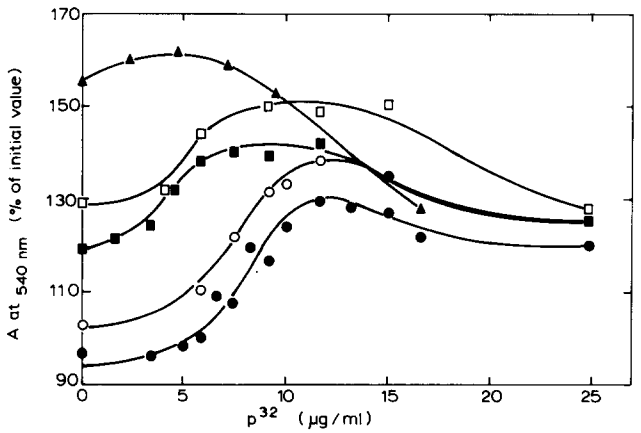


Fig. 8. Effect of p32 on chromaffin granule aggregation in the presence of synexin. Chromaffin granule aggregation was measured in the presence of varying concentrations of p32 and 0 (●), 1.6 (○), 3.2 (■), 4.8 (□), or 47.6 (▲) μg/ml synexin at pCa 3.0 as described under Materials and Methods. Data are from a single representative experiment, repeated at least once with a different batch of chromaffin granules with similar results.

chromaffin granule aggregation by p67 alone. Investigation into the mechanism of the inhibitory action of p67 failed to show that it acted by displacing membrane bound p32. For example, although high concentrations of p67 did displace some p32 from the membrane, the extent of inhibition of granule aggregation did not correlate with the amount displaced (data not shown). Furthermore, the kinetics of chromaffin granule aggregation in the presence of p32 and p67 were different from those seen with reduced p32 concentrations alone: Granule aggregation displays biphasic kinetics with a rapid first phase (less than one minute) and a prolonged second phase (ten to twenty minutes; both phases are proportionally smaller with reduced p32 concentrations, while p67 antagonism selectively inhibits the slower phase of aggregation. The initial jump in turbidity correlated with the amount of p32 present and may represent the initial binding of p32 to the membrane. P32 can also function as an inhibitor of synexin-induced chromaffin granule aggregation, but only at high synexin concentrations (Fig. 8). At lower synexin concentrations p32 partially substitutes for synexin and enhances the synexin-induced response (Fig. 8).

Discussion

This paper presents a detailed comparative study of the interactions of synexin, p32 and p67 with chromaffin granule membranes resulting in annexin binding and membrane aggregation. An analysis of the Ca^{2+} requirements for activating these proteins revealed the biphasic nature of this process, with one phase titrating between pCa 5 and 3.5 and the other at pCa < 3.5. The physiological significance of the membrane binding and aggregation observed in this study may be questioned on the basis of the rather high Ca^{2+} levels required, especially for the second 'phase' of the response. However, it is important to realize that Ca^{2+} may be compartmentalized in the cell and exist in rather high local concentrations close to the plasma membrane or endoplasmic reticulum in juxtaposition to Ca^{2+} channels [53]. Furthermore, it remains to be seen whether these Ca^{2+} requirements reflect the in vivo situation where various cofactors and conditions may exist to modify the proteins' Ca^{2+} affinity [5,52,54,55]. Indeed, we have demonstrated that free fatty acids lower the threshold Ca^{2+} concentration required for annexin activation into the low (1–10 μM) range reported to exist in some stimulated cells [62]. This fatty acid effect is potentially physiologically important since free fatty acids are liberated from membrane phospholipids by phospholipase activation in a variety of stimulated cells, and have been postulated to function as second messengers [44,56]. It is possible that these free fatty acids both recruit annexin molecules to their site of generation and also enhance annexin function by synergizing with Ca^{2+}. Although the concentrations of free fatty acids effective in this study appear rather high, they are similar to levels reported for C-kinase activation [44], and may well be in the range of local concentrations existing in certain areas of the membrane near their sites of production.

The mechanism by which free fatty acids alter the Ca^{2+} sensitivity of the annexin proteins is unclear. Since the proteins' Ca^{2+} affinities appear to be sensitive to the lipid environment of the membrane, free fatty acids could act by modifying this substrate. However, a second mode of action also appears possible because we have previously shown that substoichiometric amounts of free fatty acid could lower the Ca^{2+} requirement for the polymerization of synexin in solution [16]. It thus appears that these lipids may have direct effects on the proteins independent of membranes.

Extensions of the Ca^{2+} titrations into the millimolar Ca^{2+} range, although clearly non-physiologic, have been included in this study to provide a more complete understanding of the proteins' behavior and to allow possible correlations to be made with the known structural features of these proteins. For example, Schlaepfer and Haigler have previously shown that maximal binding of lipocortin, a related annexin protein, to PS vesicles correlated with binding of two Ca^{2+} ions to the protein [18]. Since these studies were not extended to Ca^{2+} concentrations greater than pCa 3.6, it is possible that the second phase of binding and membrane aggregation occurring at Ca^{2+} concentrations greater than pCa 3.5 in this study reflects occupancy of the two additional Ca^{2+} binding sites detected on these proteins in equilibrium dialysis experiments [18,52].

Although Ca^{2+} is the apparent intracellular regulator of the annexin proteins, we observed that Ba^{2+} and Sr^{2+} could partially replace Ca^{2+} in activating these proteins. This sensitivity to Ba^{2+} clearly distinguishes these proteins from calmodulin which is not activated by this ion [63]. Although the biological function of the annexin proteins is unknown, the immunochemical localization of calelectrin to synaptic vesicles [64] and calpactin to chromaffin granules and chromaffin cell plasma membranes [65], as well as the ability of calpactin to reactivate exocytosis in permeabilized chromaffin cells depleted of cytosolic proteins [66], has suggested a role for these proteins in exocytosis. The fact that the efficacy series, $Ca^{2+} > Sr^{2+} > Ba^{2+}$, for annexin-induced chromaffin granule aggregation is the same as that for promoting exocytosis of synaptic vesicles at the neuromuscular junction [67] is further support for this theory.

It has been suggested that the Ca^{2+} binding site of the annexin proteins forms a ternary complex with phospholipid [34], which accounts for the higher Ca^{2+} binding affinity of these proteins in the presence of phospholipid. We have demonstrated that the

Ca^{2+}requirment for binding p32 varies with the nature of the membrane substrate, consistent with this idea and with reports demonstrating varying Ca^{2+} dependence for binding vesicles of differing phospholipid composition [5,54]. This property may provide a means of enabling these proteins to bind selectively to certain subcellular membranes on the basis of locally available Ca^{2+} concentrations, and explain their predominant subplasmalemmal location based on the higher ambient Ca^{2+} concentration in this region. Based on the analysis of each protein's calculated number of binding sites and competition between these sites, it appears that each annexin binds to a distinct but partially overlapping population of 'receptor sites'. Recognition of distinct membrane features may also contribute to the differential cellular localization and possible function of these proteins. The maximum number of receptor sites on the chromaffin granule membrane for an annexin determined in this study was 16 nmol/mg for p32 at 1 mM Ca^{2+}. Assuming this protein would occupy approx. 16 nm^2 of membrane surface area, it can be estimated [9] that only 6.4% of the membrane surface area is occupied by this protein under saturating conditions.

It remains to be determined precisely what membrane features are recognized by each annexin protein. However, annexin binding appears to be more complex than interaction with the headgroups of several phospholipids, as suggested by the biphasic binding isotherms seen by both p32 and p67 under certain conditions. One of the few types of model which could explain the decline in binding sites at the upper end of the calcium titrations incorporates the assumption that at certain Ca^{2+} levels and protein concentrations small amounts of annexin are able to bind in such a way as to perturb the membrane structure and eliminate other annexin binding sites. It is known that under certain conditions at least some, if not all, annexins behave as integral membrane proteins [12,65]. Perhaps this membrane insertion might disrupt the membrane structure to prevent annexins from binding as extrinsic membrane proteins, and also inhibit phospholipase activity as has been observed in several model systems [22,24].

At the higher Ca^{2+} concentrations used in this study annexin binding displayed decreased affinity, increased capacity, positive cooperativity, and synergistic interactions between different members of the annexin family. It is conceivable that all these changes may be due to the self-association of annexin molecules which can occur at these Ca^{2+} levels (Zaks and Creutz, unpublished data). Such a self-association event might also be responsible for the membrane aggregation at $pCa^{2+} >$ 4.0, while at higher Ca^{2+} levels p32 and synexin are capable of bridging membranes as monomers under some conditions (Zaks and Creutz, unpublished data). It is possible that the decrease in granule aggregating ability of p32 observed at concentrations saturating membrane receptors is due to the inability of the protein to bridge membranes as a monomer under these conditions, instead necessarily relying on weaker annexin–annexin interactions to perform this function.

Each of the three annexins appears to have a different intrinsic ability or efficacy at promoting chromaffin granule aggregation, with synexin > p32 > > p67, which may reflect their tendency toward self-association. For example, the fact that the synexin concentration dependence for chromaffin granule aggregation reaches a stable plateau while that for p32 decreases suggests that synexin–synexin interactions responsible for membrane aggregation are stronger than p32–p32 interactions. It is also possible that these different tendencies toward self-association are preserved in heterologous interactions and explain the nature of the interactions between different family members. For example, p67, by forming weak contacts with synexin or p32, would disrupt synexin or p32 aggregates on the membrane surface necessary for chromaffin granule aggregation, and hence function as an inhibitor of synexin or p32. P32 on the other hand, by forming stronger contacts with synexin, would facilitate synexin and p32 co-polymerization at low synexin concentrations while disrupting structured synexin aggregates at higher synexin concentrations, accounting for the synergistic and inhibitory activity of p32 at low and high synexin concentrations, respectively.

In summary, we have shown that although synexin, p32 and p67 share the ability to aggregate membranes, there are distinct differences between them. They have slightly different Ca^{2+} requirements, they bind different membrane 'receptors' and they have different intrinsic membrane aggregating abilities, behaving as partial agonists in combination. Membrane lipids and free fatty acids may play an important role in regulating their Ca^{2+} affinities, and self-association may play a role in both the membrane binding and aggregating activities of these proteins.

Acknowledgements

We are indebted to Lisa G. Dowling and David C. Sterner for technical assistance, and to the latter for helpful discussions concerning free fatty acids. This study was supported by grants from the National Institutes of Health (DK33151) and the National Science Foundation (PCM8206435). WJZ was supported by the N.I.H. Medical Scientist Training Program and CEC by an Established Investigator Award from the American Heart Association with funds contributed in part by the Virginia Affiliate.

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