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Annexin-mediated membrane fusion of human neutrophil plasma membranes and phospholipid vesicles

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Membrane fusion was studied using human neutrophil plasma membrane preparations and phospholipid vesicles approximately 0.15 μm in diameter and composed of phosphatidylserine and phosphatidylethanolamine in a ratio of 1 to 3. Liposomes were labeled with *N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl (NBD) and lissamine rhodamine B derivatives of phospholipids. Apparent fusion was detected as an increase in fluorescence of the resonance energy transfer donor, NBD, after dilution of the probes into unlabeled membranes. 0.5 mM Ca^{2+} alone was sufficient to cause substantial fusion of liposomes with a plasma membrane preparation but not with other liposomes. Both annexin I and des(1–9)annexin I caused a substantial increase in the rate of fusion under these conditions while annexin V inhibited fusion. Fusion mediated by des(1–9)annexin I was observed at Ca^{2+} concentrations as low as approximately 5 μM, suggesting that the truncated form of this protein may be active at physiologically low Ca^{2+} concentrations. Trypsin treated plasma membranes were incapable of fusion with liposomes, suggesting that plasma membrane proteins may mediate fusion. Liposomes did not fuse with whole cells at any Ca^{2+} concentration, indicating that the cytoplasmic side of the membrane is involved. These results suggest that annexin I and unidentified plasma membrane proteins may play a role in Ca^{2+}-dependent degranulation of human neutrophils.

Introduction

Degranulation of human neutrophils plays an important role in the inflammatory response to infection [1]. When neutrophils engulf foreign particles by phagocytosis, they degranulate by fusion of cytoplasmic granules with the phagosome or plasma membrane [2–6]. Soluble stimuli, such as concanavalin A, phorbol myristate acetate, and externally added calcium in the presence or absence of A23187 [7–10], can also cause degranulation of neutrophils. Intracellular mechanisms of degranulation may include an increase in cytosolic free Ca^{2+} [9,11,12]. There is evidence that the highest local concentration of Ca^{2+} following phagocytosis by human neutrophils is in the periphagosomal region [13], where it may participate in phagosome-granule fusion. Thus, intracellular Ca^{2+} may interact with proteins and/or lipids at the surface of granule, phagosome or plasma membranes to induce fusion in neutrophils.

Proteins in the annexin class have been implicated in intracellular membrane fusion [14]. Annexin VII (synexin) [15], which binds to and aggregates chromaffin granules at greater than approximately 4–6 μM Ca^{2+} [15,16], mediates apparent fusion of the aggregated granules when certain free fatty acids are added [17], or at low pH when chromaffin granules have been taken through several freeze-thaw cycles [18]. Annexin VII also accelerates the rate of Ca^{2+}-induced aggregation of liposomes [19] leading to increased overall fusion rates at as little as 10 μM Ca^{2+} [20], while other Ca^{2+}-binding proteins have no effect or are inhibitory [21,22]. Free fatty acids also assist the fusion of synexin-aggregated liposomes [23]. Another member of the annexin family, annexin II (calpactin), mediates

Abbreviations: PS, phosphatidylserine from bovine brain; PE, phosphatidylethanolamine (transesterified from egg phosphatidylcholine); NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, lissamine rhodamine B-PE; DFP, diisopropylfluorophosphate; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino-ethanesulfonic acid; Hepes, 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid.

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fusion of chromaffin granules [24] and secretion by permeabilized chromaffin cells [25] at micromolar Ca^{2+}. Recently, annexin I (lipocortin I) has been shown to promote Ca^{2+}-dependent fusion of liposomes [26]. Annexins or synexin-like proteins have been previously demonstated in the human neutrophil system [27–29], where *in vitro* assays suggest that they may promote Ca^{2+}-dependent fusion of neutrophil membranes [28].

We have studied the role of the plasma membrane in degranulation of human neutrophils by monitoring fusion with phospholipid vesicles of similar diameter to neutrophil specific granules. These data show possible roles for annexins as well as proteins of the neutrophil plasma membrane in Ca^{2+}-dependent, fatty acid-independent membrane fusion.

Materials and Methods

Bovine brain phosphatidylserine (PS), phosphatidylethanolamine (PE) (transesterified from egg phosphatidylcholine), *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). KCl, NaCl, $CaCl_2$, Hepes, and *N*-ethylmaleimide were purchased from Fisher Scientific (Medford, MA). DFP, Percoll, Tes, trypsin (from bovine pancreas, type XIII), and soybean trypsin inhibitor were obtained from Sigma (St. Louis, MO). Chelex 100 was purchased from Bio-Rad (Richmond, CA) and polycarbonate filters were from Nucleopore (Pleasanton, CA). All reagents were of 97% purity or greater.

Immunofluorescence of whole neutrophils was performed using rabbit antisera against human annexin I and V generously supplied by Dr. R. Blake Pepinsky. Briefly, whole neutrophils were suspended in phosphate buffered saline at $5 \cdot 10^6$ cells/ml, placed on a glass slide and fixed in 4% paraformaldehyde for 1 h followed by washes of 0.2% Tween 20 in buffer and buffer alone. Cells were then treated with methanol to aid permeabilization followed by incubation with the primary antiserum overnight. After washes with Tween 20 and buffer, the sample was incubated 1 h at 37 °C with fluoresceinated goat anti-rabbit anbtibody and washed again.

Neutrophil plasma membranes were isolated according to the procedure described by Borregaard et al. [30], with the following modifications: The nitrogen bomb cavitate was collected over Chelex beads. The beads were removed by centrifugation at $500 \times g$ for 10 min at 4 °C before layering onto discontinuous Percoll gradients as previously described. The gradients were spun at 4°C for 20 min at $30\,000 \times g$. The highest, or least dense, of the three distinct bands contained the plasma membranes (designated as the γ fraction). After addition of Percoll, the γ fractions were spun at $150\,000 \times g$ for 45 min. The sedimentable biologic material, layered on a hard packed Percoll pellet, was then resuspended in 1 ml of 130 mM KCl, 5 mM NaCl, and 10 mM Hepes, pH 7.0 (buffer A) or in 100 mM KCl, 50 mM Hepes, 1 mM EGTA, pH 7.0 (buffer B). Enzyme marker assays [30] show that this preparation is at least 95% free of azurophil or specific granules or nuclei. Markers for mitochondria were too low to measure.

Fusion of plasma membranes with liposomes was measured using an assay of lipid mixing. Large unilamellar vesicles were prepared by the reverse-phase evaporation method [31]. To obtain a uniform size distribution, vesicles were extruded successively through polycarbonate filters with pore sizes of 0.2 and 0.1 μm [32], giving an average diameter of approx. 0.15 μm, similar to the diameter of specific granules (see Ref. 30). The aqueous buffer was either buffer A or B.

In these assays, a fluorescent lipid probe was used to monitor fusion, under conditions where fluorescence was quenched by energy transfer to another probe. Since the fluorescence of the energy transfer donor is inversely proportional to the concentration of the energy transfer acceptor, dilution of the acceptor results in more fluorescence of the donor. When both donor and acceptor are incorporated into the same liposome, dilution of the probes into an unlabeled membrane due to fusion causes an increase in donor fluorescence. In the system we employed [48], the energy transfer donor, NBD-PE , and the acceptor Rh-PE, were incorporated into liposome membranes in equimolar quantities (each at 0.75 mol% of the phospholipid) by inclusion of the probes in the lipid mixture during liposome preparation in buffer A. To assay fusion, 3 μM liposomal phospholipid was mixed with 50 μg/ml plasma membrane protein, or with 24 μM unlabeled PS/PE liposomes, in 2 ml final volume of buffer A. Fusion, as elicited by Ca^{2+}, was then monitored as the increase in fluorescence of NBD-PE at an excitation wavelength of 450 nm and emission of 530 nm. The maximum fluorescence (F_{max}) was set by adding Triton X-100 (0.1% w/v) to dissolve the membranes into micelles which effectively abolished energy transfer between the two probes, but also quenched the fluorescence somewhat. The normalized fluorescence intensity for the same probes diluted into PS/PE vesicles by the estimated amount of total lipid in the assay systems is approximately 30% higher than the fluorescence in the presence of Triton X-100.

When annexins were used, they were added to the mixture of liposomes and plasma membranes in 2 ml buffer A or B and incubated for 5 min prior to the addition of Ca^{2+}. Annexin I (lipocortin I), des(1–9)annexin I, and annexin V (lipocortin V) were kindly provided by Dr. R. Blake Pepinsky of Biogen Corporation (Cambridge, MA) and were of greater than 99%

purity as determined by one dimensional electrophoresis.

The phospholipid concentrations for all liposomes were determined using a phosphate assay [33]. The plasma membranes were assayed for phosphate concentration after being subjected to an extraction procedure [34], and for protein concentration using the bicinchoninic acid method [35]. The amount of protein used (100 μg, in a final volume of 2 ml) for fusion assays was estimated to be 24 μM phospholipid.

Ca^{2+} activities were estimated by use of a Ca^{2+} electrode (Orion). Activities were the same as the nominal Ca^{2+} concentration above approximately 10 μM. Free Ca^{2+} in Fig. 2 was estimated by the method of Bers [47] for comparison with previously reported data.

All experiments, except for those in Fig. 2, were performed at 25°C using a Perkin-Elmer model LS-5 fluorometer. All data shown are representative of two or more experiments. Variation of fluorescence at any given time point was within $\pm$10%. Data in Fig. 2 were obtained using an SLM 8000 C fluorometer.

Trypsinization. Aliquots of plasma membranes were treated with trypsin. Trypsin was prepared at a stock concentration of 400 μg/ml (4800 units of *N*-α-benzoyl-L-arginine ethyl ester activity per ml) and plasma membranes were incubated at 25°C in a final concentration of either 100 μg/ml or 10 μg/ml as specified in the text. Samples were removed at various times and added to 2 ml solution of buffer A with both labeled liposomes and soybean trypsin inhibitor. The final concentration of trypsin was approx. 2 μg/ml, and the concentration of trypsin inhibitor in vast excess, 40 μg/ml final concentration.

Results

The presence and distribution of annexins in human neutrophils was first studied by immunofluorescence. When fixed human neutrophils were treated with rabbit antiserum to annexin I (lipocortin I) and a second fluoresceinated anti-rabbit antibody, a diffuse cytoplasmic fluorescence was observed (data not shown), indicating the expected cytoplasmic localization. A peripheral ring of fluorescence was also observed, suggesting a plasma membrane bound form of annexin I in neutrophils as previously observed in other cells [36,37]. In contrast, treatment of fixed cells with annexin V antiserum and the second antibody yielded no observable fluorescence over background.

In order to elucidate the role of annexins in neutrophils, we investigated the effects of various annexins on fusion between human neutrophil plasma membranes and phospholipid vesicles as models of specific granule membranes. The vesicles were composed of PS/PE (1:3, molar ratio) labeled with the fluorescent membrane probes NBD-PE and Rh-PE. The increase in fluorescence of NBD-PE, indicating decrease of energy transfer between the fluorescent probes as a consequence of lipid dilution, served as an indicator of fusion between labeled liposomes and unlabeled plasma membranes. No increase in fluorescence (i.e. fusion) was observed prior to the addition of Ca^{2+}. Fusion was observed after the addition of 0.5 mM calcium (Fig. 1). The extent of fusion after 14 min in this setting was approx. 10% of the maximal fluorescence (as defined in Methods). Addtion of EDTA in excess of the Ca^{2+} did not reverse the increase in fluorescence (data not shown). The inclusion of annexin I (6 μg/ml) significantly enhanced the initial rate of fusion, such that 10% maximal fluorescence was reached after only 4 min, and the overall extent of fusion after 20 min was increased by approximately 2-fold. A preparation of annexin I lacking amino acids 1–9 of the N-terminus, des(1–9)-annexin I, produced an even greater enhancement of fusion at a lower concentration, with a greater than 4-fold increase in the extent of fusion after 15 min. Annexin V, however, completely inhibited fusion (Fig. 1). N-terminal cleaved versions of annexin I have been shown to have a higher Ca^{2+} sensitivity for membrane binding than the uncleaved versions [44,45]. In order to investigate the Ca^{2+} dependence of this phenomenon, we carried out the same lipid dilution assays in a Ca^{2+} buffered system (Fig. 2). Significant rates of fusion were observed at Ca^{2+} concentrations as low as the 5 μM range.

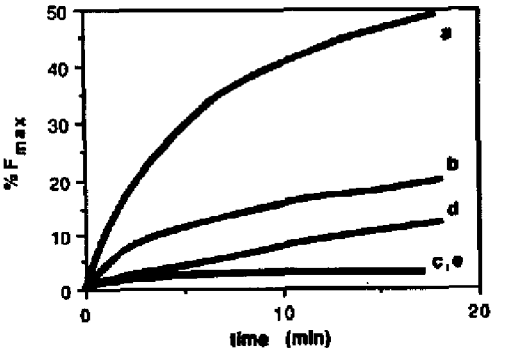

Fig. 1. Effects of annexins on fusion of neutrophil plasma membranes and PS/PE liposomes at high Ca^{2+} concentrations. Liposomes composed of PS/PE (1:3) (3 μM phospholipid) and labeled with NBD-PE and Rh-PE were mixed with neutrophil plasma membranes (50 μg protein/ml) and were preincubated for 5 min with 4 μg/ml des(1–9)annexin I (a), 6 μg/ml annexin I (b), 5 μg/ml annexin V (c), or no added protein (d). Liposomes composed of PS/PE (1:3) (3 μM phospholipid) and labeled with NBD-PE and Rh-PE were mixed with liposomes composed of PS/PE (1:3) (24 μM phospholipid) (e). 0.5 mM Ca^{2+} was added at time 0 to initiate fusion. Fusion in all cases is expressed as the percentage of the maximal fluorescence obtained as described in Methods. All experiments were performed in buffer A at 25°C at a final volume of 2 ml as detailed in Methods.

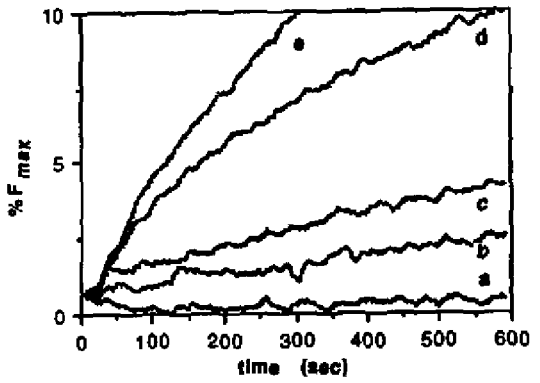

Fig. 2. Ca^{2+} concentration dependence of fusion mediated by des(1–9)-annexin I. Liposomes composed of PS/PE (1:3) (3 μM phospholipid) and labeled with NBD-PE and Rh-PE were mixed with neutrophil plasma membranes (100 μg protein/ml) and 10 μg/ml des(1–9)annexin I. Fusion was initiated at 30 s with 0 (a), 2 (b), 6 (c), 25 (d) or 150 (e) μM free Ca^{2+} measured as described in Methods. Fusion is expressed as the percentage of the maximal fluorescence obtained by the addition of 0.1% Triton-X 100. All experiments were performed in buffer B at 25°C in a final volume of 1.5 ml as detailed in Methods.

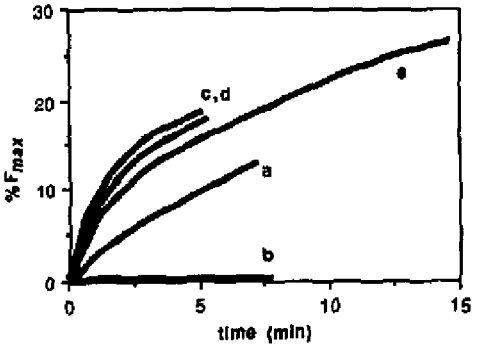

Fig. 3. Effect of pretreatment of neutrophil plasma membranes with trypsin on fusion of membranes with liposomes composed of PS/PE (1:3). A suspension of plasma membranes (3.9 mg/ml) was incubated with 100 μg/ml trypsin for 1 min (a), or 12 min (b); or with 10 μg/ml trypsin for 1 min (c), or 12 min (d); or with no addition (e). At the time intervals noted, aliquots of each suspension estimated to contain 106 μg membrane protein were removed and added to liposomes composed of PS/PE (1:3) (3 μM phospholipid) and labeled with NBD-PE and Rh-PE in 2 ml of buffer A containing 40 μg/ml soybean trypsin inhibitor. In (e) the membranes were added to the labeled liposomes (as above) in buffer A without soybean trypsin inhibitor. 1 mM Ca^{2+} was added to initiate the reactions in each case. Fusion is expressed as the percentage of the maximal fluorescence obtained as in Methods. All incubations and assays were performed at 25°C, with a final assay volume of 2 ml as detailed in Methods.

The data in Fig. 1 also showed that fusion between plasma membranes and liposomes occurred more readily, i.e. at lower Ca^{2+} concentrations, than fusion between liposomes alone (Fig. 1 d,e). This finding suggested that factors in the neutrophil plasma membrane were responsible for the lower Ca^{2+} concentration dependence. This possibility was investigated by treating the plasma membranes with trypsin for varying periods (Fig. 3). The treated membranes were then added to a buffer containing soybean trypsin inhibitor to stop the reaction.

In Fig. 3, assays were performed as before by monitoring the increase in fluorescence on mixing labeled liposomes with trypsin-treated neutrophil membranes. The reactions were initiated with the addition of 1 mM Ca^{2+}. For purposes of comparison, a control assay containing labeled liposomes and unlabeled neutrophil membranes, but neither trypsin nor trypsin inhibitor, is shown. The extent of fusion was 25% at 10 min. When the membranes were incubated with trypsin at a concentration of 100 μg/ml, the amount of fluorescence was decreased by 50% after only 1 min of incubation and was completely inhibited after 12 min. Trypsin inhibitor itself had no effect on the rate of fusion. When 24 μM unlabeled PS/PE liposomes were substituted for neutrophil membranes, trypsin had no effect on fusion (data not shown). This suggests that the target or targets of these protein-modifying agents were localized to the neutrophil plasma membranes and were indeed proteins. It is possible that products of trypsinolysis inhibit fusion. However, these products are probably not abundant as no change can be detected in the pattern of a one dimensional polyacrylamide gel of the the plasma membrane preparations before or after trypsin treatment under the conditions described (not shown).

As previously observed [39], no fusion with intact

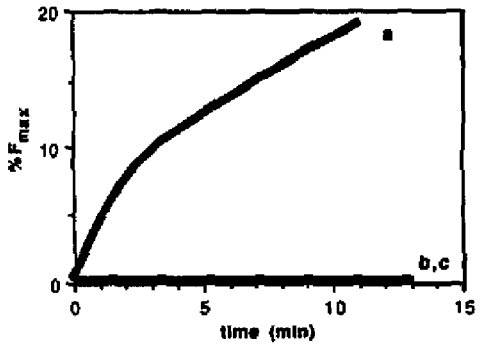

Fig. 4. Characterization of fusion between neutrophil plasma membranes and PS/PE liposomes. NBD-PE and Rh-PE labeled liposomes (3 μM phospholipid) were mixed with neutrophil plasma membranes (a) (50 μg protein/ml in the final volume of 2 ml) or whole neutrophils, at concentrations of $1.3 \cdot 10^6$ cells in 2 ml final volume (b) or $4.2 \cdot 10^6$ in 2 ml final volume (c). 1 mM Ca^{2+} was added at time 0 to initiate the reaction. Fusion is expressed as the percentage of the maximal fluorescence ($\%F_{max}$) obtained on the addition of 0.1% Triton-X 100. All experiments were performed in buffer A at 25°C at a final volume of 2 ml as detailed in Methods.

neutrophils was induced by Ca^{2+} (Fig. 4), suggesting that the cytoplasmic side of the membrane is involved in the observed fusions.

Discussion

In order to model degranulation in human neutrophils with a system that is more physiological than liposome-liposome fusion, yet well defined enough to identify individual factors involved in the process, we have monitored the Ca^{2+}-dependent fusion of liposomes with plasma membrane vesicles from human neutrophils as a model of specific granule-plasma membrane fusion. In this system, we were able to demonstrate apparent fusion between the plasma membranes and PS/PE vesicles. The fusion event was not spontaneous, but was initiated by des(1–9)annexin I and Ca^{2+} concentrations as low a few micromolar, in keeping with the rise of intracellular free Ca^{2+} to submillimolar levels elicited by stimuli of neutrophil degranulation [38]. A previous study [39] on the fusion of small sonicated liposomes with a neutrophil plasma membrane preparation suggested a micromolar Ca^{2+} dependence in the absence of annexins. More Ca^{2+} is required in our system, perhaps because the vesicles used in our study have an average diameter of 0.15 μm and are not as susceptible to fusion due to high membrane curvature as are small sonicated liposomes (see, for example Refs. 40 and 41). While the concentrations of Ca^{2+} required were higher than peak concentrations (averaged over the whole cytosol) measured in intact neutrophils [38], Ca^{2+} concentrations at sites of fusion may be significantly higher than bulk cytosolic levels [13]. The Ca^{2+} threshold may also be lowered by co-mediators not present in this highly purified model.

Annexin I enhanced the rate and extent of fusion between neutrophil plasma membranes and PS/PE liposomes. It was also enhanced by des(1–9)annexin I, a preparation of annexin I in which the first nine amino acids of the N-terminus are absent. Interestingly, annexin V, which is highly homologous to annexin I [42,43], inhibited fusion in this system. Thus it seems that although the annexins share certain properties and structural characteristics, their functional roles may be specialized. Other annexins that have been implicated in promoting membrane fusion are annexin VII [17,21], II [24,25] and I [26]. While annexin VII (synexin) promotes aggregation of isolated specific granules from neutrophils and increases the overall rate of Ca^{2+}-induced fusion of phosphatidate(PA)/PE liposomes with specific granules, it requires fatty acids and apparently more free Ca^{2+} [28] than des(1–9)annexin I requires in this system. Calpactin (or annexin II) has also been shown to mediate Ca^{2+}-dependent fusion in the chromaffin cell system at micromolar Ca^{2+} concentrations, but may require low pH in the cell free system [24,25].

The data showing the Ca^{2+} dependence of fusion mediated by des(1–9)annexin I (Fig. 2) are particularly interesting. Des(1–12)annexin I and des(1–26)annexin I have been shown to have a heightened Ca^{2+} sensitivity for membrane binding [44]. There is evidence for production of truncated forms of annexin I by an endogenous proteinase in some cells [45], which makes our findings of particular relevance, suggesting that the truncated form of annexin I is more likely to be active at physiological Ca^{2+} levels.

In addition to elucidating a potential role for cytosolic annexins, we have also provided evidence that neutrophil plasma membrane-associated proteins may mediate Ca^{2+}-dependent fusion. Our results suggest that a trypsin-sensitive factor (or factors) in the neutrophil plasma membrane could be essential for Ca^{2+}-dependent fusion, assuming that products of trypsinolysis do not inhibit fusion. The identity of this factor is as yet unknown, but it could be related to a 51 kDa protein isolated from plasma membranes of chromaffin cells on the basis of its binding to chromaffin granules [46]. Since our immunofluorescence results suggest a plasma membrane localization for some annexin I, as has been demonstrated in other cells [36,37], it is also tempting to speculate that at least one of the trypsin sensitive factors may be the membrane-bound form of annexin I. Although a wide range of membrane proteins may be affected by trypsin, our findings do serve to underscore the possible role of membrane proteins as mediators of fusion, and to suggest avenues of future research.

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