Development of an aqueous-space mixing assay for fusion of granules and plasma membranes from human neutrophils

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Several models have been developed to study neutrophil degranulation. At the most basic level, phospholipid vesicles have been used to investigate the lipid interactions occurring during membrane fusion. The two major forms of assays used to measure phospholipid vesicle fusion are based either on the dilution of tagged phospholipids within the membrane of the two fusing partners or the mixing of the aqueous contents of the vesicles. Although problems exist with both methods, the latter is considered to be more accurate and representative of true fusion. Using 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) as a fluorescent marker, we have taken advantage of the quenching properties of *p*-xylenebispyridinium bromide ('DPX') to develop a simple aqueous-space mixing assay that can be used with any

sealed vesicle. We compared our new assay with more conventional assays using liposomes composed of phosphatidic acid (PA) and phosphatidylethanolamine (PE), obtaining comparable results with respect to Ca^{2+} -dependent fusion. We extended our studies to measure the fusion of neutrophil plasma-membrane vesicles as well as azurophil and specific granules with PA/PE (1:3) liposomes. Both specific granules and plasma-membrane vesicles fused with PA/PE liposomes at $[\text{Ca}^{2+}]$ as low as $500\ \mu\text{M}$, while azurophil granules showed no fusion at $[\text{Ca}^{2+}]$ as high as 12 mM. These differences in the ability of Ca^{2+} to induce fusion may be related to differences observed in whole cells with respect to secretion.

INTRODUCTION

Central to neutrophil function is degranulation, a process whereby specific and azurophil granules fuse with the plasma membrane, releasing their contents into the extracellular or periphagosomal space. Although several cellular events are temporally linked with degranulation, their relationship and significance to degranulation, especially with respect to the actual fusion of membranes, has never been established. The rise in intracellular Ca^{2+} following the metabolism of phosphatidylinositol 4,5-bisphosphate by phospholipase C is thought to play an integral role in membrane fusion [1–7]. In fact, liposomes can be induced to fuse by the presence of Ca^{2+} alone. However, high millimolar $[\text{Ca}^{2+}]$ are required, depending on the phospholipid composition of the vesicles [8]. Current attempts to model neutrophil degranulation using liposomes have employed a variety of factors, including phospholipid composition [8], non-esterified fatty acids [9,10], ionic strength [11], pH [12,13] and external proteins [14–18], all designed to lower Ca^{2+} requirements into the micromolar range.

Although liposomes serve as good initial models for the study of degranulation, they are far less complex than neutrophil granules. Thus any information generated using liposomes must be extended through the use of biological membranes. Unfortunately, few fusion systems currently lend themselves for use with azurophil and specific granules. In particular, none of the existing assays are capable of measuring the mixing of the internal contents of granules with their fusion partners.

The most widely used fusion systems either take advantage of the dilution of a fluorescent labelled phospholipid [11,19] or fatty acid [20] within the membrane of the liposome or the mixing of

a fluorochrome and a quencher within the combined aqueous space formed by the fusion of two liposomes [8,11,21]. The latter method is considered to be a better representation of true fusion because, when properly established, a signal is generated only when both the outer and inner leaflets of two vesicles fuse and their contents mix together. Assays that measure the dilution of a lipid or fatty acid within the membrane of a vesicle will generate a fusion signal when either the outer or the inner leaflet of the membranes fuse. 'Hemifusion', or outer-leaflet fusion is incomplete, since the inner leaflets do not necessarily mix [22]. Under these circumstances, the contents of the two vesicles do not combine and true fusion, as in degranulation, has not occurred. However, such circumstances can often generate spurious fusion 'signals' with lipid-mixing assays. Currently, there are no existing models that take advantage of granular contents as a means of measuring fusion of internal spaces.

For the purpose of studying the parameters that influence the fusion of granules with the plasma membrane we have developed a universal contents-mixing assay that can be used in conjunction with almost any other sealed vesicle. Because both the quencher and the fluorochrome are in a single liposome, its fusion partner serves to dilute or dequench the quenched complex, permitting any intact vesicle to be employed as a target. By exploiting the quenching properties of *p*-xylenebispyridinium bromide (DPX) on 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) fluorescence, our assay can be calibrated to estimate the average number of fusion events which take place. Its sensitivity can be adjusted to distinguish high versus low levels of fusion, yielding comparable results with existing assays. We used this assay to measure fusion of azurophil and specific granules as well as plasma-membrane vesicles with liposomes. Using this model, we

Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; DPX, *p*-xylenebispyridinium bromide; PA, phosphatidic acid; PE, phosphatidylethanolamine; LUV, large unilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE; Rh-PE, *N*-(lissamine rhodamine B sulphonyl)-PE; DFP, di-isopropyl fluorophosphate.

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verified that azurophil granules were extremely resistant to fusion, requiring more than 12 mM Ca^{2+} , while plasma membrane vesicles and specific granules fused at approx. 500 μM Ca^{2+} .

MATERIALS AND METHODS

Chemicals

Egg phosphatidic acid (PA) and phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids, Birmingham, AL, U.S.A. CaCl_2 hydrate (> 99.99 % pure) was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. EGTA (puriss grade, > 99 %) was obtained from Fluka Chemical Co., Ronkonkoma, NY, U.S.A. ANTS and *p*-DPX were obtained from Molecular Probes, Inc., Eugene, OR, U.S.A. HPLC-grade water was obtained from Fisher Scientific, Pittsburgh, PA, U.S.A. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Ca^{2+} buffers

A stock solution of Ca^{2+} was prepared in buffer A (50 mM Hepes/100 mM KCl/1 mM EGTA, pH 7.0) such that dilutions would give a range of final free Ca^{2+} concentrations as predicted using the system of Bers [23]. Ca^{2+} concentrations of serial dilutions ranged from 100 nM to 14 mM and were measured with a Ca^{2+} -sensitive electrode (Orion Research, Inc., Boston, MA, U.S.A.).

Preparation of liposomes containing both ANTS and DPX

Liposomes composed of PA/PE (1:3) were prepared as previously described [15] with a minor modification. Phospholipids redissolved in diethyl ether were emulsified in buffer containing 10 mM Hepes, 55 mM ANTS and 50 mM DPX. Alternatively, 50 mM DPX could be replaced by 20 mM DPX and 30 mM KCl to increase sensitivity of the assay. Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation. Following evaporation, liposomes were extruded twice through a 0.1 μm -pore-size filter to ensure uniform size, and the unincorporated ANTS and DPX were removed on a Sephadex G-25 column equilibrated with buffer A.

Fusion measured by lipid dilution

Fusion measured by lipid dilution was performed as previously described [15]. Briefly, LUV (PA/PE, 1:3) labelled with 1 % *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE (NBD-PE) and 1 % *N*-(lissamine rhodamine B sulphonyl)-PE (Rh-PE) were formed in buffer A by reverse-phase evaporation. At these concentrations of NBD-PE and Rh-PE the distance between NBD and Rh was sufficiently close that Rh quenched NBD fluorescence. Labelled and unlabelled LUV (1:9; 180 μg total phospholipid) were combined in a cuvette (final volume 1 ml) and fusion was initiated by the addition of increasing $[\text{Ca}^{2+}]$. Fusion between a labelled and an unlabelled LUV resulted in an increase in the distance between NBD and Rh and a decrease in quenching. The resulting increase in NBD fluorescence was detected by an SLM 8000 spectrofluorimeter (excitation at 450 nm, emission at 530 nm).

Fusion measured by aqueous space mixing

Aqueous-space mixing during fusion was measured by taking advantage of fluorescence properties of ANTS and the quenching properties of DPX. ANTS fluorescence was based on the total concentration of ANTS in the solution, while DPX quenching was determined by its local concentration in the liposome [24]. When fusion occurred between a liposome containing both

ANTS and DPX and an unlabelled liposome, DPX was diluted, ANTS unquenched and increased fluorescence detected.

LUV containing both ANTS and DPX were combined in a stirred cuvette with unlabelled PA/PE (1:3) LUV at a ratio of 1:9 (180 μg total lipid). Fusion of liposomes was initiated by the addition of increasing concentrations of Ca^{2+} . Fluorescence of ANTS was measured continuously on an SLM 8000 spectrofluorimeter (excitation wavelength, 370 nm; emission wavelength, 530 nm). Fusion was measured as initial rate of fusion and total fusion in 90 s.

Initial rate of fusion = % maximal change in fluorescence/min = $5 \times$ (change in fluorescence in 12 s/maximal change in fluorescence)

Total fusion = change in fluorescence in 90 s/maximal change in fluorescence

For liposomes containing both ANTS and DPX, the maximal change in fluorescence was measured as the difference between the quenched (intact liposomes) and unquenched ANTS signal (lysed with 0.1 % Triton X-100 in the absence of external DPX). This change in fluorescent signal represented a theoretic maximal unquenching within our system and was only indirectly related to individual fusion events (see the Results section). However, we have arbitrarily set this scale at 100 % as a means of comparing individual experiments and different fusion systems. The signal due to leakage was controlled for using free DPX in the fusion buffer (a detailed account of the method is described in the Results section).

The standard ANTS/DPX assay was performed as previously described [8].

Isolation and preparation of granules and plasma-membrane vesicles

Neutrophils were isolated from the fresh venous blood of healthy donors using the method of Boyum [25] and suspended in 100 mM KCl/3 mM NaCl/3.5 mM MgCl_2 /10 mM Pipes/1 mM ATP, pH 7.3 (1×10^8 /ml). Following treatment with di-isopropyl fluorophosphate (DFP, 2 mM), the neutrophils were disrupted by nitrogen cavitation [26] and the nuclei removed by low-speed centrifugation in a Beckman J6 centrifuge (1000 *g* for 10 min at 4 °C). The resulting supernatant was layered on a discontinuous sucrose gradient (15, 43, 53 and 60 % layers) and centrifuged for 1 h at 93 000 *g* in a Beckman L5-50B ultracentrifuge. Fractions were removed from the interfaces between each layer and assayed for lactic dehydrogenase (cytosol) [27], alkaline phosphatase (plasma membrane) [8], lactoferrin (specific granules) [28], and β -glucuronidase (azurophil granules) [29]. Sucrose was removed from the particulate fractions by gel filtration on a Sephadex G-25 column equilibrated in buffer A. Experiments measuring latent alkaline phosphatase activity revealed that 71 ± 1 % (S.E.M., $n = 5$) of the plasma-membrane fraction was both sealed and oriented inside-out. Because azurophil granules bound to the Sephadex G-25 column, they were diluted in buffer A and re-centrifuged on to a 60 % sucrose cushion prior to harvest and resuspension in buffer A. (Previous studies in our laboratory showed that up to 20 % sucrose had no effect on the fusion assay.) Granules were used at an A_{450} of 0.17 to ensure comparable amounts of phospholipids (12 μg) between preparations.

RESULTS AND DISCUSSION

Characterization of the ANTS/DPX dequenching assay

Studies on the fusion of neutrophil granules have been hampered by the lack of suitable contents-mixing assays. We took advantage

Figure 1 Concentration-dependent quenching of ANTS fluorescence by DPX

ANTS (500 nM) and various concentrations of DPX were combined in a cuvette and fluorescence monitored on an SLM 8000 spectrofluorimeter (excitation at 370 nm, emission at 530 nm). Results are from a single representative experiment.

of the quenching properties of DPX on ANTS fluorescence to develop a fusion assay capable of measuring the mixing of the aqueous-space contents of any sealed vesicle. We reasoned that, if the aqueous space of one set of liposomes contained ANTS quenched by DPX, fusion with an unlabelled vesicle would dilute the concentration of DPX in the resulting combined aqueous space. Decreased quenching by the diluted DPX would enhance the detectable signal from the ANTS. Such an assay would be universal. Not only could this assay be used with liposomes and plasma-membrane vesicles, but it should also measure the fusion of specific and azurophil granules.

As shown in Figure 1, DPX quenched ANTS fluorescence in a concentration-dependent manner, with more than 90% quenching obtained with 25 mM DPX. Thus dilutions of DPX would result in an enhancement of the quenched ANTS signal. These assays employed 500 nM ANTS, which was the highest final concentration of this fluorochrome that we used in subsequent fusion assays. Similar quenching curves were also observed with lower concentrations of ANTS. We prepared liposomes composed of PA/PE (1:3) in buffer containing 55 mM ANTS, 50 mM DPX and 10 mM Hepes. Thus the aqueous space of these liposomes contained ANTS quenched by DPX. We determined empirically that self-quenching by ANTS did occur, but that it was dependent on the mass of ANTS in the optical path (results not shown). ANTS encapsulated into the liposomes used in fusion assays was low in mass (even though the local concentration in the liposomes was high). Hence, under the conditions employed, self-quenching of ANTS was not a problem. This was determined by the fact that liposomes loaded with 55 mM ANTS yielded the same fluorescence before and after lysis with Triton X-100 (results not shown).

As shown in Figure 2, the fluorescence properties of ANTS/DPX liposomes were simply and directly related to the amount of lipid added to the system. (Because our liposomes were extruded to ensure uniform size, phospholipid mass was directly related to liposome number.) Furthermore, lysis of the liposomes with Triton X-100 resulted in a complete dequenching of the ANTS signal and an increase in fluorescence (Figure 2). Thus quenching by DPX could only be maintained within the confines of the liposomes where DPX concentration was high. There were linear relationships between quenched and unquenched signals and the difference between them ('net'), which was dependent on

Figure 2 Fluorescence is directly proportional to the mass of liposomes containing ANTS/DPX

Liposomes composed of PA/PE (1:3) were formed by reverse-phase evaporation and contained 55 mM ANTS plus 50 mM DPX. Various amounts of phospholipid vesicles were added to a cuvette, and fluorescent emission of ANTS was determined on an SLM 8000 spectrofluorimeter before and after lysis with 0.1% Triton X-100. Net change in fluorescence was determined as the difference in signal between intact and lysed liposomes.

Figure 3 Calibration of external DPX to quench ANTS signal due to leakage

This Figure shows conceptually how external DPX was used to eliminate any signal attributable to external ANTS (and hence from vesicle lysis). Following a brief equilibration period, either 0.1% Triton X-100 or 30 mM DPX was added to each sample. Lysis with Triton X-100 produced a maximal ANTS fluorescence signal, while DPX alone quenched external ANTS. The liposomes which received Triton initially were then quenched by DPX. The external DPX concentration was adjusted such that the fluorescence, before and after lysis with Triton X-100, was the same. This concentration was determined on a daily basis and ranged from 25 to 30 mM.

the lipid concentration. It should be noted that because background fluorescence was not set at zero, the linear regression lines did not necessarily run through zero.

We expected that the fusion between an unlabelled and an ANTS/DPX-containing liposome would result in a decrease in the local DPX concentration, and hence an increased ANTS signal. Unfortunately, lysis of the liposome would also be expected to give an increased signal, which might be confused with that generated by fusion. To eliminate the effect of vesicle lysis, we added external DPX to our system to control for residual exterior ANTS as well as ANTS released by lysis. Operationally, we calibrated the amount of external DPX for each experiment, such that the intact and lysed signals remained constant (Figure 3). The difference in ANTS fluorescence ob-

Figure 4 Extent of fusion is directly related to the ratio of unlabelled to labelled (ANTS/DPX-containing) liposomes

Liposomes composed of PA/PE (1:3) labelled with 55 mM ANTS and 50 mM DPX (7.5, 22.5, and 67.5 μ M final [phospholipid]) were mixed with various amounts of unlabelled liposomes composed of PA/PE (1:3) in buffer A (final volume 1 ml). Fusion was initiated with the addition of 1 mM Ca^{2+} , and fluorescence was monitored on an SLM 8000 spectrofluorimeter (excitation at 370 nm; emission at 530 nm). The results are expressed as the change in fluorescence/maximal change in fluorescence, which were plotted against the ratio of unlabelled to labelled (ANTS/DPX-containing) liposomes.

tained between intact liposomes and lysed liposomes (in the absence of external DPX) defined the signal range or maximal potential change in fluorescence. The decrease in fluorescence of intact liposomes in the presence of external DPX determined the fluorescence due to unencapsulated ANTS. When the external DPX concentration was adjusted such that the ANTS signal before and after lysis were equal, any effect of lysis was eliminated from the assay. The external DPX concentration required was usually close to the internal DPX concentration.

Ca^{2+} -dependent fusion of phospholipid vesicles

To set up the fusion systems, we first chose to look at interactions between liposomes alone. It was important to determine how both the ANTS signal and fusion itself were related to total number of vesicles in the system (phospholipid concentration) and to the ratio between labelled and unlabelled liposomes. Using a relatively high concentration of Ca^{2+} (1 mM), which preliminary experiments had shown to be optimal for fusion, we looked at these two aspects separately. For a signal to occur, a liposome labelled with ANTS/DPX must fuse with an unlabelled liposome. Otherwise there would be no change in the internal DPX concentration. Both the probability and extent of fusion would depend on the availability of unlabelled target vesicles. This was thus an important parameter. We determined that the percentage maximal fusion was directly related to the ratio of labelled to unlabelled liposomes (Figure 4). Because background fluorescence was not set at zero, the linear regression lines did not necessarily run through zero. The data in Figure 4 are a compilation of results from three runs, each with a different concentration of labelled liposomes. (Final ANTS concentration was also determined by concentration of labelled phospholipids and ranged from approx. 50–500 nM). As can be seen by the broken lines, the extent of fusion was dependent only on the ratio of unlabelled to labelled liposomes, not upon the starting concentration. Since, for a single value of this ratio, starting concentration directly determined total lipid concentration, it

Figure 5 Comparison between the new ANTS/DPX dequenching assay, the standard ANTS/DPX and NBD-Rh-PE fluorescence transfer assay

PA/PE (1:3) containing liposomes were prepared by reverse phase evaporation and suspended in buffer A. For both the new ANTS/DPX (described in the present) and NBD-Rh-PE fluorescent transfer assay, a 1:9 labelled/unlabelled liposome (180 μ g total phospholipid) ratio was maintained. For the standard ANTS/DPX assay, there was a 1:1 ratio between ANTS-containing liposomes and DPX-containing liposomes. Phospholipid vesicle fusion was initiated by the addition of Ca^{2+} and monitored on an SLM 8000 spectrofluorimeter [excitation at 370 nm (ANTS) or 450 nm (NBD); emission at 530 nm]. Results are expressed as initial rate of fusion (a) and percent maximal fusion (b).

was also evident that the percentage of maximal fusion signal was independent of total lipid concentration. In order to permit comparisons between different experimental conditions, we would have to maintain a constant labelled/unlabelled liposome ratio. We have chosen a ratio of 1:9 (labelled/unlabelled) to maximize the fusion signal and at the same time conserve phospholipids.

We determined the Ca^{2+} requirement for the fusion of liposomes composed of PA/PE (1:3) using the new fusion assay. Aqueous-space mixing was first detected at approx. 500 μ M Ca^{2+} . With increasing $[\text{Ca}^{2+}]$, there was an increase in both the rate and extent of fusion (Figures 5a and 5b). However, at high $[\text{Ca}^{2+}]$, a decrease in fluorescence attributable to lysis (and quenching due to external DPX) occurred. To determine whether the new fusion assay gave results comparable with those obtained by pre-existing methods, we compared the fusion of liposomes composed of PA/PE (1:3) using the new dequenching ANTS/DPX assay, a standard ANTS/DPX assay [12,24] and an NBD-Rh fluorescence transfer assay [11,19]. Using all three systems, we found that PA/PE liposomes were relatively fusogenic. Threshold levels of fusion could first be detected at approx. 500 μ M Ca^{2+} , while near optimal rates of fusion were reached by 1–3 mM Ca^{2+} (Figure 5). Although all three assays produced

Figure 6 Relationship between hypothetical fusion events, internal DPX concentration, and the fusion signal

Using Figure 1 (concentration-dependent quenching of ANTS fluorescence by DPX) we calculated the predicted ANTS fluorescence resulting from each hypothetical fusion event with an unlabelled liposome. The percentage of maximal signal (predicted change in fluorescence/maximal change in fluorescence) was then plotted against hypothetical fusion events. Data presented compare fusion of liposomes containing 20 versus 50 mM DPX.

fusion curves with similar Ca^{2+} dose-response characteristics, the apparent magnitude of fusion using our assay was less. This is because each assay has a different baseline and sensitivity compared with its maximal obtainable signal. However, these are properties that can be manipulated in the new fusion assay.

In a standard ANTS/DPX quenching assay there is a 1:1 ratio between ANTS- and DPX-containing liposomes. If each liposome were involved in one, and only one, random fusion event, we would expect that approximately half of the ANTS-containing liposomes would fuse with DPX-containing liposomes, resulting in a loss of signal. The other half of both the ANTS- and DPX-containing liposomes would fuse with similar partners, resulting in no change in signal. We would expect that this would result in a net loss of 50% maximal signal change. Using the new assay, a single fusion event would be expected to decrease the internal DPX concentration from 50 mM to 25 mM, resulting in only a 5–7% increase in fluorescence. Assuming uniform size (which is appropriate for our extruded LUV), we can predict the effect of each hypothetical fusion event on the ANTS signal (Figure 6) using the data from the DPX quenching curve (Figure 1). Furthermore, by lowering the internal concentration of DPX to 20 mM, we can increase the expected signal obtained with a single fusion event to almost 20% of the maximum signal

(Figure 6). Thus, by adjusting the internal DPX concentration, we can increase or decrease the sensitivity of the assay to measure both high and low fusion frequencies. When multiple fusion events are predicted, high internal concentrations of DPX will result in minor changes in fluorescence for the first few fusion events. Conversely, increased sensitivity can be obtained using lower concentrations of internal DPX. If vesicle size is known and/or controlled, we can use this fusion system to quickly and easily estimate the average number of fusion events occurring, again increasing our understanding of membrane-membrane interactions.

Fusion of liposomes with granules and plasma-membrane vesicles

In order to look at fusion with subcellular fractions from neutrophils, it was first necessary to select an appropriate method providing relatively high yield and purity. The two most common methods for the isolation of granules from human neutrophils utilize either a discontinuous Percoll gradient [29] or a continuous sucrose gradient [30] following cellular disruption. Although the Percoll gradient yields relatively pure concentrated fractions, it is impossible to remove residual Percoll from the isolated fractions. This residual Percoll may interfere with fusion. Sucrose, on the other hand, is easily removed, but usually results in dilute membrane and granule preparations with relatively low yields and purity.

We developed our own technique for isolating granules and plasma-membrane vesicles, obtaining relatively pure fractions of cytosol, plasma-membrane vesicles, and azurophil and specific granules as determined by lactate dehydrogenase, alkaline phosphatase, β -glucuronidase and lactoferrin respectively (Table 1). Neutrophils were disrupted by N_2 -cavitation followed by low-speed centrifugation to remove nuclear material. The postnuclear supernatant (99% free of nuclear material as determined by DNA analysis [31]) was applied to a discontinuous sucrose gradient (15, 43, 53 and 60%) and ultracentrifuged for 60 min at 93000 g. The cytosolic fraction separated above the 15/45% interface, containing 98% of the lactate dehydrogenase activity (Table 1). The 15/43% interface was rich in plasma-membrane vesicles, containing 75% of the alkaline phosphatase activity and less than 12% of any of the other markers (Table 1). Latency studies revealed that 71.9 ± 0.9 ($n = 5$) of the plasma-membrane vesicles were inside-out and sealed. This high yield of inside-out vesicles will be used in future studies of granule-plasma-membrane fusion. The 43/53% interface was rich in specific granules, containing approx. 80% of the lactoferrin, while the 53/60% interface was rich in azurophil granules, containing 70% of the β -glucuronidase activity (Table 1).

Having characterized the new fusion assay with liposomes, we next examined the fusion of plasma-membrane vesicles as well as

Table 1 Recovery of marker enzymes in subcellular fractions

Following subcellular fractionation on our discontinuous sucrose gradient, each interface was isolated and assayed for lactate dehydrogenase, alkaline phosphatase, lactoferrin, and β -glucuronidase activity. Cytosol was isolated above the 15% interface. Plasma-membrane vesicles were isolated from the 15/43% interface. Specific granules were isolated from the 43/53% interface. Azurophil granules were isolated from the 53/60% interface. Results are means $\pm$ S.E.M. for at least five experiments.

Fraction	Lactic dehydrogenase	Alkaline phosphatase	Lactoferrin	β -Glucuronidase
Cytosol	97.6 ± 3.4	16.6 ± 1.3	0.9 ± 0.7	11.5 ± 4.9
Plasma membrane	2.1 ± 3.0	73.7 ± 3.0	11.2 ± 6.1	11.1 ± 8.4
Specific granules	0.3 ± 0.4	4.2 ± 1.6	83.4 ± 5.8	10.6 ± 4.2
Azurophil granules	0.0 ± 0.0	5.5 ± 1.7	4.4 ± 4.1	69.1 ± 11.2

Figure 7 Aqueous-space mixing occurs between PA/PE (1:3)-containing liposomes and both specific granules and plasma-membrane vesicles derived from human neutrophils

Azurophil granules, specific granules and plasma-membrane vesicles were isolated from human neutrophils in a manner identical with that used in Table 1. Fusion assays were performed in a manner identical with those described in Figure 4, with the exception that the unlabelled liposomes were replaced with granules or plasma-membrane vesicles derived from human neutrophils. PA/PE (1:3) liposomes containing 55 mM ANTS, 20 mM DPX and 30 mM Hepes, pH 7.0, were used as the labelled fusion partners. Fusion was initiated by the addition of Ca²⁺ and monitored on an SLM 8000 spectrofluorimeter (excitation at 370 nm; emission at 530 nm). Results are expressed as initial rate of fusion (a) and percentage maximal fusion (b).

azurophil and specific granules derived from human neutrophils with liposomes composed of PA/PE. These studies were performed identically with those examining liposome-liposome fusion with the exception that plasma-membrane vesicles or azurophil or specific granules replaced the unlabelled liposomes. Again, external DPX was calibrated to eliminate any signal generated by lysis. Neither specific granules nor membrane vesicles interfered with either ANTS-fluorescence or DPX-quenching characteristics. While intact azurophil granules exhibited some autofluorescence, this was not a factor at the wavelengths used for ANTS fluorescence. Also DPX quenching was unaffected. Although we cannot determine what effect concentrated granule contents could have on our fluorochrome, these effects could only be local and were only possible after fusion has occurred (no interaction was observed after lysis). Thus such putative anomalies would themselves be indications of fusion such that our system would remain qualitatively valid.

Fusion between specific granules isolated from human neutrophils and liposomes composed of PA/PE (1:3), as determined by aqueous-space mixing, was first detected at 300–500 μ M Ca²⁺ (Figure 7). Increase in [Ca²⁺] increased both the rate and extent of fusion (Figure 7). At extremely high [Ca²⁺], vesicle leakage occurred, reflected in a decrease in apparent fusion. This was not

an unexpected problem with this system, but is a common observation with contents-mixing assays [32]. Since leakage cannot be observed with lipid-mixing assays, such findings confirm the usefulness of assays based on contents mixing. As was observed with specific granules, plasma-membrane vesicles derived from human neutrophils fused with liposomes composed of PA/PE (1:3). Again, fusion was first detected at 300–500 μ M Ca²⁺. Increasing [Ca²⁺] resulted in an increase in the rate and extent of vesicle fusion (Figure 7). Interestingly, although plasma-membrane vesicles have a threshold [Ca²⁺] similar to that of specific granules, they appear to be slightly more fusogenic. As previously reported [24,33], azurophil granules isolated from human neutrophils do not fuse with PA/PE liposomes at [Ca²⁺] as high as 12 mM.

Previous studies using lipid-mixing assays have shown that fusion can occur between liposomes and specific granules or plasma-membrane vesicles [24,34]. However, one critical aspect lacking in all previous studies was a clear demonstration that 'true fusion', that is, the mixing of the contents of the phospholipid vesicles with the contents of the granules or plasma-membrane vesicles, had occurred. Stahl and co-workers have developed techniques of loading and measuring endosome-lysosome fusion [35,36]. Unfortunately these techniques are not applicable to neutrophil granules. In trying to develop a fusion assay for measuring the fusion of granules, we also produced a universal contents-mixing assay that can be used to measure fusion between liposomes and any other intact vesicle.

Like many previous studies using liposomes, the Ca²⁺ requirements for fusion were beyond the physiological range. However, we have developed a universal aqueous-space-mixing assay that can now be used in conjunction with other models for membrane fusion. For example, the Ca²⁺ requirements for the fusion of specific granules with liposomes composed of PA/PE can be lowered to the low-micromolar range with annexin I and arachidonic acid [33]. Using our new assay, we should be able to label plasma-membrane vesicles. This should provide a contents-mixing assay capable of measuring the fusion of granules with the plasma membrane.

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