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Annexin I interactions with human neutrophil specific granules: fusogenicity and coaggregation with plasma membrane vesicles

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The interactions of annexin I with specific granules isolated from human neutrophils were investigated. Unfractionated cytosol induced Ca^{2+}-dependent granule self-aggregation and fusion of granules with model phospholipid vesicles. High Ca^{2+} concentrations were required for these processes (500–600 μM for the half-maximal rate of granule self-aggregation; 100–200 μM for the half-maximal rate of fusion with phospholipid vesicles). These activities were inhibited by a monoclonal antibody specific for annexin I and immunodepletion of cytosol by this antibody greatly reduced activity, implicating annexin I as the major mediator of these processes in neutrophil cytosol. The fact that the Ca^{2+} concentration dependences differed for different membranes suggests that specificity may be contolled by the type of intracellular membrane involved and the local Ca^{2+} concentration. Trypsin treatment of granules enhanced the rate of fusion of phospholipid vesicles with granules, suggesting that access to phospholipids in the granule membrane may be modulated by granule proteins or that a fusogenic protein factor in the granule membrane is activated by trypsin treatment. Coaggregation of specific granules with plasma membrane vesicles mediated by Ca^{2+} and annexin I was suggested by the fact that granules preincubated with Ca^{2+}, cytosol and plasma membrane vesicles blocked the fusion of subsequently added phospholipid vesicles with the plasma membrane vesicles. These data suggest a role for annexin I as part of a multiprotein system involved in membrane–membrane contact necessary for exocytosis of specific granules in human neutrophils.

Introduction

An important component of the inflammatory response in humans is the activation and degranulation of neutrophils at the site of inflammation. In this process some cytoplasmic granules fuse with the plasma membrane and release material to the exterior of the cell. A rise in neutrophil cytosolic Ca^{2+} from extracellular entry (induced by ionophores or permeabilization of the cell) is sufficient to stimulate degranulation of specific granules [1,2]. Ca^{2+} and GTP appear to play synergistic, but possibly separate, roles in this process [3,4]. Since fusion must eventually involve direct interaction of specific granules with plasma membranes, proteins that can mediate membrane–membrane interactions in a Ca^{2+}-dependent manner are of particular interest.

Annexin I is an abundant protein in the cytosol of human neutrophils. One of the putative functions of annexins is assisting intracellular membrane fusion [5–12]. Annexin I has previously been shown to be the major mediator of Ca^{2+}-dependent vesicular aggregation in neutrophil cytosol [13,14] and to mediate Ca^{2+}-dependent fusion of liposomes with other liposomes [15] or with the cytoplasmic face of neutrophil plasma membrane vesicles [13,16]. As a further step in elucidating the mechanism of human neutrophil degranulation, the Ca^{2+}-dependent interactions of annexin I with specific granules have been investigated. Results are presented showing Ca^{2+}-dependent interaction of annexin I with specific granules and its activity as the major cytosolic factor in the Ca^{2+}-dependent self-aggregation of these granules, their fusion with model membranes and, most importantly, coaggregation with

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Abbreviations: pyrene-PC, 3-palmitoyl-2-[1-pyrenedecanoyl]-L-α-phosphatidylcholine; PS, bovine brain phosphatidylserine; PE, phosphatidylethanolamine transesterified from egg PC; NBD, *N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl; Rh, rhodamine B; EDTA, ethylenediaminetetraacetate; EGTA, [ethylene bis(oxyethylenenitrilo)]tetraacetic acid; Tes, *N*-tris(hydroxylmethyl)methyl-2-aminoethanesulfonic acid.

plasma membrane vesicles. Ca^{2+} dependence of self-aggregation and fusion are also compared.

Methods

Octadecylrhodamine B was obtained from Molecular Probes (Eugene, OR). Bovine brain phosphatidylserine (PS), phosphatidate (derived from egg phosphatidylcholine) (PA), phosphatidylethanolamine (transesterified from egg phosphatidylcholine) (PE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B-PE (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). EGTA (puriss grade) and $Na_2S_2O_4$ (sodium dithionite) were from Fluka (Ronkonkona, NY). $CaCl_2$ (> 99%), KCl (> 99%) and NaCl (> 99%), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were from Fisher (Pittsburgh, PA). Protein G-Sepharose was from Sigma (St. Louis, MO). 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 [17].

Vesicle preparation

Large unilamellar vesicles of PS/PE (1:3) labeled with NBD-PE and/or Rh-PE (0.75 mol% each) were prepared by the reverse-phase evaporation method [18] as modified by Wilschut et al. [19] 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 [20]. This method generates mostly unilamellar vesicles [21].

For some experiments these vesicles were treated with 2 mM sodium dithionite for 30 min at 0°C. Reduction of approx. 50% of the NBD groups was observed under these conditions by disappearance of NBD fluorescence, presumably due to outer monolayer reduction only [13,22]. Dithionite was subsequently removed by chromatography on Sephadex G-75.

Phospholipid concentrations were determined using a phosphate assay as described in Kingsley and Feigenson [24] modified from Chen et al. [25], Bartlett [26] and Morrison [27].

Protein preparation

Recombinant human annexin I (lipocortin I) was generated as described from a pET9d plasmid generously supplied by Dr. Joel Ernst [50] and purified to an estimated 95% purity by phospholipid vesicle affinity chromatography [9].

Neutrophil preparation and fractionation

Human neutrophil cytosol, plasma membrane and specific granule fractions were prepared as previously described [13,16]. All cell preparations are always treated with diisopropyl fluorophosphate (DFP, 5 μl/10^9 cells) and washed before fractionation. All specific granules were passed through Sephacryl S-1000 after fractionation to help remove residual Percoll. The phospholipid content of specific granules was assayed by extraction of the granule fraction [28], followed by phosphate assay. The granules contained 93 ± 1 nmol of phospholipid per mg of protein. Total protein of neutrophil fractions was assayed by the bicinchoninic acid protocol [29] in 0.1% SDS. Granule contents were prepared by ten freeze-thaw cycles followed by removal of membrane by sedimentation at $160\,000 \times g$ for 2 h.

For octadecylrhodamine-labeled plasma membranes [30], $2 \cdot 10^9$ 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 [16,31]. The plasma membrane is virtually exclusively labeled as shown previously [13].

Immunodepletion of cytosol was performed by incubation of 300 μg of cytosol protein with 20 μg of anti-annexin I monoclonal antibody for 1 h at 4°C. This mixture was then gently shaken with approx. 50 μl of protein G-Sepharose beads (approx. 100 μg of protein G). Buffer was added and the beads were separated by centrifugation for 2 min at approx. $10\,000 \times g$. For controls antibody was omitted.

Fluorescence measurements

Fluorescence measurements were made using a SLM 8000C fluorometer (Urbana, IL). NBD excitation was at 450 nm with emission monitored at 530 nm. Fusion asays [32] were performed as previously described [13]. Maximal fluorescence was arbitrarily defined as the level in the presence of 0.1% Triton X-100, though NBD is partially quenched by this detergent. Small rapid (a few seconds) decreases of fluorescence, at least partly due to dilution upon Ca^{2+} addition, were corrected in the time courses. Turbidity was measured with a photomultiplier tube in a position to accept light passing directly through the cuvette at 450 nm. The turbidity readings were in percent transmittance so that vesicular aggregation resulted in a lower reading. All error bars represent standard deviation of either duplicates or triplicates.

All samples were maintained at 25°C in buffer A. 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 [34]. 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.

Results

The ability of annexins to mediate aggregation of secretory vesicles has been taken as evidence of a possible role in secretion (e.g., Refs. 5 and 11). Therefore, the ability of annexin I to mediate Ca^{2+}-dependent aggregation of specific granules was first investigated. Whole cytosol was added to specific granules and vesicular aggregation was monitored by turbidity as manifest by a decrease in percent transmittance of the sample (Fig. 1). Aggregation was clearly dependent on the presence of cytosol. Treatment of cytosol with a monoclonal antibody specific for annexin I eliminated vesicular aggregation. A residual small rapid decrease in percent transmittance was probably due to binding of annexin I to the surface of the granules, a process which would not be inhibited by this antibody [13]. Annexin I is therefore probably the major factor in human neutrophil cytosol that mediates Ca^{2+}-dependent aggregation of specific granules, in agreement with recently published data [14].

The dependences of specific granule aggregation on Ca^{2+} and cytosol protein concentration are shown in Figs. 2A and B. Half maximal aggregation required approx. 500–600 μM Ca^{2+} and approx. 50–100 μg/ml of cytosol protein under the conditions of these experiments, although some aggregation could be observed at Ca^{2+} activities as low as 100–200 μM. Phospholipid extractions showed that for the 50 μg/ml specific granule protein used in Fig. 2B, the phospholipid concentration is approx. 4.6 μM. If we assume an active annexin I content of approx. 1.5% of total protein (roughly based on the apparent specific activity of cytosol [13]), then the annexin I-to-phospholipid (outer monolayer only) ratio at the half-maximal aggregation rate is 1 to 126, and the maximal rate is reached near 1 annexin per 59 phospholipids. This latter number is close to the approximate surface area required for binding of other annexins [33,35] and probably indicates a binding saturation effect. These data suggest that granule phospholipids are the major binding site for annexin I. These data also show that a minimum of one annexin I molecule per 347 phospholipids is required for granule aggregation. The ratio of total cytosol protein to specific granule protein at the point where the maximal rate of aggregation is reached is approx. 4:1, close to the ratio recovered in the human neutrophil fractionations.

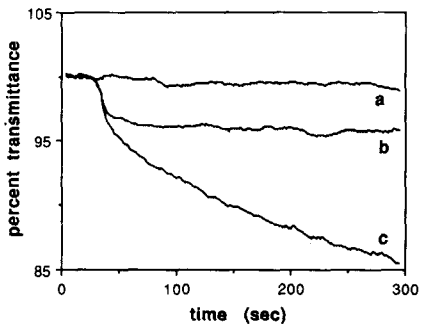

Fig. 1. Cytosol-mediated aggregation of specific granules and the effect of a monoclonal antibody to annexin I. Samples all contained 50 μg/ml total specific granule protein with no added protein (a), or 200 μg/ml cytosol protein and 2 μg/ml anti-annexin I monoclonal antibody (b), or 200 μg/ml cytosol protein alone (c). Ca^{2+} was added at 30 seconds to give a final activity of aproximately 1 mM in buffer A. Specific granule aggregation was indicated by a decrease in percent transmittance. All experiments were performed at 25°C.

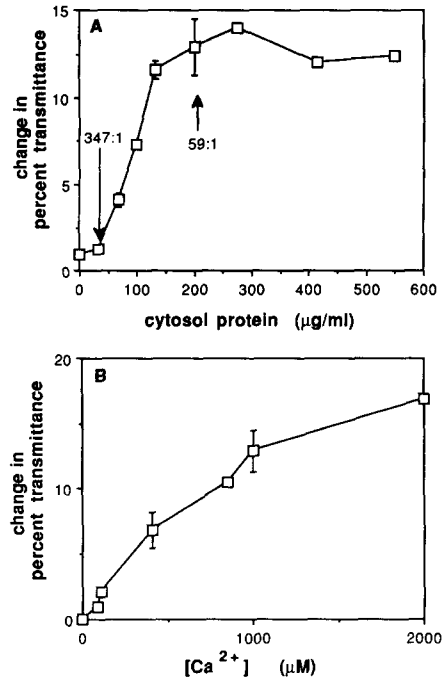

Fig. 2. Cytosol protein (A) and Ca^{2+} (B) dependence for aggregation of specific granules. Samples in plot A contained 50 μg/ml specific granule total protein and the indicated amount of cytosol protein. Aggregation was monitored as the decrease in percent transmittance 270 seconds after adding Ca^{2+} to give an activity of 1 mM in buffer A. In plot B the conditions were the same except that cytosol protein concentration was held constant at 200 μg/ml and the Ca^{2+} activity was varied. All experiments were performed at 25°C.

The fusogenicity of the specific granules was next assessed by determining the ability of liposomes composed of PS and PE to fuse with the granules in a cytosol and Ca^{2+}-dependent manner. A lipid dilution assay involving the fluorescence resonance energy transfer pair NBD and rhodamine was used [32]. In these assays, an increase in NBD fluorescence intensity results from fusion and lipid dilution. Fluorescence of the labeled vesicles was not affected by the internal contents of specific granules either in the presence or absence of Ca^{2+} (Fig. 3A). Fusion of vesicles with NBD on either both monolayers, or only the inner

monolayer, of phospholipids was observed (Fig. 3A), indicating that the increase in NBD fluorescence intensity was probably due to true fusion, and not the result of outer monolayer exchange. The fusion under these conditions was not strongly dependent on fatty acids (not shown), as previously observed over a limited set of conditions for synexin [9], but was strongly dependent on the presence of cytosol (Fig. 3A). The effect of immunodepletion of annexin I was also tested in this system. Cytosol was immunodepleted of annexin I by incubation with the specific monoclonal antibody followed by removal as a complex with protein G beads. Immunodepletion greatly reduced the rate of fusion to nearly zero (Fig. 3B), indicating that annexin I is the

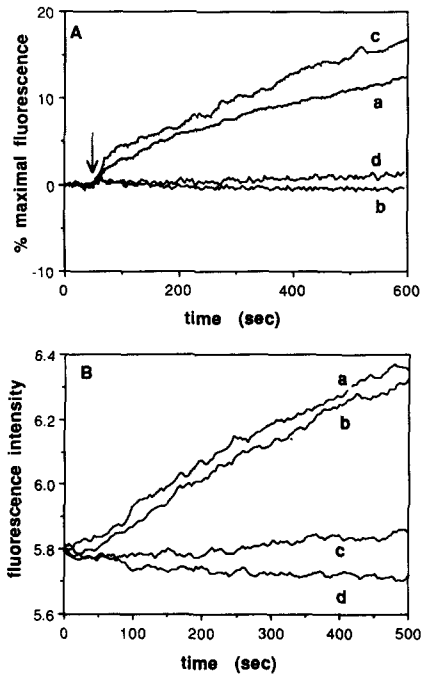

Fig. 3. Fusion of PS/PE vesicles with specific granules. (A) Effect of cytosol and specific granule contents. PS/PE (1:3) vesicles with 0.75 mol% each of NBD-PE and Rh-PE (3 μM total phospholipid) and specific granules (300 μg/ml protein) were mixed with 200 μg/ml cytosol protein (a and c) or no cytosol (b). 1 mM Ca^{2+} was added at the arrow to initiate fusion. Maximal fluorescence was defined as in Materials and Methods. PS/PE vesicles in curve c had been dithionite treated to reduce outer monolayer NBD groups (Materials and Methods). In curve d, granule contents equivalent to 300 μg of intact granule protein were added to labeled PS/PE vesicles (as above) and Ca^{2+} was added at the arrow. (B) Effect of immunodepletion. 300 μg of cytosol was incubated with no additions (a), protein G beads (see Methods) (b), or with anti-annexin I antibody followed by protein G beads (see Methods) (c). A final concentration of 270 μg/ml of these cytosol preparations were added to labeled PS/PE vesicles (3 μM total phospholipid) and specific granules (300 μg/ml protein). Curve d contained granules and PS/PE vesicles, but no cytosol. A final concentration of 1 mM Ca^{2+} was added at time zero to initiate fusion. Results are expressed in terms of arbitrary NBD fluorescence intensity units and are corrected for a small artifactual rapid decrease in fluorescence upon Ca^{2+} adddition. All fusion experiments were performed at 25°C.

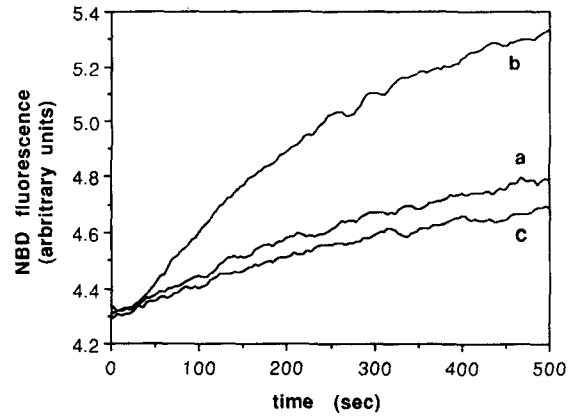

Fig. 4. Effect of trypsin treatment on fusion of PS/PE vesicles with specific granule preparations. Specific granules (300 μg total protein) were incubated with buffer alone (a) or 100 μg/ml trypsin (18 μg total) (b) for 10 min in 180 μl total volume of buffer A at 25°C. This material was added to buffer A containing soybean trypsin inhibitor (80 μg/ml final concentration), cytosol protein (200 μg/ml final concentration) and NBD,Rh-PS/PE vesicles (3 μM total phospholipid final concentration) to give a final volume of 1 ml. A final Ca^{2+} activity of 1 mM was then added at time zero to initiate fusion. Sample c contained 300 μg/ml specific granules, NBD,Rh-PS/PE (3 μM total phospholipid) vesicles and both 18 μg/ml trypsin and 80 μg/ml soybean trypsin inhibitor before addition of 1 mM Ca^{2+}.

major mediator of vesicular aggregation in this system. Addition of the antibody to the fusion mixture also strongly inhibited the rate of fusion, while a mouse monoclonal antibody specific for HLA class I antigen had no effect (not shown).

It was necessary to use a relatively large excess of granule lipid to liposomal lipid to observe fusion by the lipid probe dilution assay. Therefore, we needed to consider the possibility that the same fusogenic vesicles previously found in the plasma membrane preparations [13,16,36] existed as an impurity in the specific granule preparation. However, differences in Ca^{2+} sensitivity (see below) suggest that this is not the case. In addition, the trypsin sensitivity of fusion was different for granules and plasma membrane preparations. Trypsin treatment was inhibitory for fusion of plasma membrane vesicles [16], while fusion of the specific granules was actually somewhat enhanced by trypsin pretreatment (Fig. 4). Therefore the fusogenic vesicles in the specific granule preparation are different from those in the plasma membrane preparations. Preliminary experiments using a fluorescence activated cell sorter to separate fused products, based on a change in the ratio of the fluorescence intensity of the NBD and rhodamine phospholipid probes, showed that these fused products contained lactoferrin, a marker for specific granules (data not shown). The trypsin data also indicate that proteolytic clearing of proteins on the granule surface and/or trypsin activation of a fusogenic granule membrane protein may be important in degranulation.

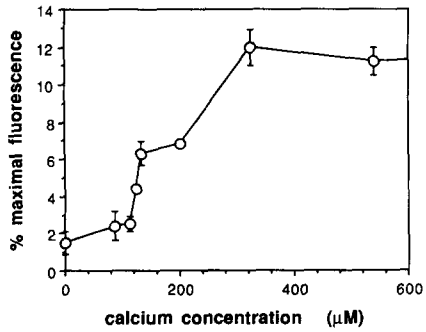

Fig. 5. Ca^{2+} dependence for cytosol-mediated fusion of specific granules with PS/PE vesicles. Samples contained specific granules (300 μg/ml total protein) NBD,Rh-PS/PE vesicles (3 μM total phospholipid) and 200 μg/ml cytosol protein. The indicated activities of Ca^{2+} were added at time zero to initiate fusion at 25°C in buffer A. The rates of fusion were calculated from the percentage of the maximal fluorescence attained at 540 seconds after Ca^{2+} addition.

The Ca^{2+} dependence for rates of fusion is shown in Fig. 5. Half-maximal rates required approx. 100–200 μM, lower than that for aggregation of specific granules (Fig. 2A), and the threshold (approx. 50–80 μM) was higher than that observed for fusion with plasma membrane vesicles (5–10 μM; Ref. 13), suggesting that the different membranes involved control the Ca^{2+} dependence rather than the annexin Ca^{2+} binding sites. Protein concentration dependence is shown in Fig. 6. Similar behavior was observed for both cytosol and purified recombinant annexin I. It appears that, as in other cases, a maximal rate of fusion is reached at a certain protein concentration and the rate decreases at least somewhat beyond that concentration.

The above data indicate that annexin I binds to specific granules, mediates aggregation of these granules and fusion of at least some fraction of the granules with other appropriately fusogenic membranes. If annexin I plays a role in degranulation of specific granules, it must minimally bring together plasma membranes and specific granules in a Ca^{2+}-dependent manner. This was tested by indirectly monitoring the coaggregation of plasma membranes and isolated specific

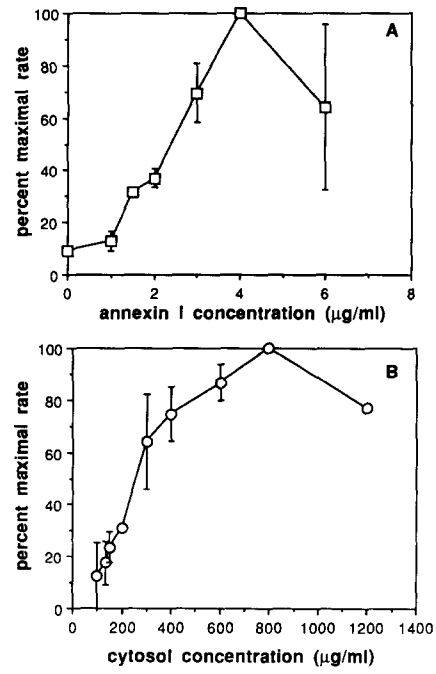

Fig. 6. Protein concentration dependence for cytosol and annexin I-mediated fusion of specific granules with PS/PE vesicles. Samples contained PS/PE (1/3) vesicles with 0.75 mol% each of NBD-PE and Rh-PE (3 μM total phospholipid), specific granules (300 μg/ml protein) and the indicated amount of annexin I (A) or cytosol protein (B). For comparision, rates were normalized to the maximal observed rate in each set of data (approx. 2.15% maximal/min for A and 1.66% maximal/min for B). Fusion was initiated with 1 mM Ca^{2+}.

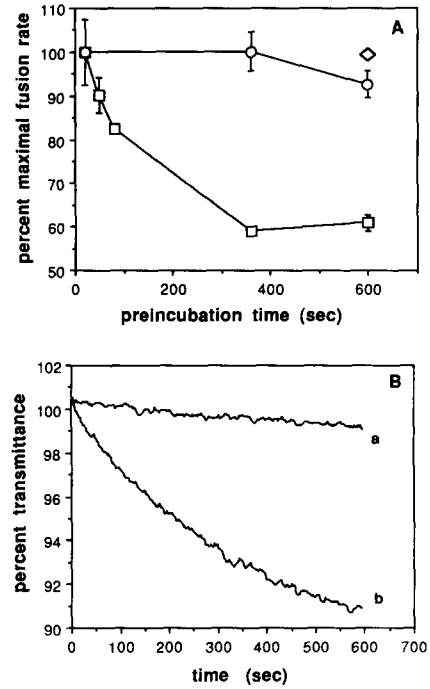

Fig. 7. Competition between specific granules and PS/PE vesicles for cytosol-mediated binding to plasma membrane vesicles. In plot A, octadecylrhodamine-labeled plasma membrane vesicles were preincubated for the indicated amounts of time with 1 mM Ca^{2+} and 200 μg/ml cytosol protein alone (circles), 50 μg/ml specific granule protein alone (diamonds) or 50 μg/ml specific granule protein plus 200 μg/ml cytosol protein (squares). 3 μM PS/PE (1:3) vesicles labeled with 0.75 mol% NBD-PE with (diamonds) or without (squares and circles) 200 μg/ml cytosol were then added at the time indicated and the rate of fusion measured by the percentage decrease in fluorescence intensity that occurred in 540 seconds. The fusion rates were normalized for each set of data by taking the rate after only 20 seconds of preincubation as the maximal fusion rate (squares, 2.83% intensity decrease/min; circles, 3.72%/min; diamonds, 2.83%/min). In plot B, the aggregation of vesicles was monitored by the change in percent transmittance at 450 nm as a function of time. Samples contained octadecylrhodamine labeled plasma membrane vesicles (50 μg/ml total protein), 200 μg/ml cytosol protein and either no other additions (a) or 50 μg/ml specific granules (b). Aggregation was initiated by addition of 1 mM Ca^{2+} at time zero.

granules. Octadecylrhodamine labeled plasma membranes were mixed with cytosol and Ca^{2+}. When NBD-PE labeled PS/PE vesicles were added to this mixture, a decrease in NBD fluorescence was observed, indicating fusion between the two types of membranes resulting in energy transfer from NBD to rhodamine. If unlabeled specific granules were present before addition of NBD-PE labeled PS/PE vesicles, the rate of fusion was initially decreased (see Fig. 7 caption), as would be expected within the first 30 seconds of preincubation due to competition from the specific granules. A much slower decrease in the fusion rate (Fig. 7A), dependent on the time of preincubation of granules and plasma membranes (with cytosol and Ca^{2+}) was also observed. The time-course of this decrease followed the time course of aggregation of specific granules and plasma membranes (Fig. 7B) under these conditions. The most likely explanantion for this phenomenon was that some sites for fusion of PS/PE vesicles with the plasma membrane vesicles were blocked by coaggregation of plasma membrane vesicles with specific granules during preincubation. Coaggregation was depedent on the presence of cytosol, as preincubation of the granules and plasma membrane

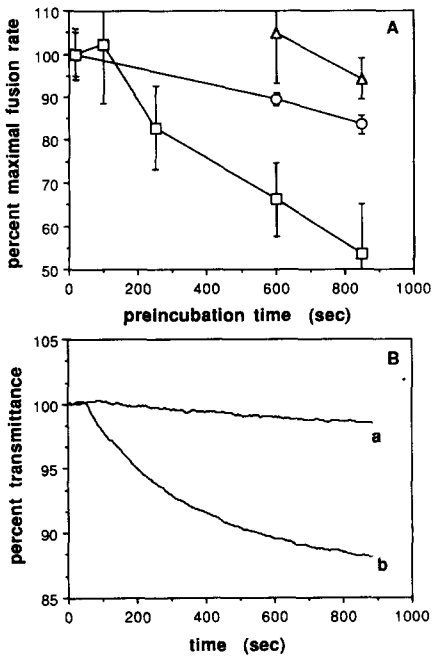

Fig. 8. Competition between specific granules and PS/PE vesicles for annexin I-mediated binding to plasma membrane vesicles. In plot A, octadecylrhodamine-labeled plasma membrane vesicles were preincubated as in Fig. 7 (triangles = diamonds) except with 3 μg/ml annexin I instead of cytosol. Data were normalized to the maximal rate for each set after only 20 seconds of preincubation (squares, 1.62% intensity decrease/min; circles, 2.12%/min; triangles, 1.62%/min). In plot B, the aggregation of vesicles was monitored as in Fig. 7 except that 3 μg/ml of annexin I was used instead of cytosol. Aggregation was initiated by addition of 1 mM Ca^{2+} at time zero.

vesicles with Ca^{2+} alone did not inhibit subsequent fusion with PS/PE vesicles (Fig. 7A). Preincubation of granules and plasma membranes did not lead to a decrease in fusion rate, showing that the specific granules preparation does not contain a factor that decreases the fusion rate in a slow time-dependent manner.

The direct action of annexin I was tested in the same assay in Fig. 8. As in Fig. 7, preincubation with specific granules and annexin I decreased the rate of fusion. A small decrease in fusion rate was also observed in this case when specific granules were omitted, but some decrease would be expected if the plasma membrane vesicle preparation preaggregates before introduction of the NBD-bearing PS/PE vesicles. As above, omission of annexin I in the preincubation precluded the time dependent inhibition of fusion rate. Therefore, it is likely that annexin I can mediate contact of specific granules and plasma membranes in a Ca^{2+}-dependent manner.

Discussion

If annexin I plays a role as a membrane crosslinker or vesicle aggregator in human neutrophil degranulation of specific granules, it obviously must interact with specific granules during the process. It had been previously shown that annexin VII (synexin) can mediate Ca^{2+}-dependent self-aggregation of specific granules and fusion with phospholipid vesicles (at Ca^{2+} concentrations as low as 120 μM; Ref. 9). By use of an anti-annexin I monoclonal antibody, it was later shown that annexin I is the major cytosolic mediator of Ca^{2+}-dependent vesicular aggregation (but not fusion per se), and, by this mechanism, mediates fusion of plasma membrane vesicles at Ca^{2+} concentrations as low as 5–10 μM [13]. Recently, similar results with monoclonal antibodies were shown for annexin I-mediated liposome-liposome fusion and self-aggregation of specific granules [14]. Here, we have presented data on the Ca^{2+} and annexin I concentration dependence of specific granule self-aggregation as well as of annexin I-mediated fusion of granules and their coaggregation with plasma membrane vesicles.

The differences in Ca^{2+} dependences are particularly interesting. The Ca^{2+} concentrations required for aggregation and fusion of specific granules are significantly higher than those shown to be required for fusion of PS/PE vesicles with plasma membranes [13,16,36], suggesting that the Ca^{2+} dependence may be dictated by the membrane involved, rather than the annexin-Ca^{2+} binding sites, at least in these cases. Differences in the effective Ca^{2+} concentration at various membrane surfaces due to electrostatic effects could be one reason for these observations. As a result, the specificity of annexin I binding and action may be

regulated by the precise level of Ca^{2+} in the cell and which particular membrane is in the vicinity. The average Ca^{2+} concentration in intact neutrophils has not been observed to reach levels much higher than 1 μM (e.g., Refs. 2,37,38,39). However, the Ca^{2+} dependence for fusion of isolated granules (this study), or isolated plasma membrane vesicles [13], with liposomes is not far from that observed for exocytosis from permeabilized human neutrophils in response to Ca^{2+} alone, as measured by release of vitamin B-12 binding protein (e.g., Ref. 4). There is evidence in human neutrophils [40] and in other secretory cells [41–45] that locally and transiently high Ca^{2+} concentrations, as high as 1 mM, occur upon stimulation and are especially found near the sites of Ca^{2+} influx into the cytosol. The location and timing of these influxes, if they exist in human neutrophils, could be a means of regulating the spatial and temporal specificity of degranulation in conjunction with a protein mediator that has a Ca^{2+} dependence higher than the μM range, such as annexin I. In permeabilized neutrophils, the control of local Ca^{2+} increases may be lost so that higher overall Ca^{2+} concentrations are required.

Previous data suggested that annexins act mainly as enhancers of vesicular aggregation, rather than directly mediating the actual fusion of vesicles [10]. Another co-factor, free fatty acids, may modulate membrane fusion [46] and appears specifically necessary for specific granule fusion involving annexin VII (synexin) [9]. In the system studied here, though some effects of fatty acids were observed in preliminary experiments, especially at short time scales, fatty acids were not found to be required for annexin I-mediated fusion. Several differences in experimental conditions could account for this finding, including a different member of the annexin family, different liposomal lipid composition, different ratios of fusing vesicles (based on an improved figure for specific granule phospholipid content; see Materials and Methods), a somewhat different method of specific granule isolation and the possibility of some free fatty acid content in the preparations. Based on our data, it is reasonable to suggest that some other lipid or protein factor, besides fatty acids, directly mediates the fusion process per se.

The fact that the Ca^{2+} dependence is influenced strongly by the type of membrane involved also suggests that the expression of proteins and lipids on the surface of the granules and plasma membrane might play an important role in degranulation. Previous experiments showed inhibition of fusion by trypsin treatment of plasma membrane vesicles, suggesting that intact integral plasma membrane proteins are required for the membrane fusion process with, or without, annexin I [13,16]. In the case of granules, maturation to a fusogenic form may involve changes in surface expression. The existence of specific granule subpopulations, apparently heterogeneous in terms of density and Ca^{2+} sensitivity [47–49], supports this idea. It is also consistent with the fact that a large excess of granule lipid (Fig. 4) to liposomal lipid is necessary to observe significant fusion, suggesting that a minority of the granule population is competent for fusion. Experiments with trypsin indicate that proteolysis of the surface of the granule membrane enhances fusion. Therefore maturation may involve exposure of lipids in the membrane and/or an important proteolytically activated fusogenic protein in the membrane. These results point to the probability that annexin I may be only one component of a multipolypeptide membrane fusion system.

Acknowledgements

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