Volume 217, number 2, 158–162 FEB 04664 June 1987

Intracellular organelle motility and membrane fusion processes in human neutrophils upon cell activation

Faustino Mollinedo and Donald L. Schneider

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756, USA

Received 23 February 1987

Release and subcellular fractionation experiments indicate that fusion of a novel tertiary granule with the plasma membrane is concomitant with human neutrophil activation. Phorbol 12-myristate 13-acetate (PMA) induced a respiratory burst in human neutrophils as well as a high release of gelatinase, a marker of the tertiary granule. Preincubation of neutrophils with cytochalasin E induced a partially activated or 'primed' state, in which cells were unable to generate superoxide anion, but showed a reduced latency period for this activity. Fusion of tertiary granules with the cell surface also occurred during priming, although to a lesser extent than in PMA stimulation. The rapid tertiary granule degranulation, preceding that of specifics and azurophilics, seems to play an important role in the functionality and secretory properties of human neutrophils.

Tertiary granule; Membrane fusion; Cell activation; Superoxide anion; (Human neutrophil)

1. INTRODUCTION

Neutrophilic polymorphonuclear leukocytes (neutrophils) constitute the first line of defense against infection, and are one of the primary mediators of the acute inflammation response. Central to these functional roles are the neutrophil's cytoplasmic granules, containing a great amount of hydrolytic enzymes and microbicidal factors that function at both intracellular and extracellular sites [1]. Two major types of granules, azurophil (primary) and specific (secondary), have been characterized in human neutrophils based on ultrastructural morphology and biochemical composition [2]. However, recent evidence indicates considerably greater granule heterogeneity than is generally accepted [3]. In this regard, we have recently reported the presence of a novel tertiary granule in human neutrophils [4].

Correspondence address: F. Mollinedo, Unidad de Biomembranas, Centro de Investigaciones Biologicas, Velazquez 144, 28006-Madrid, Spain

This tertiary granule is highly enriched in cytochrome *b* and ubiquinone [4], two putative components of the superoxide-generating system, and contains gelatinase as an enzyme marker [4,5].

Upon exposure to ingestible particles or to several soluble stimuli (e.g. PMA), neutrophils undergo profound changes in oxygen metabolism after a characteristic time lag [6]. This metabolic alteration, termed 'respiratory burst', results from the activation of an oxidase system which catalyzes the reduction of molecular oxygen to superoxide anion (O_2^-), which is subsequently converted into hydrogen peroxide and other reactive oxidants [7]. Other early events readily observed after cell stimulation include proton release to the surrounding medium [8], and degranulation, i.e. fusion of cytoplasmic granules with the plasma membrane (exocytosis) or with the endocytic vacuole (endocytosis).

Here, we show evidence favoring the functional involvement of the novel tertiary granule in the respiratory burst activity of activated neutrophils. Furthermore, we find that this tertiary granule

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50 © 1987 Federation of European Biochemical Societies

fuses readily with the plasma membrane upon cell activation and precedes degranulation of specific and azurophilic granules.

2. MATERIALS AND METHODS

2.1. *Isolation of neutrophils*

Neutrophils were prepared from fresh human peripheral blood after removal of erythrocytes by sedimentation at unit gravity through dextran as described [4].

2.2. *Cytochalasin E and PMA treatments*

Freshly isolated, resting cells were resuspended in 5 ml Locke's solution (0.9 g NaCl, 0.024 g $CaCl_2$, 0.042 g KCl, 0.02 g $NaHCO_3$, 0.1 g glucose and water to make 100 ml of solution) per 0.1 g packed neutrophils. The cell suspension was incubated with cytochalasin E (5 μM) or PMA (3.2 μM) for 6 min at 37°C. Stock solutions in dimethyl sulfoxide were 2 and 0.2 mg/ml, respectively. After rapid cooling to 4°C, the cells were collected and washed with cold saline by centrifugation at 800 × *g* for 3 min.

For measurement of enzyme release, the cell pellets and washes obtained after cytochalasin E and PMA treatments were assayed for marker activities as described below.

2.3. *Subcellular fractionation*

Cells were disrupted by hypotonic shock and homogenization as in [4]. The postnuclear supernatant or extract fraction was fractionated on a 14.3–34.5% (w/w) continuous sucrose gradient by centrifugation at 70000 × *g* for 15 min at 4°C as reported in [4].

2.4. *Miscellaneous*

Oxygen consumption was determined using a Gilson model K-ICC-H oxygraph with glucose or NADPH as substrates for intact or disrupted cells, respectively, as described [9].

Protein and markers for cytosol (lactate dehydrogenase), plasma membrane (5′-nucleotidase), tertiary granules (gelatinase), specific granules (lysozyme) and azurophilic granules (myeloperoxidase) were assayed according to [10]. Cytochrome *b* and ubiquinone were determined as previously described [11].

3. RESULTS

3.1. *Effect of cytochalasin E on the neutrophil respiratory burst*

Preincubation of human neutrophils with heat and cytochalasin E did not induce O_2^- production, but diminished the latency period upon PMA stimulation (table 1). These results suggest that the respiratory burst oxidase can exist in a partially activated or 'primed' state that has yet to acquire oxidase activity.

Table 1

Effect of cytochalasin E on phorbol myristate acetate-induced respiratory burst in human neutrophils

Preincubation conditions	Respiratory burst (time lag after PMA addition)
4°C	10 min
37°C	2 min
37°C, 5 μM cytochalasin E	1 min

Human neutrophils (~3 mg cell protein) were preincubated for 15 min as indicated in the presence and absence of 5 μM cytochalasin E, and then the time lag for oxygen consumption was determined after addition of 3.2 μM PMA

3.2. *Enzyme release in cytochalasin E- and PMA-treated neutrophils*

As shown in table 2, there is a progressive increase in gelatinase release from neutrophils treated with heat, cytochalasin E and PMA. These results suggest that tertiary granules fuse with the plasma membrane during the above cell treatments. Simple heating and cytochalasin E diminished substantially the time lag required for the induction of the respiratory burst (see table 1), but did not activate the system by themselves. PMA is a well known activator of the respiratory burst by itself [6], and induced a high release of gelatinase activity (table 2). Thus, there is a correlation between the degree of tertiary granule fusion with the cell surface and the capacity to affect the respiratory burst parameters. Interestingly, the release of lysozyme (specific granules) paralleled that of gelatinase, but to a lesser extent.

Table 2

Extracellular release of cytoplasmic granule markers during priming and activation of human neutrophils

Preincubation conditions	Enzyme secretion (%)[a]	
	Lysozyme	Gelatinase
4°C	1.1 ± 0.1	4.0 ± 0.7
37°C	2.9 ± 0.3	15.4 ± 3.9
37°C, 5 μM cytochalasin E	12.1 ± 4.3	31.5 ± 10.9
37°C, 3.2 μM PMA	36.6 ± 2.1	65.0 ± 17.5

[a] Enzyme secretion is shown as percentage of total cell activity. Human neutrophils were preincubated for 6 min as indicated, and enzyme secretion was assayed as described in section 2. The means ± SE for at least three determinations are shown

3.3. *Subcellular localization of tertiary granule components in cytochalasin E- and PMA-treated human neutrophils*

We have previously found that cytochrome *b* and ubiquinone are mainly localized in novel gelatinase-rich tertiary granules in resting human neutrophils [4]. Other secondary locations of these components were also reported [4]. In this context, it is worthy of note that there is an additional peak of ubiquinone in an undefined region of the gradient ([4] and fig.1). Comparison of the subcellular distribution of cytochrome *b*, ubiquinone and 5′-nucleotidase (AMPase) in control and cytochalasin E-treated cells indicated a shift of these markers to deeper positions in the gradient after cytochalasin E treatment (fig.1), suggesting fusion of plasma membrane with tertiary granules. Sucrose gradient centrifugation of PMA-activated human neutrophils showed oxygen uptake activity in the membrane fraction as well as in dense parts of the gradient (fig.1). Concomitantly, cytochrome *b* and ubiquinone were shifted to these positions in the gradient. The plasma membrane marker, 5′-nucleotidase, was also shifted to denser parts in the gradient (fig.1). This shift does not represent cell aggregation or non-specific aggregation inasmuch as other markers, such as lactate dehydrogenase (cytosol) and peroxidase (azurophilic granules), were not affected. A portion of lysozyme was slightly shifted (fig.1). The cytochalasin E- and PMA-induced shifts in cytochrome *b*, ubiquinone and 5′-nucleotidase are more evident in the relative activity difference plots shown in fig.2. The similar shifts of cytochrome *b* and ubiquinone to the fractions active in respiratory burst activity further support the involvement of cytochrome *b* and ubiquinone in the oxidase activity.

4. DISCUSSION

Release and subcellular fractionation studies suggest that fusion of tertiary granules with the plasma membrane is concomitant with priming and activation of the respiratory burst activity. This process is depicted in fig.3, wherein extensive and rapid fusion of tertiary granules with the plasma membrane of forming endocytic vesicles occurs before pinching off from the cell surface. A sequential order in degranulation following tertiary, specific and azurophilic granules was observed in human neutrophils (table 2, and not shown). Rapid fusion of tertiary granules with the plasma membrane, and the presence of disrupted and intact vesicles may account for the above-described cytochrome *b* and ubiquinone shifts in the sucrose gradient upon cell activation (see fig.2). This fusion process takes place even during the formation of enucleated neutrophils [12], which are devoid of intracellular granules and are able to generate O_2^- upon stimulation. These results are in agreement with recent evidence demonstrating cytochrome *b* translocation after neutrophil stimulation from an internal pool to a light membrane fraction, putatively plasma membrane [13–15]. Manara and Schneider [16], studying the temperature effects on oxygen uptake by stimulated neutrophils, found a correlation between the gelatinase and lysozyme release and the respiratory burst activity. On these grounds, it is reasonable to suggest that membrane fusion between tertiary granules and plasma membrane is a necessary, but not sufficient condition for activation of the superoxide-generating system in human neutrophils. The time lag observed between addition of a stimulus and generation of O_2^- could be due in part to the time required for the membrane fusion process. In this regard, a membrane fusion process has been proposed for O_2^- generation in neutrophils [17], which might bring together components of a putative electron transport chain, partially located in the plasma membrane and in the

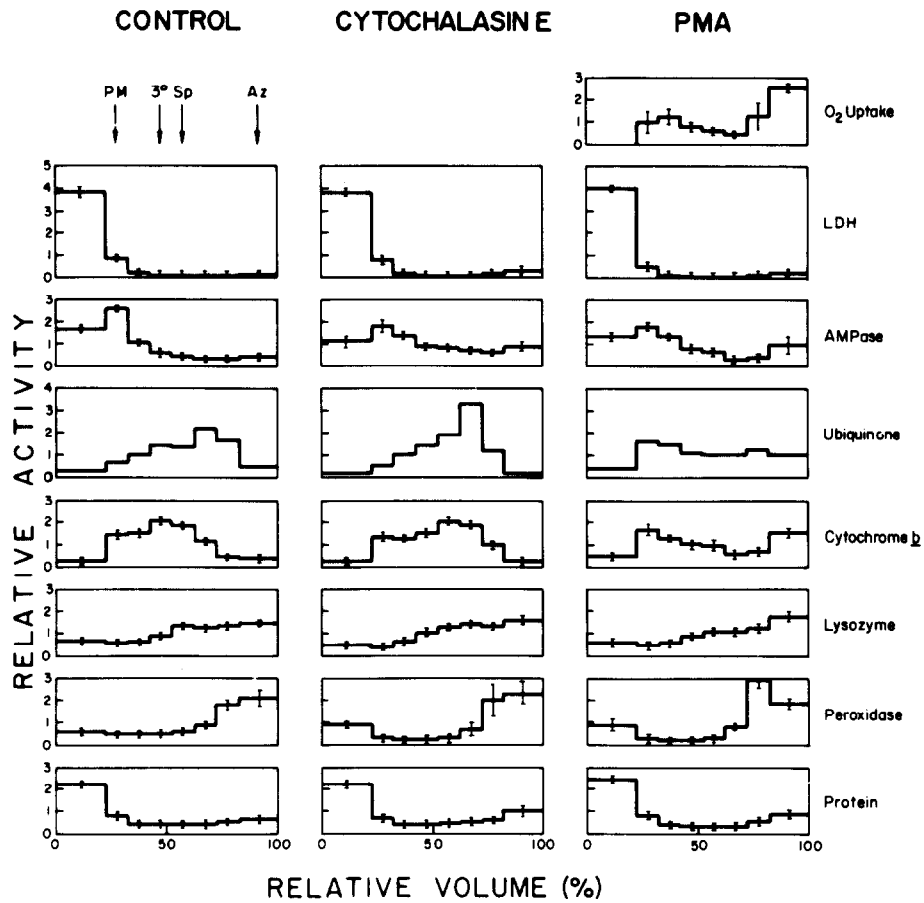

Fig.1. Subcellular distributions of cytochrome *b*, ubiquinone, and marker enzymes after gradient centrifugation of neutrophil extracts. Extracts from resting (left), cytochalasin E-treated (middle) and PMA-treated (right) human neutrophils were fractionated on continuous sucrose gradients as described in section 2. Plots of relative activity vs percent volume are given, where relative activity is the percent activity in a fraction divided by the percent volume collected in that fraction. Values are shown as means ± SE of at least three independent determinations. PM, plasma membrane; 3°, tertiary granule; Sp, specific granule; Az, azurophilic granule. Recovery for oxygen uptake (oxidase) was 60%. Recoveries of the remaining activities were over 85%.

membrane of an intracellular organelle, to constitute an active oxidase.

On the other hand, we have recently localized an H^+-ATPase in the tertiary granule of human neutrophils [10]. The dominant location of acidification activity in tertiary granules that very readily degranulate presumably has significant implications for the importance of low pH in cidal events and the inflammatory process [10]. Furthermore, the rapid fusion of tertiary granules with the cell surface suggests a potential role of this organelle and its contents in mediating inflammatory reactions. It is also plausible to postulate that the membrane fusion process herein described may facilitate the replenishment of some plasma membrane components of the respiratory burst machinery. This latter mechanism has been reported for the *N*-formyl-methionyl-leucyl-phenylalanine receptor [18] and the surface glycoprotein Mo1 [19].

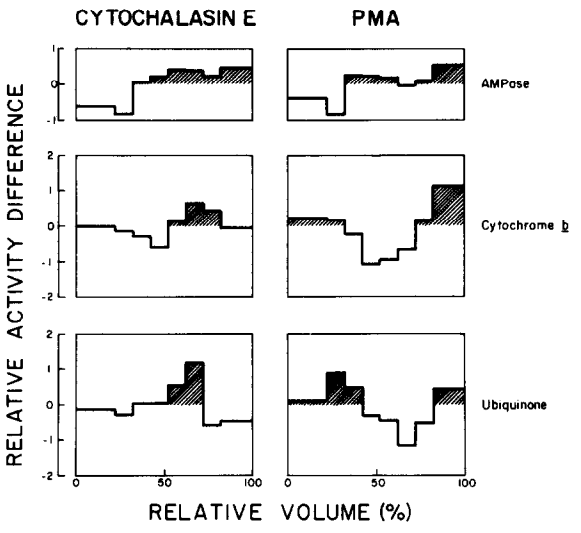

Fig.2. Relative activity difference distributions of 5′-nucleotidase (AMPase), cytochrome *b* and ubiquinone after gradient centrifugation of neutrophil extracts from cytochalasin E- and PMA-treated cells. Plots of relative activity difference vs percent volume are given, where relative activity difference is the difference of percent activity in a fraction (resulting from subtracting the percent activity in the absence of any addition from that in the presence of cytochalasin E or PMA) divided by the percent volume collected in that fraction. Data are calculated from values taken from fig.1. Hatched zones indicate relative increments of the respective markers.

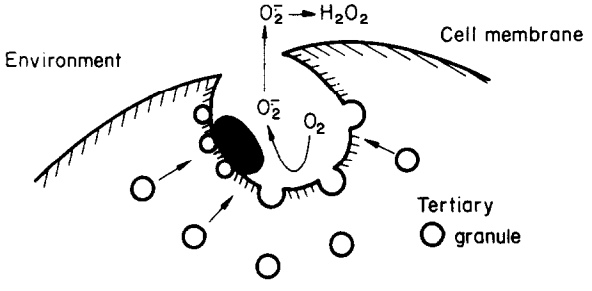

Fig.3. Hypothetical mechanism of fusion of tertiary granules with the plasma membrane during activation of the respiratory burst in human neutrophils. Tertiary granules fuse readily with the cell surface of forming endocytic vesicles, even before they are pinched off from the plasma membrane.

ACKNOWLEDGEMENT

F.M. kindly thanks the United States-Spanish Joint Committee for Scientific and Technological Cooperation for a fellowship.

REFERENCES

[1] Spitznagel, J.K. (1984) in: Contemporary Topics in Immunobiology (Snyderman, R. ed.) vol.14, pp.283–343, Plenum, New York.
[2] Wright, D.G. (1982) in: Advances in Host Defense Mechanisms (Gallin, J.I. and Fauci, A.S. eds) vol.1, pp.75–110, Raven, New York.
[3] Rice, W.G., Kinkade, J.M. jr and Parmley, R.T. (1986) Blood 68, 541–555.
[4] Mollinedo, F. and Schneider, D.L. (1984) J. Biol. Chem. 259, 7143–7150.
[5] Dewald, B., Bretz, U. and Baggiolini, M. (1982) J. Clin. Invest. 70, 518–525.
[6] McPhail, L.C. and Snyderman, R. (1984) in: Contemporary Topics in Immunobiology (Snyderman, R. ed.) vol.14, pp.247–281, Plenum, New York.
[7] Klebanoff, S.J. (1982) in: Advances in Host Defense Mechanisms (Gallin, J.I. and Fauci, A.S. eds) vol.1, pp.111–162, Raven, New York.
[8] Van Zwieten, R., Wever, R., Hammers, M.N., Weening, R.S. and Roos, D. (1981) J. Clin. Invest. 68, 310–313.
[9] Crawford, D.R. and Schneider, D.L. (1983) J. Biol. Chem. 258, 5363–5367.
[10] Mollinedo, F., Manara, F.S. and Schneider, D.L. (1986) J. Biol. Chem. 261, 1077–1082.
[11] Crawford, D.R. and Schneider, D.L. (1982) J. Biol. Chem. 257, 6662–6668.
[12] Mollinedo, F. (1986) Biochim. Biophys. Acta 861, 33–43.
[13] Borregaard, N., Heiple, J.M., Simons, E.R. and Clark, R.A. (1983) J. Cell Biol. 97, 52–61.
[14] Ohno, Y., Seligmann, B.E. and Gallin, J.I. (1985) J. Biol. Chem. 260, 2409–2414.
[15] Borregaard, N. and Tauber, A.I. (1984) J. Biol. Chem. 259, 47–52.
[16] Manara, F.S. and Schneider, D.L. (1985) Biochem. Biophys. Res. Commun. 132, 696–701.
[17] Sloan, E.P., Crawford, D.R. and Schneider, D.L. (1981) J. Exp. Med. 153, 1316–1328.
[18] Fletcher, M.P. and Gallin, J.I. (1980) J. Immunol. 124, 1585–1588.
[19] Todd, R.F., Arnaout, M.A., Rosin, R.E., Crowley, C.A., Peters, W.A. and Babior, B.M. (1984) J. Clin. Invest. 74, 1280–1290.