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Annexin I-Mediated Vesicular Aggregation: Mechanism and Role in Human Neutrophils†

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ABSTRACT: Whole cytosol isolated from human neutrophils was found to accelerate the Ca^{2+}-dependent fusion of phospholipid vesicles with neutrophil plasma membranes as measured by several fluorescence resonance energy transfer lipid dilution assays or by the fate of an encapsulated aqueous soluble fluorophore. The Ca^{2+} (threshold of 2–10 μM) and protein concentration dependencies for fusion mediated by purified human neutrophil annexin I (lipocortin I), recombinant annexin I and des(1–9)annexin I showed behavior similar to that of whole cytosol. A monoclonal antibody against the N-terminal region of annexin I strongly inhibited the action of isolated annexins as well as whole cytosol, indicating that annexin I is the major activity of this type in whole neutrophil cytosol and that it functions even in this complex mixture of proteins. Residual Ca^{2+}-dependent fusion activity in the absence of cytosol or annexin I was not inhibited by several antibodies against annexin I, implicating an as yet unknown protein. Kinetic analysis of liposomal fusion showed that annexin I, as in the case of synexin, accelerates aggregation of vesicles but not the actual fusion event per se. The disposition of annexin I in liposomal aggregates was studied by monitoring binding of the protein with a pyrene-phospholipid and by simultaneously monitoring vesicular aggregation by turbidity. An antibody to the N-terminus of annexin I inhibited vesicular aggregation but not binding, suggesting that initial binding of annexin I is similar to that of annexin V. A relatively small proportion of the bound annexin was involved in intervesicular linkage, and no exchange of bound annexin to subsequently added vesicles was observed. The lack of extensive contact between lipids of aggregated vesicles was supported by a lack of energy transfer between phospholipid probes on separate aggregating vesicles. Covalent linkage of maleimidyl or photoaffinity phospholipid derivatives with annexin I in vesicular aggregates did not allow complete disaggregation of vesicles by EDTA, suggesting that monomers of annexin I can contact two membranes simultaneously at the point of intervesicular linkage. These data are discussed in terms of possible models for the structure of this site.

The annexins (Geisow et al., 1987) are a class of proteins that bind to phospholipid membranes in a Ca^{2+}-dependent manner and may be involved in intracellular fusion processes such as exocytosis, a hypothesis supported by the ability of many of the annexins to aggregate and mediate fusion of various vesicles (Creutz et al., 1978; Hong et al., 1981, 1982a,b; Meers et al., 1987, 1988a,b; Drust & Creutz, 1988; Ali et al., 1989). Annexin I has recently been shown to mediate Ca^{2+}-dependent fusion of phosphatidylserine liposomes alone (Blackwood & Ernst, 1990) and fusion of phosphatidylserine/phosphatidylethanolamine liposomes with neutrophil plasma membranes (Oshry et al., 1991). Since there are other annexins in whole neutrophil cytosol (Meers et al., 1987; Ernst et al., 1990) as well as potential inhibitors, it is important to establish the function of annexin I in this complex system. Of the annexins, only synexin has been characterized kinetically (Meers et al., 1988a) to determine that it accelerates Ca^{2+}-

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dependent aggregation of vesicles alone but not fusion per se. This distinction is of utmost physiological importance as it may indicate whether a particular annexin relies on lipids or other proteins to complete the overall fusion process.

The strong homologies between members of the annexin family are manifest in similar Ca^{2+} and phospholipid binding characteristics. Annexin V, highly homologous to annexin I, appears to bind by Ca^{2+} bridges between the annexin and the membrane in which conserved annexin loops make contact with the phospholipids (Huber et al., 1990; Meers, 1990; Meers et al., 1991b). The N-terminus of annexin V is probably located on the opposite side of the annexin molecule from the Ca^{2+} binding loops. The N-terminus of bound annexin I is accessible to proteases (Glenney, 1986; Huang et al., 1987), and a tryptophan near the N-terminus exhibits little interaction with phospholipids (Meers, 1990), indicating that it too may orient away from the bilayer surface.

Despite similarities in membrane binding, the annexins differ greatly in terms of their respective abilities to aggregate phospholipid vesicles and to self-associate. For instance, annexin V differs from annexin I in that it is inhibitory to vesicular aggregation and fusion (Blackwood & Ernst, 1990; Oshry et al., 1991). Recent data suggest that the first repeated 60–70-amino acid segment of annexin I imparts vesicle-aggregating activity when it is incorporated into annexin V (Ernst et al., 1991). The structure of annexin-mediated vesicular aggregates has not been well studied. It is not known, for instance, whether a single annexin I molecule can mediate vesicular aggregation by simultaneous interaction with two bilayers or if annexin–annexin interactions are necessary.

We report the results of experiments that were designed to address these problems. The data help elucidate the role of annexin I in the complex mixture of neutrophil cytosol proteins and characterize the mechanism of the vesicle-aggregating and fusion-promoting activities of annexin I with a focus on the role of individual annexin molecules in the interaction of two membrane bilayers. Portions of this work have been presented previously in abstract form (Meers et al., 1991c).

Materials and Methods

3-Palmitoyl-2-(1-pyrenyldecanoyl)-L-α-phosphatidylcholine (pyrene-PC),[1] neutral rhodamine B dextran (10 000 average molecular weight) (Rh-dextran), octadecylrhodamine B, *N*-(4-(4-maleimidylmethyl)cyclohexyl-1-carbonyl)dipalmitoyl-L-α-phosphatidylethanolamine (MMCC-PE), and succinimidyl *p*-azidobenzoate were obtained from Molecular Probes (Eugene, OR). Bovine brain phosphatidylserine (PS), phosphatidate (derived from egg phosphatidylcholine) (PA), egg phosphatidylcholine (PC), phosphatidylethanolamine (transesterified from egg phosphatidylcholine) (PE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE), and lissamine rhodamine B-PE (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) and 1,3-bis[tris(hydroxymethyl)methylamino]propane (BIS-TRIS propane) were obtained from Sigma (St. Louis, MO). EGTA (puriss grade) and $Na_2S_2O_4$ (sodium dithionite) were from Fluka (Ronkonkoma, NY). $CaCl_2$ (>99%), $MgCl_2$ (>99%), and NaCl (>99%), and *N*-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES) were from Fisher (Pittsburgh, PA). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

A mouse monoclonal antibody to the N-terminus of annexin I was obtained from Zymed (La Jolla, CA). The properties of this antibody have been previously characterized (Glenney & Zokas, 1988). A mouse monoclonal antibody to human HLA class I antigen was obtained from Biodesign, Inc. (Kennebunkport, ME). Ascites fluid with antibodies against the first repeat segment of annexin I were generously supplied by Dr. Joel Ernst (University of California, San Francisco) and purified by protein A affinity chromatography.

Vesicle Preparation. Large unilamellar vesicles of PS/PE (1/3) labeled with NBD-PE or Rh-PE (0.75 mol % each) were prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) as modified by Wilschut et al. (1980) in 100 mM KCl, 50 mM HEPES, 1 mM EGTA, pH 7.0 (buffer A). Liposomes were extruded through 0.2 μm and then 0.1 μm polycarbonate filters during this procedure (Szoka et al., 1980). This method generates mostly unilamellar vesicles (Düzgünes et al., 1983).

For some experiments, these vesicles were treated with 2 mM sodium dithionite for 30 min at 0 °C. Reduction of approximately 50% of the NBD groups was observed under these conditions by disappearance of NBD fluorescence, presumably due to outer monolayer reduction only [see also McIntyre and Sleight (1991)]. Dithionite was immediately removed by chromatography on Sephadex G-75. Thin-layer chromatography (silica gel, with a solvent of chloroform/methanol/water 65/25/4) analysis showed no significant changes in the pattern of spots from the same vesicles without fluorescent phospholipids, indicating that the unlabeled lipids remained intact. The rate of Ca^{2+}-dependent fusion of dithionite-treated labeled vesicles was nearly identical to the rate of fusion of untreated labeled vesicles with unlabeled PS/PE vesicles. After incubation of 7.7 μM total phospholipid of dithionite-treated PS/PE vesicles containing 0.083 mol % NBD-PE with 200 μg/mL cytosol and 1 mM free Ca^{2+} at 25 °C for 10 min followed by addition of 2 mM EDTA, latency of further reduction by 2 mM dithionite at 0 °C remained 96 ± 0.7% of controls. If incubation at 25 °C included 50 μg/mL plasma membrane protein, latency was 82 ± 6%. These experiments indicated that little probe "flip-flop" from inner to outer monolayer was induced by the incubation conditions used in fusion assays, and leakage may in fact account for the small losses in latency.

Vesicles containing Rh-dextran were prepared as above but without lipid fluorophors. Rh-dextran was encapsulated at a concentration of 1 mg/mL in buffer A. Encapsulated dextran was separated from free dextran by chromatography on Sephadex G-75.

PS vesicles with pyrene-PC or egg PC that were used for binding and aggregation studies were also large unilamellar vesicles, but they were prepared by the extrusion method of Mayer et al. (1986) in 100 mM NaCl, 10 mM TES, 0.1 mM EDTA, at pH 7.4 (buffer B), with 10 freeze-thaw cycles and 10 extrusions. MMCC-PE/PC (1/3) vesicles were produced by the same extrusion method in 100 mM NaCl, 10 mM PIPES, 0.1 mM EDTA, at pH 6.5 (buffer C), with only three cycles of freeze-thaw and three extrusions and used immediately after preparation to avoid hydrolysis of the maleimide. For vesicles containing an azidobenzoyl derivative of PE

[1] Abbreviations: pyrene-PC, 3-palmitoyl-2-(1-pyrenyldecanoyl)-L-α-phosphatidylcholine; PS, bovine brain phosphatidylserine; PC, egg yolk phospatidylcholine; PA, phosphatidate derived from egg yolk; PE, phosphatidylethanolamine transesterified from egg PC; AB-PE, (*p*-azidobenzoyl)-PE; MMCC-PE, *N*-(4-(4-maleimidylmethyl)cyclohexyl-1-carbonyl)dipalmitoyl-L-α-PE; NBD, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl); Rh, rhodamine B; Rh-dextran, rhodamine B dextran (10 000 molecular weight average); EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

(AB-PE/PC vesicles), PE/PC (1/3) vesicles were prepared in 20 mM sodium borate, 90 mM NaCl at pH 8.5 by extrusion as described for PS vesicles. Five micromoles of total phospholipid in 1 mL was then reacted in the dark with a 10 mM final concentration of succinimidyl *p*-azidobenzoate added as concentrated stock in DMSO. Final DMSO concentration was approximately 1%. After the sample was stirred for 30 min, 0.5 mL of 100 mM Tris, pH 8, was added, and the sample was stirred for another 30 min. Vesicles were separated from the original buffer by chromatography on Sephadex G-75 in the dark and eluted with buffer B. Phospholipid concentrations were determined using a phosphate assay as described in Kingsley and Feigenson (1979) modified from Chen et al. (1956), Bartlett (1959), and Morrison (1964).

Protein Preparation. Recombinant human annexin I (lipocortin I) and des(1–9)annexin I were kindly provided by Dr. R. Blake Pepinsky of Biogen Corporation (Cambridge, MA). These proteins were at least 98% pure by single-dimension gel electrophoresis. Native annexin I from human neutrophil cytosol was purified by a modificiation of a phospholipid vesicle affinity chromatography method (Meers et al., 1987) followed by ion-exchange chromatography (Ernst et al., 1990). Annexin I applied to a Mono Q column in 20 mM BIS-TRIS/propane at pH 6.8 and eluted by a salt gradient at 0.5 mL/min appeared mainly in the void volume, free of significant contaminants. In some preparations, a significant amount of slightly lower molecular weight contaminant was observed. This was separated from annexin I by subsequent chromatography on the same column at pH 8 in 20 mM Tris and may be a proteolytic product of annexin I. Two-dimensional electrophoresis of annexin I (isoelectric focussing followed by SDS electrophoresis) demonstrated a single overloaded spot by silver stain (Merrill et al., 1981) with a molecular mass of 35–40 kDa and a high isoelectric point (>7). Impurities were estimated to constitute <5% of the protein and were found in several faint spots of lower molecular weight and isoelectric point than those of annexin I. The identity of the isolated annexin I was verified by strong binding on Western blots to a specific monoclonal antibody against the N-terminus of annexin I. It is not known if the annexin I thus isolated is phosphorylated, but two-dimensional electrophoresis shows that it is not composed of multiple isoelectric forms. Approximately 100 mg of cytosol yielded approximately 0.5 mg of purified native annexin I.

Neutrophil Preparation. Human neutrophil cytosol and plasma membrane fractions were prepared as previously described (Oshry et al., 1991). For octadecylrhodamine-labeled plasma membranes (Hoekstra et al., 1984), 2×10^9 cells were added to a dispersion in 4 mL of 18 μM octadecylrhodamine. After incubation at 0 °C for 30 min with gentle shaking, labeled cells were separated from free octadecylrhodamine by chromatography on coarse Sephadex G-50 at 4 °C. Cells were then fractionated as described (Oshry et al., 1991; Borregard et al., 1983). The presence of the fluorophore in the plasma membrane, specific granule, and azurophil granule fractions was assessed by measuring the fluorescence per total protein. Fluorescence was undetectable in the granule fractions where the detection limit would be approximately 10 000 times less fluorophore than that found in the plasma membrane fraction.

To test for exchange to other membranes within the plasma membrane fraction under conditions used for experiments, labeled (80 μg/mL final protein concentration) and unlabeled (4 mg/mL final protein concentration) preparations were mixed and incubated at 4 °C in buffer A for 14 h. Since the octadecylrhodamine was partially self-quenched in the labeled plasma membrane preparations, any substantial exchange to unlabeled membranes should result in relief of quenching. Fluorescence before and after addition of 0.1% sodium deoxycholate was used to assess self-quenching. After the 14-h incubation, the deoxycholate-induced increase in fluorescence was (1.68 ± 0.22)-fold, while the original labeled sample without unlabeled membranes increased in fluorescence by (1.69 ± 0.9)-fold.

Fluorescence Measurements. Fluorescence measurements were made using an SLM 8000C fluorometer (Urbana, IL). NBD excitation was at 450 nm wih emission monitored at 530 nm, while rhodamine was excited at 560 nm and emission was measured at 590 nm. For the pyrene-PC probe, excitation was at 344 nm. Emission was measured at 377 nm for time courses. Turbidity was measured with a photomultiplier tube in a position to accept light passing directly through the cuvette at either 344 nm or 450 nm. The turbidity readings were in the percentage of transmittance so that vesicular aggregation resulted in a lower reading. Lipid dilution assays for fusion used in Figures 1, 2, 3, and 4 were modifications of Struck et al. (1981) as described in Meers et al. (1987) or in this paper.

All samples were maintained at 25 °C in buffer A, B, or C. Additions of materials to initiate annexin binding, such as Ca^{2+}, were made with small aliquots of a concentrated stock. All Ca^{2+} activities were determined by the method of Bers (1982) when buffer A was used. In some cases, the free Ca^{2+} activity was measured directly in the samples used for experiments to verify that the Ca^{2+} buffering was as expected when all the components of the experiment were mixed. For buffer B, Ca^{2+} electrode measurement showed that the free Ca^{2+} concentration (if >10 μM) was approximately the same as the excess Ca^{2+} over EDTA.

Results

Establishment of Cytosol-Mediated Fusion. The cytosol of human neutrophils was tested for promotion of Ca^{2+}-dependent membrane fusion of liposomes with neutrophil plasma membrane preparations. The liposomes were composed of PS/PE (1/3) labeled with the phospholipid probes NBD-PE and Rh-PE, and fusion was monitored by a fluorescent assay that depends on lipid dilution due to fusion [see Materials and Methods and Struck et al., (1981) and Oshry et al. (1991)]. When 1 mM Ca^{2+} was added to vesicles and plasma membrane alone, a low rate of fusion was observed (Figure 1A, curve a). In the presence of increasing amounts of cytosol, the rate of fusion was significantly enhanced (Figure 1A, curve b), indicating that a cytosolic factor mediates fusion.

These fluorescent phospholipid probes do not transfer easily through the aqueous phase (Düzgünes et al., 1987), but it is important to eliminate semifusion or aggregation-mediated lipid exchange (Düzgünes et al., 1987) as a mechanism that increases NBD fluorescence in this system. Therefore, the fate of rhodamine B-dextran as a marker of the aqueous contents was comapred with that of the Rh-PE probe in separate experiments. Table I shows that when plasma membranes, liposomes, and cytosol were incubated with Ca^{2+} followed by sedimentation, 60–80% of the fluorescence of either the Rh-PE or the Rh-dextran was associated with the plasma membrane pellet. EDTA addition at the end of incubation, before sedimentation, showed that 30–40% of the fluorescence was irreversibly associated with the pellet using either type of liposome. The ratio of NBD fluorescence to Rh fluorescence in the pellet material involving the lipid probes increased by a factor of 3.6 over that with untreated liposomes, indicating that fusion and lipid dilution had occurred. This fact along with the fact that the amounts of Rh-dextran and Rh-PE

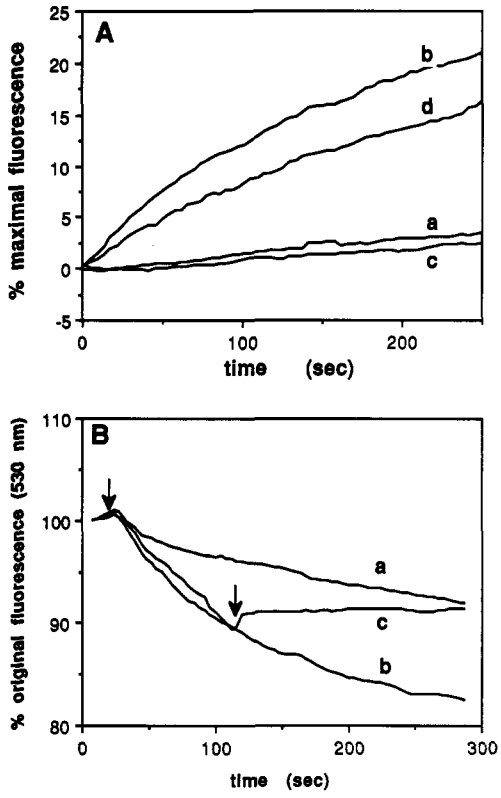

FIGURE 1: Fusion of PS/PE vesicles and neutrophil plasma membrane mediated by neutrophil cytosol. In panel A, 50 μg/mL plasma membrane protein and phospholipid vesicles composed of NBD-PE, Rh-PE (0.75 mol % each), and PS/PE (1/3) were induced to fuse by addition of Ca^{2+} to an excess of 1 mM over the EGTA in buffer A. Untreated (a, b) or dithionite-reduced vesicles (c, d) were used at a total phospholipid concentration of 3 μM and were suspended in buffer A (see Materials and Methods) at 25 °C. Samples also contained either no addition (a, c) or 200 μg/mL cytosol protein (b, d). In panel B, fusion was monitored by using octadecylrhodamine-labeled plasma membranes (labeled as whole cells) at 50 μg/mL protein and untreated liposomes composed of NBD-PE (0.5 mol %) and PS/PE (1/3) at a final concentration of 3 μM phospholipid. At 30 s (first arrow from left), Ca^{2+} was added to all samples in an excess of 1 mM over EGTA to initiate fusion. In curve c, 3 mM EDTA was added at 100 s (second arrow from left).

Table I: Sedimentation of Membrane Fusion Products Involving Fusion of PS/PE Vesicles with Plasma Membranes[a]

PS/PE liposomes (μM)	plasma membrane protein (μg/mL)	Ca^{2+} (mM)	subsequent EDTA (mM)	% fluorescence in pellet
3	50	1	1	36.5 ± 1.7
3	50	1		78.5 ± 0.5
3	50			3.5 ± 3.5
3		1	1	4
Rh-dextran				
3	50	1	1	31.7 ± 1.5
3	50	1		62 ± 0
3	50			0

[a] Plasma membrane preparations, 200 μg/mL cytosol protein, and liposomes were mixed in a total of 1 mL of buffer A. Liposomes labeled PS/PE contained 0.75 mol % NBD-PE and 0.75 mol % Rh-PE as well. Samples labeled Rh-dextran were PS/PE (1/3) with encapsulated Rh-dextran as described in Materials and Methods. Any Ca^{2+} additions (1 mM excess over EGTA) were made at the beginning of a 30-min incubation period (25 °C). Any EDTA additions were made at the end of the incubation period, just before centrifugation for 20 min at 10000*g*. Pellets from all samples were resuspended in 1 mL of buffer A to measure rhodamine fluorescence as a percentage of the total fluorescence in both pellet and supernatant.

pelleted were nearly the same in each case indicated that most or all of the observed fluorescence increase was probably due to fusion with mixing of aqueous contents. The only nonfusion possibility left was Ca^{2+}-dependent but EDTA-irreversible binding of 30–40% of the liposomes (in 30 min) accompanied by Ca^{2+}-dependent outer monolayer exchange.

We attempted to rule out this possibility by monitoring the behavior of the inner monolayer phospholipids after reducing the NBD moiety on the outer monolayer (McIntyre & Sleight, 1991). This procedure does not modify the Ca^{2+}-dependent fusion behavior of PS/PE liposomes with other (unlabeled) PS/PE liposomes (not shown). In Figure 1A, it is clear that dilution of the inner monolayer phospholipids also occurs when PS/PE liposomes and plasma membranes are treated with Ca^{2+} (compare curves a and c to curves b and d). Latency to dithionite treatment after fusion was too high for inner-to-outer monolayer translocation followed by probe transfer to account for the observed fluorescence change (see Materials and Methods). Though the rate of fluorescence increase is somewhat lower (approximately 75%) than observed for inner plus outer monolayer fusion, it is clear that a least a major part of the fluorescence response is due to true fusion of PS/PE membranes with the plasma membrane.

The plasma membrane preparation utilized in these experiments has been shown to have little or no contamination with nuclear membranes, mitochondria, azurophil granules, or specific granules (Borregaard et al., 1983). However, the presence of other cellular membranes could contribute to the observed fusion. To eliminate this possibility, plasma membrane was labeled in whole neutrophils with octadecylrhodamine (see Materials and Methods). When labeled cells were fractionated, the fluorophore was undetectable in the granule fractions. The probe also did not exchange when incubated with an unlabeled plasma preparation (see Materials and Methods), indicating that only the plasma membranes were labeled. Octadecylrhodamine-labeled plasma membrane preparations were then mixed with liposomes containing an NBD-PE probe under conditions nearly identical to those described above. If fusion occurred, quenching of the NBD fluorescence would be expected due to resonance energy transfer to the rhodamine probe. Fusion was demonstrated by the decrease in NBD fluorescence induced by addition of Ca^{2+} (Figure 1B). Cytosol also accelerated this fluorescence change (Figure 1B, curve b). When EDTA was added during fusion, the fluorescence decrease completely stopped, but it did not reverse, indicating that fusion had occurred and that the fluorescence response was not due to energy transfer between reversibly aggregated vesicles containing the two types of probes.

Annexin I as the Predominant Cytosolic Mediator of Ca^{2+}-Dependent Fusion. Since it is known that neutrophils contain annexins (Meers et al., 1987; Ernst et al., 1990) and that recombinant annexin I and des(1–9)annexin I can increase the overall rate of Ca^{2+}-dependent fusion of liposomes with neutrophil plasma membranes (Oshry et al., 1991), the characteristics of fusion mediated by cytosol were compared to those of fusion mediated by these isolated annexins, as well as native annexin I isolated from human neutrophil cytosol. In Figure 2A is shown the Ca^{2+} dependence for fusion in the 1–70 μM range. The fusion rate increased gradually with Ca^{2+} concentration with a threshold of 2–10 μM for native annexin I, recombinant annexin I (not shown) and des(1–9)annexin I (not shown) as well as whole cytosol.

The protein concentration dependence at excess Ca^{2+} is shown in Figure 2B. Cytosol as well as purified annexins exhibited an optimal protein concentration, where the rate of fusion was enhanced from 3- to 20-fold. Above this concen-

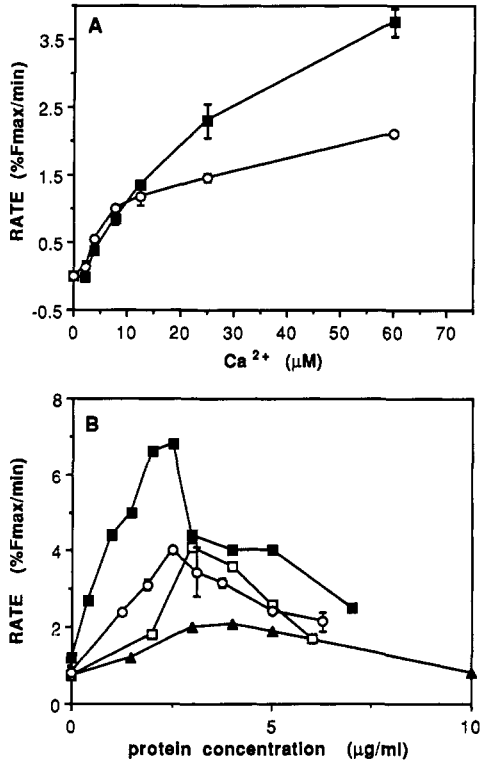

FIGURE 2: Characteristics of neutrophil cytosol-mediated fusion compared to purified annexin I. (A) Ca^{2+} dependence of fusion. Final Ca^{2+} activities were measured as described in Materials and Methods. Samples contained 200 μg/mL cytosol (○) or 2 μg/mL native annexin I (■) in buffer A. Other conditions were as in Figure 1A for curve a. The rate of fusion was defined by measuring the percentage of maximal fluorescence increase at 250 s and converting to units of inverse minutes. All experiments were performed in buffer A at 25 °C. (B) Protein concentration dependence of fusion. Experiments were performed under the same conditions as above with the indicated protein concentrations and symbols. Other annexin I derivatives were 5 μg/mL recombinant annexin I (□) and 5 μg/mL des(1–9)annexin I (▲). Fusion was initiated by addition of Ca^{2+} in 1 mM excess over EGTA. The protein concentration of whole cytosol was multiplied by 0.015 for comparison with purified annexins.

tration, the apparent rate of fusion decreased, eventually reaching a level the same as or lower than the rate in the absence of protein. The amount of cytosolic protein necessary to reach the optimal concentration was approximately 50–100 times the amount of purified annexin I or des(1–9)annexin I. This corresponds to the percentage of annexin I estimated to exist in the cytosol calculated from purification yields. The ratio of cytosol to plasma membrane where the cytosol effect was maximal in Figure 2B was estimated to be close to the actual ratio in whole neutrophils, suggesting that the large amounts of annexin I in neutrophil cytosol may be relevant for mediation of fusion. The specific activities of the purified annexins differed. Native annexin I exhibited the highest specific activity while the recombinant and des(1–9)annexins exhibited lower specific activities. A lower maximal rate of fusion for cytosol compared to annexin I isolated from the neutrophils (Figure 2B) suggests that the cytosol may contain some inhibitors but not enough to completely stop fusion.

Specificity for various phospholipids was also tested showing that whole cytosol, as well as des(1–9)annexin I, enhanced fusion with vesicles containing phosphatidate in place of phosphatidylserine (data not shown). Hence, though negatively charged phospholipids are required, there is apparently little specificity. Cytosol-mediated fusion was unaffected by 10 mol % phosphatidylcholine, but high amounts (e.g., 75%) were highly inhibitory to fusion.

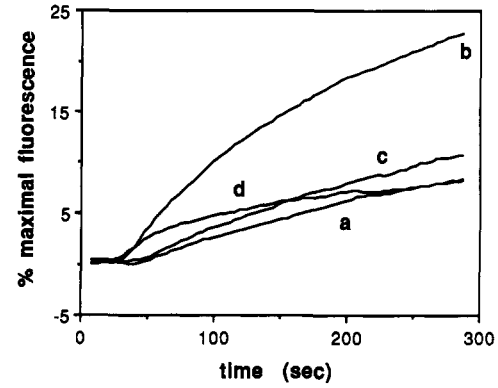

FIGURE 3: Effect of monoclonal antibody against the N-terminus of annexin I. Fusion of PS/PE liposomes and plasma membranes was measured in the presence of no additions (a), 200 μg/mL cytosol (b), 1 μg/mL antibody to annexin I (c), or 200 μg/mL cytosol and 1 μg/mL antibody to annexin I (d). Ca^{2+} was added at 30 s to initiate fusion. Ca^{2+}, plasma membrane, and phospholipid concentrations and all other conditions except for indicated cytosol concentrations were the same as in Figure 1A for curve a.

In order to further identify the cytosolic factor of interest as annexin I, the effect of a specific monoclonal antibody against the N-terminus of the annexin I was compared to its effect on whole cytosol (Figure 3). This antibody inhibited fusion mediated by all the purified annexin I derivatives, as well as by whole cytosol (curve d versus curve b), but not fusion in the absence of added protein (curve c versus curve a). As an added control, an equal amount of mouse anti-human HLA class I antigen had no effect on the cytosol-mediated rate of fusion (not shown). Hence, it is likely that the activity observed in whole cytosol is in fact that of annexin I or a derivative of annexin I.

The presence of annexin I in the cytosol was confirmed by Western blot analysis of fractionated neutrophils (data not shown). Annexin I was sometimes detected in small amounts in EDTA-washed plasma membrane fractions, consistent with previous immunofluorescence observations (Oshry et al., 1991). The effects of monoclonal antibodies to the N-terminus of annexin I were tested to determine whether the intrinsic Ca^{2+}-dependent fusogenicity of the neutrophil plasma membranes was due to residual annexin I in the membrane preparations. In curve c in Figure 3, it is evident that addition of the monoclonal antibodies does not inhibit Ca^{2+}-dependent fusion in the absence of cytosol or purified annexin. Two other antibodies against the first repeat of annexin I (see Materials and Methods) similarly had no activity in this case but strongly inhibited the effect of purified annexin I or cytosol (data not shown). Therefore, either the epitopes tested are inaccessible or unimportant in the plasma membrane associated form of annexin I or a different protein imparts Ca^{2+}-dependent fusogenicity to neutrophil plasma membrane preparations.

Annexin I Enhances Vesicular Aggregation Rather Than Fusion Per Se. Since it has been shown that at least one member of the annexin family, synexin, enhances the overall rate of fusion solely by increasing the rate of vesicular aggregation, it was of interest to determine if annexin I also follows this mechanism. Fusion of PS vesicles was chosen as a well-defined system to delineate effects on aggregation and fusion (Meers et al., 1988a). Fusion induced by 3 mM Ca^{2+} alone is mainly aggregation rate limited, and it can be seen from the data in Figure 4 that annexin I does significantly increase the rate of aggregation. If fusion is induced by 4 mM Ca^{2+} and 5 mM Mg^{2+}, the overall process is largely limited by the rate of the fusion step itself. Under these conditions, annexin I is actually somewhat inhibitory to the overall fusion

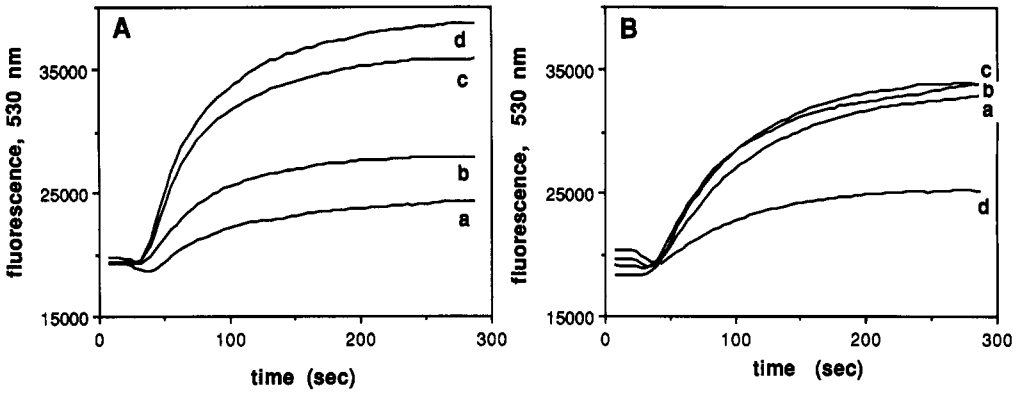

FIGURE 4: Fusion of PS vesicles as mediated by annexin I. (A) PS vesicles (1 μM total phospholipid concentration) containing 0.75 mol % each NBD-PE and Rh-PE were mixed with unlabeled PS vesicles (9 μM total phospholipid), and a final concentration of 3 mM Ca^{2+} was added at 30 seconds. (B) The same concentrations of vesicles as in part A were used, and 4 mM Ca^{2+} with 5 mM Mg^{2+} was used to initiate fusion at 30 s. In both parts A and B, samples contained 0 (a), 0.5 (b), 1 (c), or 2 (d) μg/mL recombinant annexin I. All experiments were performed in buffer B at 25 °C.

process. Therefore, it is likely that annexin I, like synexin, mediates Ca^{2+}-dependent aggregation of vesicles but not the actual fusion event.

Bilayer Interactions in Annexin I–Vesicle Aggregates. Since annexin I is mainly a Ca^{2+}-dependent aggregator of vesicles, aggregation was monitored directly by changes in light scattering or turbidity as manifest by a decrease in the percentage of transmittance. We chose vesicles that bind to annexin I and are aggregated by this protein but do not fuse readily because of large amounts of the inhibitory phospholipid, PC. Aggregation of PS/PC (1/1) vesicles (also containing NBD-PE or Rh-PE) mediated by annexin I, avidin, or Mg^{2+} were compared in Figure 5. Under the conditions in Figure 5, the rate of aggregation and size distribution of aggregates induced by these three factors were assumed to be nearly the same on the basis of nearly identical turbidity changes (Figure 5A) and 90° light scattering curves (not shown). Simultaneous observation of the fluorescence of NBD-PE-containing vesicles aggregated in the presence of excess Rh-PE-containing vesicles showed major differences. Mg^{2+}, which may be expected to mediate close contact between bilayers (see Discussion), initiated a large drop in NBD fluorescence presumably from close contact between NBD-PE headgroups and Rh-PE headgroups, leading to resonance energy transfer from the NBD donor to the Rh acceptor. When either avidin or annexin I (with Ca^{2+}) was used to initiate aggregation, almost no energy transfer between NBD and Rh was observed, indicating few points of contact between the phospholipid bilayers and/or a minimal area of contact and/or large distance between membranes, where contact occurs. This is consistent with previous observations of avidin-mediated vesicular aggregates (see Discussion).

Mode of Binding of Annexin I to Phospholipid Vesicles. Vesicular aggregation monitored by turbidity was also correlated with annexin I binding as measured by changes in the fluorescence of a pyrene-phospholipid probe (Meers et al., 1991a) in Figure 6. When annexin I was preincubated with a monoclonal antibody against the N-terminus, membrane binding was unaffected, but vesicular aggregation was abolished (Figure 6A, curve b). This suggests that most or all of the annexin I molecules bind initially such that the N-terminal region or concave face of the molecule orients away from the membrane. In Figure 6, it is also clear that binding as monitored by the pyrene fluorescence is much more rapid (faster than the mixing time in the cuvette) than the aggregation of vesicles. Preincubation with unlabeled vesicles before Ca^{2+} addition completely abolished the fluorescence change

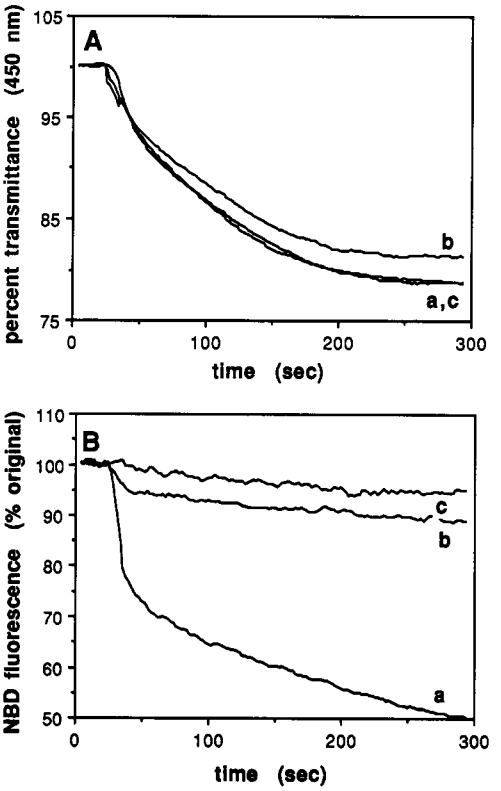

FIGURE 5: Comparison of aggregation and extent of contact between aggregated bilayers. In part A, vesicular aggregation was monitored by turbidity changes at 450 nm while the same samples were simultaneously monitored for interactions of bilayers by quenching of NBD fluorescence at 530 nm (part B). All samples contained vesicles composed of PS/PC (1/1) with 2 mol % NBD-PE at 2.5 μM total phospholipid and vesicles composed of PS/PC (1/1) with 2 mol % Rh-PE at 47.5 μM total phospholipid. Sample c also contained 5 μg/mL recombinant annexin I. Aggregation of vesicles was initiated by 30 mM Mg^{2+} (a), 3.9 μg/mL avidin (b), or a free concentration of 1 mM Ca^{2+} (c). All experiments were performed in buffer A at 25 °C.

by competition (Figure 6B, curve c'). When the unlabeled vesicles were added after the pyrene-PC-containing vesicles had aggregated for 60 s, there was no significant exchange of bound annexin I to the unlabeled vesicles as evidenced by the lack of change in pyrene fluorescence (Figure 6B, curve a, second arrow). Simultaneous observation of turbidity (corrected for the addition of more vesicles) showed that, despite the lack of exchange, there was a subsequent further aggregation of the unlabeled vesicles (Figure 6A, curve a,

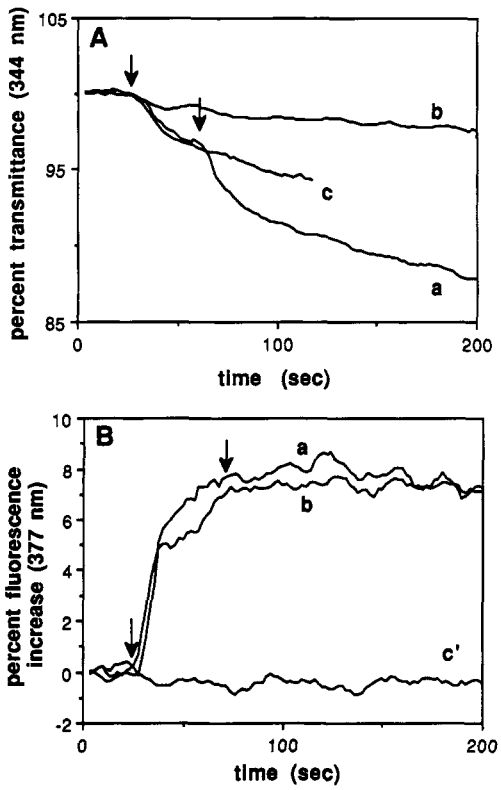

FIGURE 6: Annexin I binding and exchange between vesicles. (A) Vesicular aggregation as measured by turbidity at 344 nm. (B) Binding of annexin I as measured by pyrene-PC fluorescence simultaneously. Samples contained vesicles composed of PS with 5 mol % pyrene-PC at a total phospholipid concentration of 10 μM, 3 μg/mL recombinant annexin I, and either no other additions (a, c) or 3 μg/mL antibody to the annexin I N-terminus (b) or vesicles (40 μM total phospholipid) composed of PS and 5 mol % egg PC (c'). At 30 s (first arrow from left), Ca^{2+} was added to a final free concentration of 200 μM in all samples. At 60 s in curve a (second arrow from the left), unlabeled PS vesicles with 5 mol % egg PC were added to a final phospholipid concentration of 40 μM.

second arrow). This aggregation was not due to residual unbound annexin as supernatants from centrifugation (180000*g* for 3 h) of the original vesicular aggregates did not mediate Ca^{2+}-dependent aggregation of the unlabeled vesicles. Hence, the aggregation of the unlabeled vesicle population was mediated by existing aggregates and an undetectably small percentage of the previously bound annexin I was involved in binding to a second bilayer and/or annexin I does not significantly change its initial mode of binding to bind a second bilayer.

Requirement of Multiple Annexin I Molecules for Intervesicular Linkage. The quantitative relationship of bound annexin I to rate of aggregation of PS vesicles (with 5 mol % pyrene-PC) was next studied in Figure 7. The rate of aggregation was not linear with the amount of added protein. Maximal aggregation rate was reached at 1 annexin I/approximately 100–200 phospholipids. Below approximately 1 annexin/900 outer monolayer phospholipids (1 μg/mL annexin I) or approximately 100 annexin molecules/vesicle, no vesicular aggregation occurred. In order to ascertain that a significant portion of the added annexin I was actually bound at this lower threshold concentration, an experiment similar to the one presented in Figure 6B was performed. It was first observed that Ca^{2+} addition induced a nearly maximal increase in pyrene-PC monomer fluorescence when labeled vesicles (2 μM total phospholipid) and 1 μg/mL annexin I were used. In the presence of 50 μM unlabeled phospholipid (5% egg PC, 95% PS) as well as the labeled vesicles, no change in pyrene

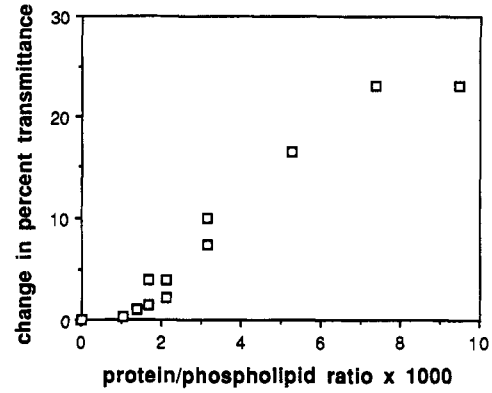

FIGURE 7: Vesicular aggregation as a function of bound annexin I. Vesicles composed of PS with 5 mol % pyrene-PC at a total phospholipid concentration of 50 μM were suspended in buffer B with the indicated amounts of native annexin I. Ca^{2+} (500 μM) was added and the change in the percentage of transmittance at 450 nm was measured after 2 min. All experiments were performed at 25 °C.

fluorescence was observed upon Ca^{2+} addition, indicating that most if not all of the annexin I had bound to the competing unlabeled vesicles. This indicates that essentially all of the annexin I was bound at 50 μM total phospholipid, the threshold conditions in Figure 7, yet no vesicular aggregation occurred. Clearly, binding of annexin I is separable from vesicular aggregation and there is positive cooperativity of vesicular aggregation in terms of bound protein concentration, suggesting that several annexin molecules may be needed to assure stable aggregation of vesicles.

Disposition of Annexin I Monomers between Aggregated Vesicles. The results of Figure 7 showing continued vesicular aggregation at saturating annexin I concentrations suggest that Ca^{2+}-dependent protein–protein interactions are necessary for vesicular aggregation, while the opposite conclusion would be consistent with the data of Figure 6 showing that prebound annexin I can mediate binding to a second bilayer. This apparent paradox would be difficult to investigate directly if only a very small percentage of the bound annexin molecules are involved in intervesicular linkage as suggested by data in Figures 5 and 6. As a first step to ascertain the importance of protein–protein interactions versus protein–lipid interactions, the ability of monomers of annexin I to make simultaneous contact with two bilayers was tested. Since annexin I was relatively nonspecific for the type of negatively charged phospholipid, it was possible to use reactive negatively charged phospholipid derivatives for these studies.

To test the topology of cross-linking on a millisecond time scale, a photoaffinity cross-linker was used. Vesicles composed of 75% PC and 25% PE were reacted with a succinimidyl derivative of azidobenzene to produce AB-PE (see Materials and Methods), a negatively charged phospholipid, on the outer surface of the vesicles. These were aggregated by Ca^{2+} as monitored by turbidity in the presence of native annexin I (Figure 8A, curve a or c1) or des(1–9)annexin I (Figure 8B, curve a or c1). Aggregation was fully reversible by excess EDTA if it was monitored at 450 nm with no exposure to ultraviolet light (Figure 8A, curve a; Figure 8B, curve a). If the sample was first aggregated and then exposed to light from a xenon arc lamp containing wavelengths in the 300–350-nm range for 1–10 min, subsequent addition of EDTA had little effect (Figure 8A, curve c2; Figure 8B, curve c2). These cross-links were shown to be through protein and not lipid–lipid cross-links by addition of trypsin to the irreversibly cross-linked sample. This protease slowly dissociated the aggregates by proteolysis such that eventually aggregation was nearly com-

Table II: EDTA Reversibility of Vesicular Aggregates Cross-Linked through Annexin I and Reactive Phospholipids

phospholipid composition	phospholipid concn (μM)	protein concentration (μg/mL)	[Ca^{2+}] (mM)	Δ% transmittance before EDTA	% recovery by EDTA before reaction	% recovery by EDTA after reaction
AB-DPPE/DPPC[a] (1/3) vesicles	100	5 (r)	2	−21	95	9
AB-DPPE/DPPA (1/3)	100	3 (r)	0.1	−28	75	11
MMCC-PE/PC[b] (1/3)	200	15 (r)	0.9	−33	100	45
	200	15 (d)	0.9	−15	100	20
	200	15 (d)	0.57	−21	100	27
	100	15 (d)	0.57	−28	100	25

[a] Incubations were initiated by addition of Ca^{2+} at the indicated concentrations. Samples were incubated for 15 min, chilled to 0–3 °C, and incubated in the presence or absence of UV light to initiate nitrene reaction. Excess EDTA was subsequently added. Percentages of recovery are the fractional return to the original transmittance reading. (d) designates des(1–9)annexin I, and (r) designates recombinant annexin I. [b] Samples were incubated at the indicated Ca^{2+} concentrations for approximately 4 h. EDTA addition to samples aggregated in the presence of 50 mM 2-mercaptoethanol allowed complete EDTA reversal (100% recovery). In the absence of 2-mercaptoethanol, reaction of the maleimidyl probe with protein occurred and was not reversible by 2-mercaptoethanol. EDTA reversibility is shown.

pletely reversed (Figure 8A, curve c2; Figure 8B, curve c3, level f). Hence this azido-phospholipid probe can access a monomer of annexin I or des(1–9)annexin I from both of two interacting vesicle bilayers. The completeness of this irreversibility, along with the temporally short and presumably nonspecific nature of the azido probe, suggests that all of the vesicular aggregation may be mediated such that annexin I monomers are able to contact two bilayers simultaneously.

The effects of cross-linking with reduced motion and lower Ca^{2+} levels were also investigated. It was possible that motions of lipids and annexins during the milliscond time scale of the reactive nitrene could lead to cross-linked products not related to the true active species. Lack of annexin exchange between aggregated bilayers (Figure 6) and lack of contact between aggregated bilayers (Figure 5 and trypsin experiment, Figure 8) suggest that this was not the case. To further rule out this possibility, gel-phase phospholipids and low temperature were used. EDTA irreversible cross-linking at 0–3 °C was observed when vesicles were composed solely of gel-phase dipalmitoyl phospholipids with outer monolayers of dipalmitoyl-PC (DPPC) and an azido derivative of DPPE (Table II). When DPPA was used instead of DPPC, vesicles could be aggregated by approximately 100 μM Ca^{2+}. Cross-linking was also irreversible in this case. In both cases, the more rigid cross-linked structures appeared more resistant to protease digestion, but they did yield to at least partial reversal of cross-linking by proteinase K.

A maleimidyl derivative, MMCC-PE (see Materials and Methods), showed similar behavior. Annexin I or des(1–9)-annexin I induced Ca^{2+}-dependent aggregation of vesicles composed of 75% PC and 25% MMCC-PE (Table II). After long incubations, partial irreversibility of aggregation by EDTA was observed. Irreversibility probably resulted from the cross-linking of a single annexin I molecule to two separate bilayers through at least two of its four thiol groups. Addition of β-mercaptoethanol during or after vesicular aggregation showed that irreversibility by EDTA was a result of reaction of the protein with the maleimide and not a result of disulfide bond formation between annexin monomers. Incubation of the reactive vesicles with the protein in the absence of Ca^{2+} did not lead to a significant amount of aggregation, suggesting that all cross-linking was specifically related to annexin-mediated Ca^{2+}-dependent vesicular aggregation.

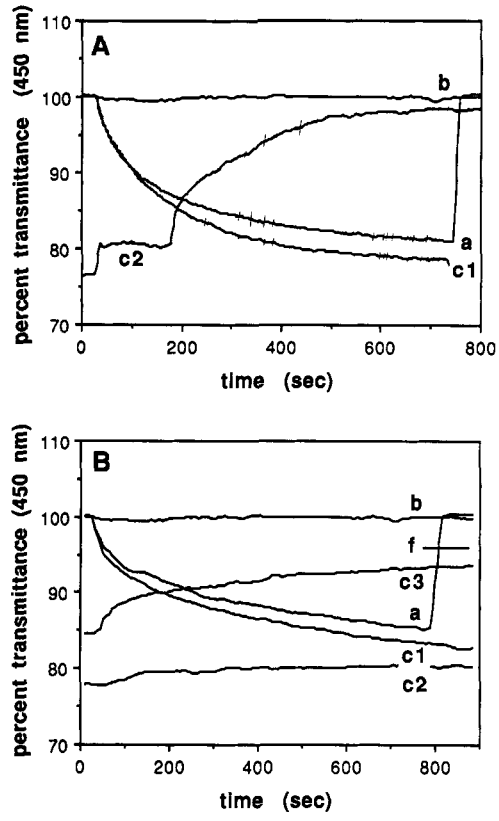

FIGURE 8: Effect of covalent linkage to azido-phospholipids on vesicular aggregation. All samples contained vesicles composed of an estimated 25% AB-PE and 75% PC (on the vesicle outer monolayers) at 100 μM total phospholipid concentration in buffer B. In panel A, samples contained 10 μg/mL native annexin I (a, c1, c2) or no protein (b). Ca^{2+} (2 mM) was added at 30 s in curves a, b, and c1. A 3 mM final concentration of EDTA was added at 750 s in curve a. Curve c2 is a continuation of curve c1 after exposure of the sample to ultraviolet light. At 30 s in c2, 3 mM EDTA was added and a 150 μg/mL final concentration of trypsin was added at 180 s. In panel B, samples contained 15 μg/mL des(1–9)annexin I (a, c1, c2, c3) or no protein (b). In curves a, b, and c1, a 2 mM final concentration of Ca^{2+} was added at 30 s. A 2 mM final concentration of EDTA was added at 800 s in curve a. Curve c2 is a continuation of curve c1 after exposure of the sample to ultraviolet light for 2 min. EDTA (2 mM) was added at 30 s in curve c2. Curve c3 is a continuation of curve c2. At 30 s in curve c3, 100 μg/mL trypsin was added and it was added again at 330 s. The line f shows the level of sample c3 after incubation for 30 min.

Discussion

Annexin I and Degranulation. Degranulation of human neutrophils has been associated with an increase in intracellular Ca^{2+} levels that may regulate this membrane fusion process (Naccache et al., 1979; Gennaro et al., 1984; Goldstein et al., 1984), possibly through Ca^{2+}-dependent proteins. There is evidence that annexins can act as intracellular Ca^{2+}-dependent mediators of membrane fusion (Creutz et al., 1978; Meers et al., 1987) and particularly that annexin II plays such a role in chromaffin cells (Ali et al., 1979, Drust & Creutz, 1988).

Annexin II is a minor component in neutrophils (unpublished data), while several other annexins including I and III are major components of the cytosol (Hirata et al., 1980; Meers et al., 1987; Ernst et al., 1990). The data presented here suggest that annexin I is the major mediator of Ca^{2+}-dependent fusion in the neutrophil cytosol in agreement with previous observations of annexin I-mediated liposomal fusion (Blackwood & Ernst, 1990) and fusion of liposomes with plasma membrane preparations (Oshry et al., 1991). Liposomes have been used as surrogates for specific granules in this work, but it has previously been shown that synexin (annexin VII) can bind and mediate fusion of specific granules (Meers et al., 1987). Demonstration of annexin I-mediated fusion of specific granules directly with the plasma membrane is the next step in substantiating its role in degranulation.

Though the relevant Ca^{2+} concentration near the site of fusion is unknown, the observed dependence may be influenced by locally higher Ca^{2+} concentrations (Sawyer et al., 1985), dependence on the concentration of fusing vesicles, and/or the absence of modulating factors as discussed previously (Meers et al., 1987). A modified form of annexin I may mediate fusion as discussed in Oshry et al. (1991). Phosphorylation near the N-terminus (Schlaepfer & Haigler, 1987) and cleavage near the N-terminus (Ando et al., 1989) have been reported to increase the Ca^{2+} sensitivity for binding, though no data on fusion have been presented. The data presented here nullify our previous speculation (Oshry et al., 1991) that des(1–9)annexin I may mediate fusion at a lower Ca^{2+} concentration than annexin I. Instead, cleavage may play a role in down regulation. The possibility that cleavage at a more internal position [e.g., des(1–29), see Ando et al. (1990)] or phosphorylation near the N-terminus may allow vesicular aggregation at lower Ca^{2+} levels remains open.

The data on annexin I concentration dependence are also interesting in that they show the high concentration inhibition of fusion observed previously for synexin (Meers et al., 1987). The inhibitory effect of increased annexin I binding may be physiologically relevant as well. Induction of annexin I synthesis has been proposed as a mechanism of the antiinflammatory activity of glucocorticoids (Hirata et al., 1980). The data showing inhibition suggest the possibility that increased bound amounts of annexin I may mediate glucocorticoid effects by inhibition of neutrophil degranulation.

Annexin I, like annexin VII (synexin) (Nir et al., 1987; Meers et al., 1988a,b), appears to mediate only vesicular aggregation and not fusion per se. This fact is supported by the observation that vesicles that cannot themselves fuse at some Ca^{2+} concentration, also will not fuse with annexin I present, but can aggregate (e.g., Figure 5). While this does not preclude a role for this protein in neutrophil degranulation or other fusion processes, it does open the possibility that other proteins, probably membrane associated, play a directly fusogenic role to assist annexin I. The fact that Ca^{2+}-dependent fusion of liposomes with the cytoslic side of plasma membranes occurs in the absence of annexin I or cytosol (Francis et al., 1990; Oshry et al., 1991) along with trypsin sensitivity of the plasma membranes (Oshry et al., 1991) suggests that one such protein may reside in the plasma membrane. Though there have been indications that annexin I may exist in a membrane-bound form not extractable by chelators (Sheets et al., 1987; Oshry et al., 1991), the data presented here suggest that the putative protein is probably not annexin I.

Mechanism of Vesicular Aggregation by Annexin I. Annexin-mediated membrane fusion entails binding to the membrane, possible oligomerization or conformational changes of the protein, aggregation of vesicles, and finally actual fusion of membranes. It is clear (e.g., Figures 4 and 7) that these are separable phenomena for annexin I. Annexin I appears to initially bind to phospholipid vesicles in the manner of annexin V [see Figure 6 data and Huber et al., (1990)]. At low surface densities, the bound annexin I is unable to mediate vesicular aggregation (see Figure 7 data). At higher densities, more than one annexin I molecule is probably necessary for intervesicular linkage, though it is not clear from our data if actual self-association of the annexin I monomers is necessary. The contact between bilayer phospholipids in these aggregates is minimal (e.g., Figure 5 data) and only a minority of the bound annexin I molecules is involved (Figure 6 data). Nonetheless, the fusion process per se is not mediated by the annexin but is dependent on the phospholipids (Figure 4). Monomers of annexin I may be able to contact more than one bilayer simultaneously, whether or not annexin self-association is involved.

Studies on other annexins have suggested that they may self-associate either on or off the phospholipid membrane (Creutz et al., 1979; Walker et al., 1983; Newman et al., 1989; Zaks & Creutz, 1991; Barbara Seaton, Boston University, personal communication). Self-association of annexin I has been observed by the isolation of a form that is covalently cross-linked through the N-terminal region (Pepinsky et al., 1990). Self-interaction involving the first repeat of annexin I has also been suggested (Ernst et al., 1991). However, no self-association of annexin I in the absence of membranes was observed (data not shown).

Prepolymerized annexin VII (synexin) retained activity in vesicular aggregation (Meers et al., 1988a), suggesting a possible role for annexin oligomers in the vesicular aggregation process. However, other studies indicate that annexin self-association is not necessarily correlated with vesicular aggregation. For example, annexin V can self-associate (e.g., annexin V; Barbara Seaton, Boston University, personal communication) but does not mediate vesicular aggregation (Blackwood & Ernst, 1990; Oshry et al., 1991). Annexin IV, VI, and VII intervesicular protein–protein interactions have been suggested to play a role in intervesicular linkage at Ca^{2+} concentrations in the 100 μM range on the basis of fluorescence energy transfer between labeled monomers bound to separate vesicles (Zaks & Creutz, 1991). But the small change in fluorescence (similar in magnitude to experiments with labeled phospholipids in Figure 5B, curve c) could not be unequivocally distinguished from random collisions between monomers.

The mechanism of the actual fusion event is still unknown, but the data presented suggest that the extensive contact between bilayers induced by Mg^{2+} and other divalent cations, observed previously as large flattened apposed surfaces (Kachar et al., 1986) and by resonance energy transfer (Düzgünes et al., 1987), may not be relevant for annexin-assisted fusion. Lack of energy transfer in the avidin experiment is consistent with our previous observations by freeze fracture electron microscopy of PS/PC (3/7) vesicles aggregated by avidin, where the vesicles in aggregates remain relatively spherical with minimal points of contact, and the bilayers seem to be held apart (not shown). The lack of contact between membranes and lack of effect on fusion per se by annexin I is also consistent with the relatively peripheral binding of annexins to the membrane (Meers, 1990; Meers et al., 1991a,b).

Some possible mechanisms for annexin-mediated vesicular aggregation are crudely modeled in Figure 9. Initial binding

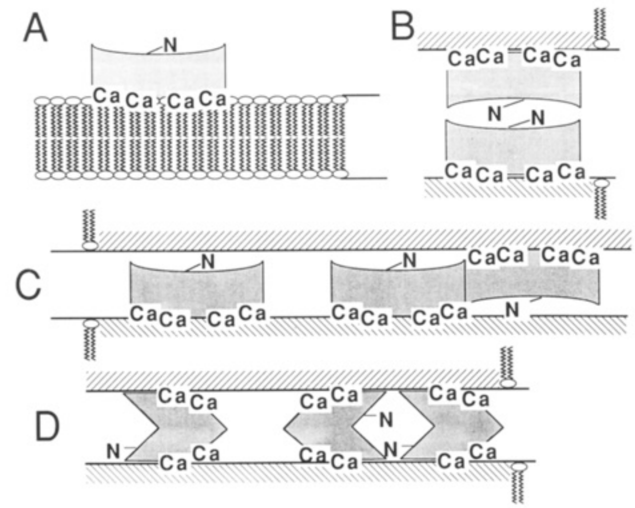

FIGURE 9: Models for annexin I binding to membranes. (A) Annexin I bound to a single bilayer on the basis of the information available from the annexin V structure (Huber et al., 1990) and its interaction with the phospholipid bilayer (Meers, 1990; Meers et al., 1991b). Key features are a group of Ca^{2+} binding sites on one side of the molecule that presumably mediate protein–lipid interactions. The N-terminus is localized away from the bilayer as discussed in the introduction. (B) Vesicular aggregation depending solely on protein–protein interactions after initial binding in the manner of annexin V. Any interaction that puts more than one protein molecule between bilayers would be equilvalent for this discussion. (C) Annexin I maintains a conformation similar to the annexin V-like conformation, but each monomer is able to make contact with more than one bilayer simultaneously. Besides the protein–Ca^{2+}–phospholipid linkages at one membrane, the second membrane linkage would be made through phospholipid interaction with the N-terminal face of the protein (left side) or through lateral protein–protein interactions (right side). (D) Binding to two bilayers via an unknown conformation that allows multiple Ca^{2+} binding sites from each monomer to contact more than one bilayer.

of annexin I is depicted in the manner of annexin V in Figure 9A. Subsequent protein–protein mediation of vesicular aggregation shown in model B is not consistent with irreversibility of vesicular aggregation by coupling to reactive phospholipids, unless the protein–protein contacts are EDTA-irreversible. Contact of annexin monomers with two bilayers probably best accounts for EDTA-irreversible cross-linking. Therefore, model B would appear to be least likely according to our data.

In model C, Ca^{2+}-independent interaction of the N-terminal (or concave) side of annexins with membrane phospholipids (left side, case C) would be inconsistent with binding reversibility. Ca^{2+}-dependent conformational change of the N-terminal face to allow lipid binding cannot be ruled out, however. The model on the right side of Figure 9C would require a restricted few of the annexin I monomers to be able to "flip" from the original membrane to be bound to a second membrane. If the "flip" were not restricted to some extent, equilibrium would entail a sustained exchange of monomers to subsequently added excess vesicles, in contrast to the lack of exchange observed (data in Figure 6).

The models in Figure 9D show that if a conformation significantly different from that of the original annexin V structure could occur then each monomer could contact two bilayers, consistent with data in Figure 8. The requirement for at least part of each annexin I monomer to remain bound to its original membrane could preclude extensive exchange, consistent with Figure 6. Only a small percentage of the bound annexin I monomers near the site of intervesicular contact would need to undergo such a transformation, consistent with Figures 5 and 6. Freeing the membrane surface area of part of an annexin could allow part of an annexin molecule from another membrane to bind. This could explain the data in Figure 7 showing that saturation of annexin I binding sites [also see Meers et al., (1991a)] does not inhibit aggregation. It is also likely that there are at least some bare membrane patches at annexin I binding saturation due to the irregular shape of the annexin monomer or oligomer [e.g., see Brisson et al. (1991)]. This is supported by the fact that though binding saturation is inhibitory to fusion, it does not completely inhibit fusion (Figure 4 and data not shown). Binding of annexin monomers to two membranes simultaneously may be enhanced by self-association as depicted on the right side of Figure 9D, consistent with data in Figure 7 and also the suggestion of self-association of other annexins at 100 μM Ca^{2+} by Zaks and Creutz (1991). Model D best accounts for the data at present, but other models cannot be definitely ruled out. Future attempts to determine the sites of the phospholipid cross-links should allow more definite discrimination between these and other possible models.

Acknowledgments

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