Vesicle Aggregation by Annexin I: Role of a Secondary Membrane Binding Site†

Milton de la Fuente* and Adriana V. Parra

Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Casilla 70005, Correo 7, Santiago, Chile

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ABSTRACT: Proteins of the annexin family bind to and aggregate secretion granules or liposomes in the presence of Ca^{2+}. We investigated the mechanism of vesicle aggregation performing experiments in which annexin I bound to PS liposomes was allowed to aggregate additional liposomes. The protein was initially bound to PS liposomes in 50−100 μM Ca^{2+} under nonaggregating conditions; then further liposomes were added, and aggregation was started by increasing Ca^{2+} to 0.5−1 mM. Coaggregation between both liposome populations was followed using resonance energy transfer (RET) and turbidimetric techniques. In RET experiments, annexin I was bound to liposomes containing *N*-(7-nitro-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE), and the second liposome population contained lissamine-Rhodamine-PS. Coaggregation was estimated from NBD fluorescence quenching. Rapid fluorescence and turbidimetric changes were observed, demonstrating coaggregation between both populations of liposomes. Therefore, annexin I molecules may bind two membranes in a bivalent fashion. Rates of coaggregation were similar to the rates of aggregation observed when all vesicles contained protein, indicating that aggregation is mediated only by bivalent annexin I molecules. Thus, membrane aggregation is due to a secondary membrane binding site in annexin I. PS liposomes containing annexin I coaggregated with phosphatidylcholine (PC) liposomes, demonstrating that membrane-bound annexin I binds PC, in contrast with soluble annexin I. Secondary binding to PC was significantly slower than secondary binding to PS, pointing to the importance of negative charge in the secondary membrane. Coaggregation with PS liposomes was stimulated by Ca^{2+} or by Mg^{2+} in the millimolar range concentration, whereas the rate of coaggregation with PC was not stimulated by Ca^{2+} above 50 μM. Annexin I binding to secondary vesicles was reversible upon addition of EGTA. Hence, secondary binding probably requires Ca^{2+} in micromolar concentrations, similarly to primary binding.

The annexins are a family of widely distributed proteins of unknown function that bind phospholipids in the presence of Ca^{2+} (Klee, 1988; Burgoyne & Geisow, 1989). These proteins share a highly conserved C-terminal core domain made of four or eight 70 amino acid repeats, but the N-terminus domain is not conserved. Some annexins promote the Ca^{2+}-dependent aggregation of secretion granules and liposomes. In the case of annexins I and II, granule aggregation is activated at a low, physiological range of Ca^{2+} concentration (Drust & Creutz, 1988; Meers et al., 1992; Blackwood & Ernst, 1990; Ali et al., 1989).

In spite of its potentially crucial biological importance, the mechanism of membrane aggregation by annexins is still not well understood. It was early observed that synexin (annexin VII) and calelectrin (annexin IV) self-aggregate in solution in the presence of the same Ca^{2+} concentrations required for aggregation (Creutz et al., 1979; Südhof et al., 1982). This led to the proposal that membrane aggregation would result from self-association of annexin molecules bound to different membranes (Zaks & Creutz, 1990; Creutz & Sterner, 1983). In turn, this implies that each protein molecule would bind only one membrane at a time. It is relevant, then, that recent crystallographic studies on annexin I and V suggest that all the Ca^{2+} sites in annexins V and I are on one face of the molecule, presumably the membrane-binding domain (Huber et al., 1990; Weng et al., 1993). Additionally, it has been found that some annexins indeed self-aggregate when bound to membranes (Zaks & Creutz, 1991; Mosser et al., 1991; Voges et al., 1994). However, the importance of self-aggregation in the formation of membrane contacts has been questioned by some recent studies. Thus, resonance energy transfer experiments showed no interactions between annexin molecules bound to opposite aggregated vesicles (Zaks & Creutz, 1991). In a recent study of the covalent linkage of phospholipid derivatives with annexin I in vesicular aggregates, it was shown that the reversible annexin-promoted aggregation of liposomes containing a photoactive derivative of phosphatidylethanolamine became irreversible upon illumination (Meers et al., 1992). The aggregates could be dissociated by trypsin. This result is consistent with the opposite hypothesis, namely, that annexin molecules support membrane aggregation by directly binding two membranes in a bivalent fashion.

The purpose of this work was to investigate the mechanism of membrane aggregation by annexin I. In particular, we wanted to learn if aggregation is mediated by protein molecules making simultaneous contact with two membrane surfaces, or if aggregation needs protein bound to both membranes, so that each protein molecule contacts only one membrane at a time. We studied interactions of membrane-bound annexin I with liposomes, using resonance energy transfer and turbidimetric techniques. The results demonstrate that annexin I molecules mediate membrane aggregation in a bivalent manner through a secondary binding site

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* To whom correspondence should be addressed. Fax: 56 2 7776916. E-mail: mdelafue@med.uchile.cl.

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that would appear after the primary Ca^{2+}-dependent binding to membranes.

EXPERIMENTAL PROCEDURES

Materials. Brain L-α-phosphatidylserine brain (PS)[1] and 1-palmitoyl-2-oleoylphosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Birmingham, Al). *N*-(Lissamine Rhodamine B sulfonyl)-1,2-dihexadecanoylphosphatidylethanolamine (Rh-PE) and *N*-(7-nitro-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoylphosphatidylethanolamine (NBD-PE) were purchased from Molecular Probes (Eugene, OR). The rest of the reagents were analytical grade.

Preparation of Liposomes. Large unilamellar vesicles were prepared by extrusion (Mayer et al., 1986). A solution of phosphatidylserine (PS) or phosphatidylcholine (PC) in chloroform was dried under a nitrogen stream and thoroughly desiccated under vacuum. The dried lipids were hydrated in 0.1 M NaCl and 20 mM Hepes, pH 7.4, for 30 min at room temperature, suspended by vortexing for 1 min, and extruded 20 times through a 0.1 μm polycarbonate filter (Costar Nucleopore, Pleasanton, CA). The phosphate content was determined in all preparations (Meek, 1986). PS or PC liposomes containing either Rh-PE or NBD-phosphatidylethanolamine were prepared in the same way, adding the fluorescent probe to the initial solution of PS at a concentration of 2 mol %. The resulting vesicles are called Rh-PS or NBD-PS or Rh-PC. Very large thin-walled liposomes loaded with 0.2 M sucrose were made according to Reeves and Dowben (1969).

Determination of Coaggregation between Different Populations of Liposomes. Annexins bind very strongly to membranes [$K_D \approx 10^{-10}$ M (Tait et al., 1989; Andree et al., 1990)] in the presence of low Ca^{2+} concentrations [micromolar range in the case of annexin I (Blackwood & Ernst, 1990; Schlaepfer & Haigler, 1987; Ando et al., 1989a)]. As a consequence, bound annexins do not exchange when new vesicles are added (Zaks & Creutz, 1991; Meers et al., 1992). The following control experiment (not shown) confirmed that annexin I indeed bound to PS liposomes quantitatively in 50 μM Ca^{2+}, the lowest concentration we used here, and that it did not exchange when new liposomes were added. We measured by SDS−PAGE the protein bound to very large liposomes (100 μM) loaded with sucrose, prepared according to Reeves and Dowben (1969). These liposomes could be quantitatively pelleted in a centrifugation run at 10000*g* for 10 min, whereas extruded liposomes did not pellet under these conditions. The experiments were carried out at 25 °C in sucrose buffer (see below). Annexin I (4 μg) bound quantitatively to the very large liposomes in 50 μM Ca^{2+} in the absence of primary liposomes. This agrees with published data indicating that annexin I binds essentially completely at this Ca^{2+} concentration (Blackwood & Ernst, 1990; Schlaepfer & Haigler, 1987; Ando et al., 1989a). However, when the protein was first bound to extruded liposomes in 50 μM Ca^{2+} and then very large liposomes were added and the system incubated for 1 min before centrifugation, no protein was detected in the latter liposomes. This demonstrates that under the conditions used the extruded liposomes bound all the protein, and that the membrane-bound protein did not exchange with the very large liposomes. If, however, aggregation was allowed by increasing Ca^{2+} to 1 mM (see below), all the protein was found in the pellet.

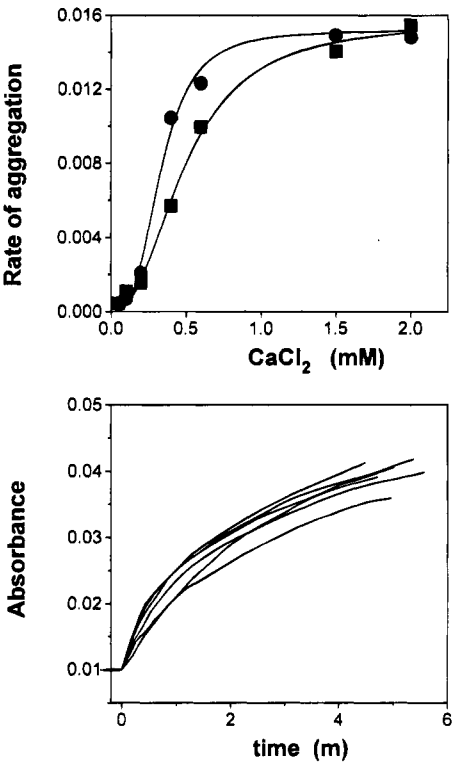

FIGURE 1: Determination of conditions to carry out coaggregation experiments. (Upper panel) Annexin I-mediated aggregation of PS liposomes requires high Ca^{2+} concentrations. Cuvettes contained 1 mL of either 0.1 M NaCl and 20 mM Hepes, pH 7.4 (closed circles), or 0.175 M sucrose and 20 mM Hepes, pH 7.4 (closed squares), plus 10 μM PS, 5 μg annexin I, and the indicated concentrations of Ca^{2+}. Aggregation was determined from absorbance. (Lower panel) Annexin I may be bound to PS liposomes without an effect on final aggregation rates. Three experiments were run in NaCl buffer and three in sucrose buffer, containing 10 μM PS and 4 μg of annexin I in 1 mL. In one experiment of each series, 1 mM $CaCl_2$ was added to bind the protein and start aggregation immediately. In the other two experiments, Ca^{2+} was added to 50 or 100 μM (nominal) to bind the protein to the liposomes. Thirty seconds later, 0.95 mM Ca^{2+} or 0.9 mM Ca^{2+} were respectively added, to start aggregation. Absorbance curves are shown superimposed so that zero time corresponds to the moment in which Ca^{2+} increased to 1 mM.

The experiments throughout this work followed coaggregation between vesicles with bound annexin I ("primary liposomes") and vesicles without the protein ("secondary liposomes"). The formation of coaggregates between both populations of liposomes was detected by resonance energy transfer (RET) and turbidity measurements. Experiments were carried out at 25 °C in either 0.1 M NaCl and 20 mM Hepes, pH 7.4 (NaCl buffer), or 0.175 M sucrose and 20 mM Hepes, pH 7.4 (sucrose buffer), as indicated in each case. Initial annexin binding was carried out using 50−100 μM Ca^{2+}, because under these conditions there is full binding (Blackwood & Ernst, 1990; Schlaepfer & Haigler, 1987) but little or no aggregation. As shown in Figure 1A, aggregation of PS liposomes by annexin I required rather high Ca^{2+}

[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; NBD-PE, *N*-(7-nitro-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoylphosphatidylethanolamine; NDB-PS, PS liposomes containing 2% NBD-PE; Rh-PS, PS liposomes containing 2% Rh-PE; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoylphosphatidylethanolamine (Rh-PE); RET, resonance energy transfer; EGTA, ethylene glycol bis-(β-aminoethyl ether).

concentrations under the conditions used ($K_{0.5}$ = 0.33 mM in NaCl buffer and $K_{0.5}$ = 0.49 mM in sucrose buffer). Rates of aggregation could be reduced further in sucrose buffer at subsaturating Ca^{2+} concentrations, so the binding step was often carried out in this buffer. Addition of secondary liposomes about 30 s after starting binding resulted in the coexistence of two populations of monomeric liposomes, one of them containing annexin I. Finally, aggregation was initiated adding extra Ca^{2+} (usually 1 mM). Figure 1B shows an experiment validating this two-step protocol. The traces in panel B are the normalized changes in absorbances after starting aggregation by increasing Ca^{2+}. The figure compares experiments in NaCl and sucrose buffers. Aggregation was performed in a single step (binding and aggregation occurred simultaneously in 1 mM Ca^{2+}) or in two stages (binding first, aggregation later, as described above). The curves superimpose, demonstrating that the initial binding step does not disturb the measurement of aggregation rates. If significant aggregation between annexin I-containing vesicles had taken place during the preliminary step, then the final aggregation rate should have been notoriously slower.

Assay of Aggregation by Resonance Energy Transfer. The method is based on the fact that proximity of NBD-PE and Rh-PE molecules cause quenching of the fluorescent emission of the NBD donor due to resonance energy transfer (RET) to Rh acceptor (Struck et al., 1981). The probe mixing version of this assay was used (Düzgünës et al.1987): each probe is placed in a separate population of liposomes.

The experiments were carried out in 2 mL of the specified buffer. PS liposomes containing NBD-PE were added at the indicated final concentration, usually 5 μM. NBD emission was followed at 520 nm (excitation at 450 nm) in a Shimadzu fluorimeter. Annexin I was added next, followed by 50 or 100 μM $CaCl_2$ (nominal final concentration). Then the Rh-PS liposomes were added, usually 5 μM, and the Ca^{2+} was raised to the value indicated in each experiment to start the aggregation. All the additions were in small volumes from concentrated stocks. Experiments were carried out in duplicate.

Assay of Aggregation by Absorbance Measurements. The turbidity of the liposome suspension was measured following the absorbance at 350 nm in a thermostated Gilford 2400 spectrophotometer. Changes in absorbance were continuously followed with a recorder (Perkin Elmer 56) or sampled using a Digidata acquisition data system. The experiments were carried out at 25 °C in 1 mL cuvettes containing 1 mL of the buffer indicated in each case. PS liposomes and annexin I were added at the concentrations indicated in each experiment, followed by 50 or 100 μM $CaCl_2$. Additional PS was added after about 30 s, followed by $CaCl_2$ to reach the final concentration indicated (usually 1 mM). Experiments were carried out in duplicate. Rates of aggregation were computed from the initial slopes of the absorbance curves and are reported as change of absorbance at 350 nm per minute.

Preparation of Annexin I from Human Placenta. Annexin I was prepared from human placenta by a modification of the procedure described by Haigler et al. (1987). The placentas were obtained just after delivery at the nearby University Hospital and transferred in ice to the lab. The tissue was freed from amnios and chorion, washed in cold 0.9% NaCl, and homogenized in a Waring Blendor in buffer A (20 mM Hepes, pH 7.4, 0.15 M KCl, 2 mM $MgCl_2$, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 4 mM iodoacetic acid, 1 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride). The homogenate was spun at 700*g* and the supernatant filtered through cheese cloth. The pellet was re-extracted and centrifuged as before, and the supernatants were combined. $CaCl_2$ was added to a final concentration of 1 mM, and after 15 min standing the suspension was centrifuged at 100000*g* for 20 min. The pellet resuspended in buffer A was washed three more times in the same fashion. The final pellet was resuspended in 50 mL of buffer A (without iodoacetamide and benzamidine) containing 3 mM EGTA. After a final centrifugation, the supernatant was transferred to a dialysis bag and subjected to an overnight dialysis against 10 mM Hepes, pH 7.4. The dialysate was clarified by centrifugation (100000*g* for 20 min), and the supernatant was applied to a DEAE-Sephacel column (15 mL) equilibrated in the same buffer. Annexin I was not retained. Unretained fractions were pooled and dialyzed against 10 mM Mes, pH 6.0, and applied to a column of CM-52 (2 mL) equilibrated in the same buffer. The column was first washed with buffer and then subjected to a gradient of 0−0.1 M NaCl in 10 mM Mes, pH 6.0. Annexin I eluted at a NaCl concentration of about 30−40 mM. Its purity was confirmed using one-dimensional SDS−PAGE.

RESULTS

In the main experiment presented in Figure 2 (trace B), annexin I was prebound (in the presence of 100 μM Ca^{2+}) to fluorescent NBD-labeled liposomes. Rhodamine-labeled PS liposomes without annexin I were subsequently added, followed by 1 mM Ca^{2+} (zero time in the figure). If aggregation required annexin in both adjoining membranes, then only NBD-labeled liposomes would aggregate, and the fluorescence would not be expected to change. Contrarily, if aggregation required annexin molecules in only one of the membranes, both populations would coaggregate and the fluorescence would be quenched as a consequence of the ensuing membrane mixing (Blackwood & Ernst, 1990). As shown in Figure 2B, a fast quenching of the NBD fluorescence was observed. Therefore, liposomes containing annexin I coaggregated with the rhodamine-labeled liposomes containing no protein. There was no quenching in the absence of annexin I (trace C) or in the absence of Rh-PS (trace D). Hence, annexin I molecules bound to one population of PS liposomes may bind secondarily another PS membrane. The rate of coaggregation, as estimated from the rate of quenching, was similar to the rate of aggregation observed when all liposomes contained annexin I (Figure 1A). This suggests that binding of membrane-bound annexin I to a second membrane is not a ancillary reaction but the mechanism leading to aggregation even when both intervening membranes contain annexin I. This conclusion was confirmed by the results displayed in the lower panel in Figure 2, showing that the rate of fluorescence quenching had the same dependency on annexin concentration independently of the protein distribution. The biphasic dependence on protein concentration is due to the fact that RET is caused by membrane mixing (Francis et al., 1992).

The capacity of membrane-bound annexin I to bind a second membrane was also assayed following the total rate of aggregation with absorbance measurements. Annexin I

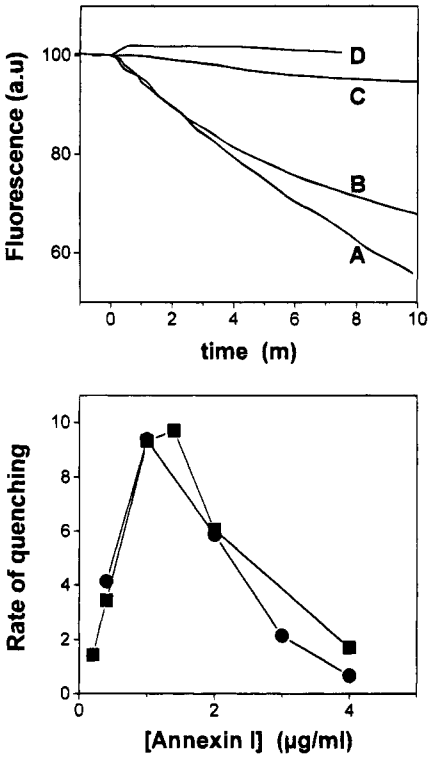

FIGURE 2: Coaggregation of liposomes containing annexin I with liposomes without protein as determined by resonance energy transfer. (Upper panel) The traces show only the aggregation stage of the experiments, which started at $t = 0$ with the addition of 1 mM Ca^{2+}. In the experiment labeled B, annexin I (1 μg) was bound to NBD-PS liposomes (5 μM) in the presence of 0.1 mM Ca^{2+} in NaCl buffer. Thirty seconds later, Rh-PS liposomes (5 μM) were added, followed by 1 mM Ca^{2+} (zero time). In experiment A, Rh-PS was added together with NBD-PS at the binding step, so annexin I bound to both NBD-PS and Rh-PS liposomes, and aggregation was started immediately with 1 mM Ca^{2+}. Experiments C and D were performed similarly to B but lacked protein and Rh-PS, respectively. (Lower panel) Effect of annexin I concentration on the rate of quenching does not depend on protein distribution. The closed circles show the rate of fluorescence quenching (initial change in fluorescence units/min) in the presence of the indicated amounts of annexin I under the conditions of experiment B in upper panel. Closed squares show similar experiments performed under the conditions of experiment A in upper panel.

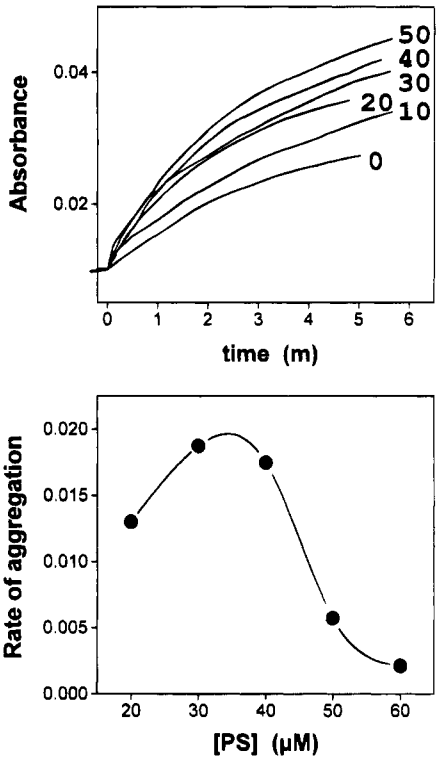

FIGURE 3: Coaggregation of liposomes containing annexin I with liposomes without protein as determined by absorbance measurements. (Upper panel) 1 μg of annexin I was prebound to 10 μM PS in 1 mL of NaCl buffer in the presence of 0.1 mM $CaCl_2$. After about 30 s, the indicated amounts of PS (final μM) were added followed by addition of 0.4 mM $CaCl_2$ at the time marked as zero in the plot. The absorbance curves were arbitrarily superimposed to the same initial absorbance in such a way that zero time corresponds to the final Ca^{2+} addition. (Lower panel) Effect of PS concentration on the rate of aggregation at constant annexin I concentration. The experiments were carried out as above, but without the addition of extra PS and in the presence of 0.5 mM $CaCl_2$.

was first bound to PS liposomes. Then, variable amounts of PS liposomes without annexin I were added, followed by 1 mM Ca^{2+} at zero time. As shown in the normalized traces of the upper panel of Figure 3, addition of extra PS liposomes increased the rate of aggregation in a saturable manner. Because PS liposomes do not aggregate measurably at 1 mM Ca^{2+} in the absence of annexin I (see, for instance, Figure 2C), the increase in rate must be caused by coaggregation with the liposomes containing annexin I.

The lower panel of Figure 3 shows the contrasting result of an experiment in which all liposomes contained annexin I. The protein concentration was constant (as in the experiment of the upper panel), and therefore the protein surface density dropped as the PS concentration increased. The rate of aggregation first raised with increasing lipid concentration, but then declined. This was probably caused by the reduction in average protein surface density, which must be higher than a critical value for aggregation to occur (Meers et al. 1992). Thus, the fact that the rate of aggregation in the experiments of the upper panel did not fall when adding further liposomes is an indication that the protein surface density did not drop after adding extra PS liposomes. Hence, annexin I bound to primary liposomes did not exchange with the liposomes without protein.

The protein density required for start aggregation would depend on the distribution of the protein. Thus, when all vesicles contain annexin I, aggregation would be supported by protein molecules from both membranes. But when only a fraction of the liposomes contain annexin I, the critical surface density should be higher in order to provide the same number of connecting molecules. Hence, there must be a range of protein density which will be enough to support aggregation between vesicles containing annexin I but not coaggregation between vesicles with and without annexin I. We tested this prediction by measuring the amount of annexin I required for start aggregation and coaggregation. As shown in Figure 4, slow but significant rates of aggregation were measured with annexin I between 0.2 and 0.6 μg/mL. No coaggregation was detected within this range (i.e., addition of extra PS liposomes did not cause an increase in rate). However, at a protein concentration over 0.7 μg/mL, coaggregation was easily demonstrated. Thus, aggregation of PS liposomes required about 140 protein molecules/vesicle when vesicles without protein associated to vesicles with annexin I, under the conditions used. This amounts to a minimum of about 70 molecules/vesicle when all liposomes contained annexin I.

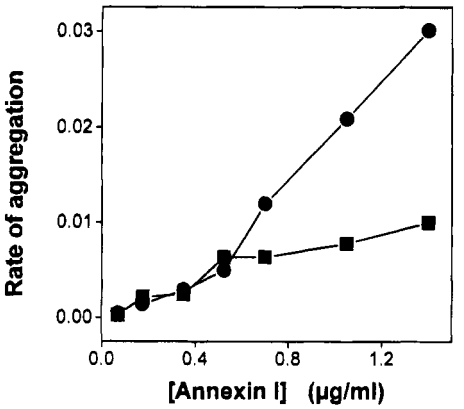

FIGURE 4: Protein concentration required to start coaggregation. The indicated amounts of annexin I were added 0.5 mL of sucrose buffer containing 20 μM PS and 100 μM Ca^{2+}. After about 30 s, 0.5 mL of NaCl buffer plus (circles) or minus (squares) 100 μM PS was added, followed by 0.9 mM $CaCl_2$ (final). Rates were computed from the initial slopes after starting aggregation. Binding was carried out in sucrose buffer in order to minimize any aggregation, and NaCl was included in the aggregation step to maximize the rate of aggregation.

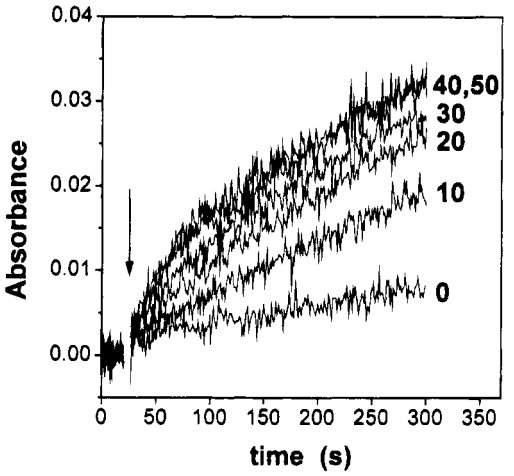

FIGURE 5: Coaggregation of PC liposomes and PS liposomes with bound annexin I. The experiments were carried out in 1 mL of sucrose buffer containing 10 μM PS, 1 μg of annexin I, 0.5 mM $CaCl_2$, and the indicated concentrations of PC (μM). Since soluble annexin does not bind to PC, prebinding of annexin I to PS liposomes was not necessary. Absorbance was sampled every second with a DigiData data acquisition system.

Since previous experiments showed that aggregation is mediated by bivalent annexin molecules, the question arises as to the properties of the secondary binding site, in particular regarding phospholipid specificity and Ca^{2+} dependence. It is well documented that soluble annexin I binds in a Ca^{2+}-dependent fashion to acidic phospholipids but does not bind to phosphatidylcholine at Ca^{2+} concentrations under 1 mM (Haigler et al., 1987; Blackwood & Ernst, 1990). We decided to test if secondary binding shares this property of primary binding. Figure 5 shows turbidity experiments carried out in the presence of variable amounts of PC liposomes. As previously observed for PS, the addition of PC increased the aggregation rate, saturating at a PC/PS ratio of about 4. No aggregation was observed when PS or annexin I were omitted (not shown). Thus, membrane-bound annexin I in contrast to soluble annexin I, has the capacity to bind and aggregate PC membranes. Secondary binding sites would have a less well defined specificity for phospholipids than primary binding. Rate of coaggregation with

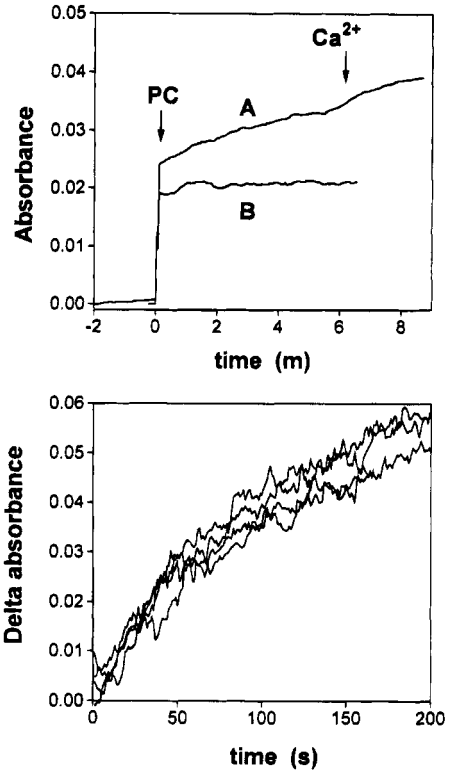

FIGURE 6: Lack of effect of Ca^{2+} ions on the coaggregation of PS liposomes binding annexin I with PC liposomes. (Upper panel) In experiment A, annexin I (1.5 μg) was bound to PS liposomes (10 μM) in the presence of 50 μM Ca^{2+}, and then PC liposomes (50 μM) and Ca^{2+} (0.4 mM) were added as shown by the arrows. Experiment B was performed similarly, except that PS was omitted. Thus, annexin I is not membrane-bound. (Lower panel) Annexin I was added at zero time to 1 mL of sucrose buffer containing 15 μM PS, 50, 100, 200, or 300 μM $CaCl_2$, and plus or minus 100 μM PC. Absorbance was sampled every second with a Digidata acquisition system. Data obtained in the absence of PC were discounted point to point from the data obtained in the presence of PC, at each Ca^{2+} concentration. The plot shows the four difference curves thus obtained. The small increases in rate with increasing Ca^{2+} observed in the original coaggregation experiments (not shown) are due to the simultaneous aggregation between PS liposomes, which is sensitive to divalent cation concentration

PC liposomes was about one-fifth the rate of coaggregation with PS liposomes, when measured under identical conditions (not shown). In fact, optimal coaggregation with PC was obtained in low ionic strength sucrose buffer, because under this condition the competing PS−PS aggregation is inhibited (not shown).

Coaggregation with PC liposomes can be demonstrated in 50 μM Ca^{2+} (Figure 6, upper panel). The reaction rate was not increased by the addition of further Ca^{2+}, in sharp contrast with PS-PS coaggregation, which does require high Ca^{2+} concentrations (see Figure 1). The upper panel also shows that soluble annexin I does not aggregate PC liposomes (neither PS alone, not shown). The effect of Ca^{2+} on PS−PC coaggregation was tested as shown in the lower panel of Figure 6. Here, the rates of coaggregation between PS·annexin and PC liposomes are expressed as the point-to-point differences between experiments with PC and experiments without PC, at four Ca^{2+} concentrations ranging between 50 μM Ca^{2+} (nominal concentration) and 300 μM Ca^{2+}. The four difference curves depicted in the panel overlap, showing that these concentrations of Ca^{2+} have no effect on the rate of PS−PC coaggregation. Therefore,

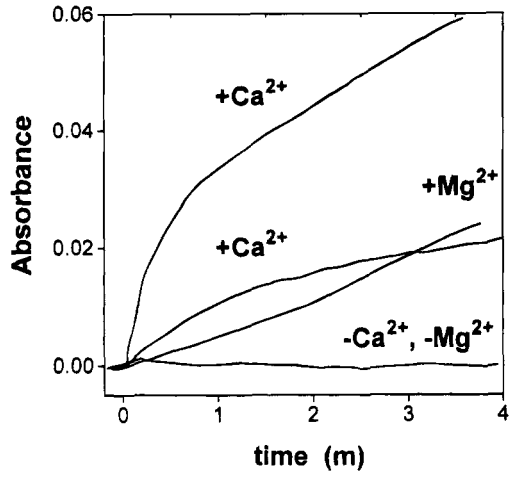

FIGURE 7: Calcium or magnesium ions may activate coaggregation with PS liposomes. Annexin I (1.7 μg) was added to NaCl buffer containing 5 μM PS and 100 μM $CaCl_2$. Aggregation was started after 30 s, by adding either 30 μM PS followed by 1 mM Ca^{2+} (upper curve), or 1 mM Ca^{2+} (middle curve), or 30 μM PS followed by 3 mM Mg^{2+}, or without adding bivalent cations. The plot shows normalized data.

coaggregation with PC does not require Ca^{2+} concentration beyond that necessary for keeping annexin I bound to the primary liposomes. This result raises the point as to whether the secondary binding site indeed requires high Ca^{2+} for binding PS, as suggested by the aggregation experiments of Figure 1. Figure 7 shows that coaggregation with PS can be supported by Mg^{2+}, in the presence of the low Ca^{2+} required to keep primary binding. Mg^{2+} cannot replace Ca^{2+} in annexin primary binding and in fact did not support by itself annexin I-promoted aggregation (not shown). Thus, secondary binding for PS does not absolutely require high Ca^{2+} concentrations. It seems probable that the high concentrations of Ca^{2+}—or Mg^{2+}—required reflect binding to PS, causing a reduction in electrostatic repulsion which facilitates close approach of the vesicles. Coaggregation with electrically neutral PC should not require additional Ca^{2+}, as it was observed.

We studied the effect of EGTA on secondary binding using very large PS liposomes prepared using the procedure of Reeves and Dowben (1969). Annexin I was bound to extruded unilamellar liposomes, which were subsequently coaggregated with the larger thin-walled liposomes increasing the Ca^{2+} concentration. The very large liposomes were collected and washed by centrifugation in an Eppendorf centrifuge (extruded liposomes are not pelleted) and analyzed by SDS−PAGE. In the presence of extruded liposomes, annexin I appeared in the thin-walled liposomes only after addition of 1 mM Ca^{2+}, as expected. Protein was not detected in the 1 mM Ca^{2+} aggregates when EGTA was added before centrifugation (not shown). Thus, secondary binding of annexin I is reversible upon Ca^{2+} chelation. This result suggests that secondary binding requires Ca^{2+} at micromolar concentrations, similarly to primary binding. The alternative possibility, that secondary binding requires no Ca^{2+} at all, has not been disproved, though.

DISCUSSION

The association between liposomes binding annexin I and liposomes without protein was followed using RET and turbidimetric techniques. In the "probe mixing" configuration used, RET experiments may in principle report aggregation and membrane mixing (Düzgünes et al., 1987). Annexin I has been demonstrated to promote membrane mixing between PS liposomes (Blackwood & Ernst, 1990). This process is apparently Ca^{2+}-dependent (Meers et al., 1992; Francis et al., 1992). We did not observe RET under 0.5 mM Ca^{2+} (not shown), and RET was inhibited by an excess of annexin I independently of the distribution of the protein (lower panel in Figure 2). However, aggregation did occur under 0.5 mM Ca^{2+} (Figure 1) and was not reduced by the same excess of protein (not shown). Thus, RET experiments reported Ca^{2+}-dependent membrane mixing in liposomes aggregated by annexin I. Very slow rates of RET were observed between PS liposomes containing annexin I and PC liposomes, suggesting that there was no significant membrane mixing in PC−PS aggregates. As shown in Figure 2 (upper panel, traces A and B, and lower panel), membrane mixing occurred at the same rate irrespective of the distribution of annexin I, indicating that aggregation of PS liposomes proceeds only through bivalent interactions.

Turbidimetric experiments showed that liposomes with bound annexin I coaggregate with liposomes without the protein (Figures 3 and 4), in agreement with the RET experiments and confirming recent results (Meers et al., 1992). Thus, two independent physical measurements suggested that annexin I molecules can bind two membranes. We have reached the same conclusion from preliminary experiments with annexin II tetramer (M. de la Fuente and A. Parra, unpublished results). Annexins IV, VI, and VII also may bind two membranes (Zaks & Creutz, 1991). Tsao (1990) reported fusion between vesicles containing an undefined lung annexin and vesicles without the protein. Thus, the capacity of monomeric binding to two membranes may be a general property among the aggregating annexins.

Characterization of the aggregation process led to the unexpected result that membrane-bound annexin I, as opposed to the protein free in solution, bound PC liposomes (Figure 5). This results extends a similar recent finding by Andree et al. (1993), who showed that an annexin I−annexin V chimera bound to a planar bilayer could bind PC liposomes. Thus, membrane-bound annexin I displays a secondary membrane binding site that has a less stringent specificity than the primary binding site. Secondary binding occurred much faster on PS liposomes, suggesting that negative charge might greatly favor this interaction, as has been recently demonstrated for primary binding in annexin V (Meers & Mealy, 1993). This, and the reversibility of secondary binding by EGTA (not shown), suggest that secondary interactions are not unspecific in nature (such as hydrophobic interactions). Our data do not allow us to conclude if the secondary binding site does not require Ca^{2+} ions, or if it has a requirement in the micromolar range. Reversion of secondary binding by EGTA would appear to favor the second alternative. However, it is possible that EGTA-induced annexin desorption from the primary membrane might revert the conformational change which presumably formed the secondary site, thus reversing a Ca^{2+}-independent secondary binding.

At this point, model D as proposed from Meers et al (1992) seems better as a rough explanation for bivalent interactions. The model assumes that primary binding would use only half of Ca^{2+}-binding sites, whereas the other half would be left exposed and would be responsible for secondary interac-

tions. Secondary binding would then require Ca^{2+} in the micromolar range. The structure of the membrane-bound annexin I would be significantly different from the crystalline structure, thus defining two independents Ca^{2+} domains which would form membrane binding sites. Another possibility (also listed by Meers et al. as model C), is that primary binding takes all the Ca^{2+} sites as suggested by the crystallographic models, thus leaving the concave face containing the N-terminal domain pointing away from the membrane. Groups from this face would then form a Ca^{2+}-independent secondary site. This model more easily accommodates a significant amount of information relating the N-terminal domain to aggregation (but not to primary binding). Thus, experiments using an annexin I−annexin V chimera have shown that the N-terminal domain of annexin I confers aggregation activity to the core of annexin V (Andree et al., 1993). Proteolytic cleavages of the N-terminus of annexin I lead to significant changes in aggregating activity (but not in Ca^{2+}-dependent membrane binding) (Wang & Creutz, 1994). Phosphorylation at the N-terminal domain by protein kinase C profoundly inhibits the aggregation activity without significantly affecting the Ca^{2+}-dependent binding to membranes (Wang & Creutz, 1992; Johnstone et al., 1993). Mutagenesis of Ser 27 or Tyr 21 has similar effects (Wang & Creutz, 1994). Finally, a monoclonal antibody to the N-terminus of annexin I inhibits aggregating activity but not Ca^{2+}-dependent binding (Meers et al. 1992).

Our data do not support the self-association hypothesis for aggregation, which requires protein in both aggregating membranes (Zaks & Creutz, 1990). Annexin V does polymerize on the plane of membranes in the presence of Ca^{2+} (Zaks & Creutz, 1991; Mosser et al., 1991; Voges et al., 1994). Except for dimerization by a transglutaminase in A431 cells (Ando et al., 1989b), there have been no reports of polymerization of annexin I. However, even if they occurred, our data indicate that such polymers would not be a direct requisite for aggregation.

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