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The lateral organization of components of the membrane skeleton and superoxide generation in the plasma membrane of stimulated human neutrophils

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Studies were performed to examine the lateral organization of the NADPH oxidase system in the plasma membrane of human neutrophils. Analysis of the subcellular fractionation of human neutrophils by isopycnic sedimentation of cavitated cell lysates suggested that there may be more than one population of plasma membrane vesicles formed upon cell disruption. One population (30–32% sucrose) contained surface accessible wheat germ agglutinin binding sites, alkaline phosphatase activity, and cytochrome *b*. Another population (34–36% sucrose) contained membrane-bound flavin and, when the cells were prestimulated with phorbol myristate acetate (PMA), NADPH-dependent superoxide generating activity. Approximately 25% of the neutrophil cytochrome *b* cosedimented with the heavy population, confirming our previous hypothesis (Parkos et al. (1985) J. Biol. Chem. 260, 6541–6547) that only a fraction of the total cellular cytochrome *b* is involved in superoxide production. The heavy plasma membrane fraction was also enriched in membrane associated actin and fodrin as detected by Western blot analysis. After extraction of the plasma membrane vesicles with detergent cocktails, the majority of superoxide generating activity remained associated with the detergent insoluble pellet. Western blot analysis demonstrated that the pellets were also enriched in actin. Further analysis of these pellets using rate-zonal detergent-containing sucrose density gradients indicated that the superoxide generating complex had an approximate sedimentation coefficient of 80 S, suggesting that the neutrophil superoxide generating system may form a complex on the plasma membrane which is associated with or somehow organized by the membrane skeletal matrix. This organization may be of functional relevance not only to the actual production of superoxide, but also to the targeting of microbicidal oxidants.

Introduction

The role of the neutrophil in the body's defense against pathogens has been well documented [1–5]. Upon exposure to a pathogenic stimulus, the neutrophil responds by generating toxic oxygen metabolites such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [6,7]. This response, which is commonly known as the oxidative burst, is primarily due to the activation of a membrane-bound NADPH oxidase that is inactive in the unstimulated cell [8]. Although the components of this oxidase system have not yet been completely defined, current evidence suggests that it may be a multicomponent electron transport system that includes a flavoprotein [9–11], which acts as an NADPH dehydrogenase, a low potential *b*-cytochrome which is thought to be the terminal component that reduces O_2 to O_2^- [12–14], and possibly a ubiquinone which acts as an electron shuttle between the flavoprotein and cytochrome *b* [15].

Support for the involvement of the *b*-cytochrome, specifically cytochrome *b*-559 (also known as cytochrome *b*-558 or cytochrome *b*-245), in the NADPH oxidase system stems from several lines of evidence: (1) neutrophils from patients with X chromosome-linked chronic granulomatous disease (X-CGD), a disease resulting from mutations in a gene known to encode one

Abbreviations: CGD, chronic granulomatous disease; PMA, phorbol myristate acetate; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); SDS, sodium dodecyl sulfate; ^{125}I-WGA, ^{125}I-conjugated wheat germ agglutinin; DPBS, Dulbecco's phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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component of the *b*-cytochrome [16,17], cannot produce superoxide when stimulated [18]; (2) *b*-cytochrome is spectrophotometrically absent in neutrophils from certain CGD patients, and these neutrophils cannot produce a respiratory burst [19]; (3) genetic complementation studies with monocyte hybrids from *b*-cytochrome positive and *b*-cytochrome negative CGD patients can regenerate oxidase activity [20]; (4) cytochrome *b*-559 copurifies with superoxide generating activity in detergent extracts of neutrophil membranes [21]; (5) it has an unusually low (−245 mM) electrochemical potential [22]; (6) it has a very high kinetic capacity to reduce molecular oxygen under aerobic conditions [14]; and (7) purified *b*-cytochrome can reconstitute superoxide generating activity when combined with detergent extracted membranes and cytosol obtained from X-linked CGD patients [23]. However, a major issue that still needs to be addressed is the precise biochemical role of the cytochrome in this process.

The primary arguments against the participation of cytochrome *b*-559 stem from the failure to achieve complete reduction of the cytochrome in broken cell preparations under anaerobic conditions [11,21], the poor recovery of cytochrome *b* in partially purified, NADPH-dependent superoxide generating activity [24,25], and the separation of most *b*-cytochrome from superoxide-generating activity in detergent extracts using a cell free system for activation of NADPH oxidase [26]. Yet, these arguments do not take into account organizational requirements for the production of superoxide in the intact cell.

In the studies presented here, we examine the organization of the components of superoxide production in the membrane of unstimulated and PMA-stimulated human neutrophils. We describe (1) the isolation of two plasma membrane microdomains (enriched or depleted in proteins of the membrane skeleton); (2) the determination of the content of cytochrome *b*-559, flavin, and superoxide generating activity in these actin/fodrin-enriched vs. depleted domains; and (3) detergent extraction of the actin/fodrin-rich membrane domains and sedimentation studies of superoxide generating activity. The results of these studies further suggested that only a fraction of the total membrane-bound cytochrome, that fraction accessible to the flavoprotein and complexed to a detergent insoluble matrix, may be involved in oxidant production. They also suggested that the active components of superoxide production are laterally organized into functional domains [27] of the plasma membrane which are characteristically enriched in cytoskeletal proteins. Finally, these studies suggested that these structural proteins may be components of a supramolecular complex with superoxide generating activity that is stable in detergent extracts.

Materials and Methods

Chemicals

Diisopropylfluorophosphate, phorbol myristate acetate (PMA), wheat germ agglutinin (WGA), 2,2-azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS), disodium adenosinetriphosphate (ATP), aprotinin, bovine serum albumin (BSA), superoxide dismutase, catalase, glycine, Lubrol WX, Triton X-100 and cytochrome C (Type VI) were purchased from Sigma (St. Louis, MO). Gelatin, EDTA, sucrose, and dithionite were purchased from Fisher (Tustin, CA). Sodium dodecyl sulfate (SDS), bisacrylamide, sodium persulfate, TEMED, Zeta-Probe blotting membranes, peroxidase conjugated goat anti-rabbit and goat anti-mouse IgG, and peroxidase color developer (4-chloro-1-naphthol) were purchased from Bio-Rad (Richmond, CA). Hepes was from U.S. Biochemical Corp. (Cleveland, OH). Ultrapure acrylamide and sucrose were purchased from Schwarz/Mann Biotech (Cleveland, OH). Normal goat serum was purchased from ICN ImmunoBiologicals (Lisle, IL). Prestained protein standards for SDS gels were purchased from Bethesda Research Laboratories (BRL) (Gaitherberg, MD). Renex 30 (polyoxyethylene tridecyl ether) was obtained directly from the manufacturer, ICI Americas, Inc. Monoclonal anti-actin antibody was purchased from Amersham (Arlington Heights, IL). Affinity purified rabbit anti-actin antibody (IgG) was a kind gift of Dr. Keigi Fujiwara (Department of Biology, Harvard University, Boston, MA). Rabbit anti-human brain fodrin antiserum was a gift of Dr. Jon Morrow (Department of Pathology, Yale University School of Medicine, New Haven, CT).

Buffer composition

Cell resuspension buffer was a modified Dulbecco's phosphate-buffered saline containing 2.68 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.1% glucose, and 0.1% BSA (pH 7.4) (DPBS (+)). Nitrogen cavitation buffer consisted of 0.34 M sucrose, 10 mM Hepes, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM disodium adenosine triphosphate, and 1/100 diluted aprotinin (pH 7.4). Gradient buffer consisted of the appropriate sucrose concentration made up in 10 mM Hepes (pH 7.4). All sucrose solutions are given in a weight/weight basis. The buffer used for colorimetric assays was identical to the cell resuspension buffer except that glucose and BSA were omitted (DPBS (−)).

Preparation and fractionation of granulocytes

The methods of granulocyte isolation, cavitation, fractionation, and the buffers used are as described elsewhere [28] with slight modifications. Neutrophils

were isolated from human blood using gelatin sedimentation followed by hypertonic lysis of any remaining red blood cells. The resulting cell suspension contained greater than 90% neutrophils as determined by visualization under light microscopy. The purified neutrophils were treated with 2.5 mM diisopropylfluorophosphate for 15 min at 4°C, washed, and resuspended in cell resuspension buffer containing catalase (250 U/ml) and superoxide dismutase (50 U/ml) at $1 \cdot 10^8$ cells/ml. The cells were then warmed to 37°C and stimulated for 3 min with 1 μg/ml PMA. The reaction was stopped by the addition of an equal volume of ice-cold DPBS (−). The cells were then washed, resuspended in nitrogen cavitation buffer and lysed by N_2 cavitation (400 psi/15 min at 4°C). The resulting homogenate was fractionated into a low speed ($1000 \times g$ for 5 min) supernatant (1KS) and foam-pellet (1KP) residue. The 1KP was resuspended in cavitation buffer and rehomogenized with 10 strokes in a glass dounce homogenizer and again fractionated into 1KS and 1KP. The 1KS fractions were pooled and fractionated either by isopycnic or discontinuous sucrose density gradient sedimentation. The isopycnic sucrose density gradients were constructed by layering a 20 ml 20–55% sucrose gradient on top of a 4.0 ml 60% sucrose cushion and allowed to set at 4°C overnight. A 1.5 ml cushion of 15% sucrose was overlayed before application of 10 ml 1KS homogenate to the gradients. The gradients were then sedimented at $163000 \times g$ for 45 min (4°C) in a Dupont-Sorvall TV850 vertical rotor. 1.5-ml fractions were collected from each gradient. Discontinuous sucrose gradients [29] were constructed by layering consecutively 5 ml 60% sucrose, 6 ml 40% sucrose, and 8 ml 15% sucrose. After layering 15 ml 1KS homogenate on top of the gradients, they were sedimented at $163000 \times g$ for 30 min (4°C) in the TV850 rotor. 1.0 ml fractions were collected and the plasma membrane fractions were combined for the bulk plasma membrane preparations.

Cell surface labelling

^{125}I-conjugated wheat germ agglutinin was prepared as described previously [28]. To label the cells, unstimulated neutrophils were incubated with ^{125}I-WGA ($6 \cdot 10^4$ cpm of ^{125}I-WGA ($2 \cdot 10^7$ cpm/μg) per 10^8 cells) in cell resuspension buffer at 0–4°C for 5 min, washed, and resuspended in the same buffer. Surface ^{125}I-WGA was assayed directly using a gamma radiation counter (Iso-Data). As reported previously [28], WGA at the levels used here does not interfere with normal stimulation of superoxide production and the cells remain functionally viable. In addition, WGA at these levels does not alter the sedimentation distributions of the other plasma membrane markers [30].

Biochemical assays

Cytochrome *b*-559 was quantitated by reduced-minus-oxidized difference spectroscopy on a Perkin-Elmer Lambda 4C dual beam spectrophotometer (Perkin-Elmer Corp., Newark, NJ) assuming an absorption coefficient of $2.16 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [31]. Samples were reduced by the addition of 5 μl of a 1.0 M solution of sodium dithionite made up in H_2O immediately before use. Protein was measured by the Bradford method [32] and the BCA method as described by Pierce (Rockford, IL) using BSA as a protein standard. Total flavin content was determined by the alkaline photohydrolysis of all flavin to lumiflavin followed by organic extraction in chloroform [33]. Alkaline phosphatase, myeloperoxidase, and other markers were measured as described by Jesaitis et al. [28].

Detergent extraction of membranes and sedimentation analysis

Membranes prepared from sucrose gradients (discontinuous) were extracted with several detergents above their respective critical micelle concentrations at detergent/protein ratios in excess of 5:1. Extraction was carried out at 4°C for 30 to 60 min in buffer originally employed by Lenk et al. [34] to isolate cytoskeletons from HeLa cells and applied to neutrophils by Jesaitis et al. [35]. Subsequent to extraction the membranes were sedimented in a Beckman 50Ti rotor at 45000 rpm ($180000 \times g$ at $r_{m,ax}$) for 2 h at 4°C. Pellets were carefully decanted by gentle aspiration of all but 100 μl of the original volume (2 ml). Pellets were then resuspended in the original extraction buffer by sonication using a Heat Systems-Ultrasonics sonicator (Heat Systems Ultrasonics, Inc., Plainville, NY) for three 5-s bursts and then analysed for superoxide generating capacity, flavin, cytochrome, actin, and protein content. The pellets were also further fractionated by sedimentation in rate zonal, detergent-containing sucrose gradients (10–40% w/w). The sedimentation was carried out in a Dupont-Sorvall TV865 vertical rotor at 45000 rpm ($180000 \times g$ at r_{av}) for 36 min. Fractions (0.3 ml) were collected for further content analysis. An estimate for the sedimentation coefficient for the superoxide generating system was derived from application of the relevant 'SEA' chart for the TV865 rotor.

Measurement of superoxide anion generation

Superoxide generation was measured in two ways. The first method employed ELISA microtiter plate wells as cuvettes and reaction chambers. 200 μl reaction buffer (0.65 mg/ml cytochrome C, 2 mM $MgCl_2$, 2 mM NaN_3, and 10 mM Hepes (pH 7.4)) with and without 100 U/ml superoxide dismutase (SOD) were aliquoted into ELISA plates. 5-μl samples of the gradient fractions were then mixed into the reaction buffer and the reaction was started by the addition of 10 μl 2.3 mM NADPH. The absorbance at 550 nm was recorded as a function of time in 1-min intervals in the 'Repeated

Reads' mode on a Biotek Model EL310 ELISA plate reader with a 550 nm interference filter. Alternatively, activity was measured in conventional 1.6-ml microcuvettes utilizing 650 μl reaction buffer and 50 μl gradient sample. The reaction was initiated by the addition of 10 μl 10 mM NADPH (140 μM final concentration) and monitored at 550 nm on the Perkin-Elmer Lambda 4C dual beam spectrophotometer. After 5 min, 10 μl of 50000 U/ml SOD were added to the reaction and the reaction was again monitored. SOD-insensitive activity was constant over the entire time interval studied, even if it was measured at $t = 0$. The rate of superoxide dismutase inhibitable cytochrome C reduction was calculated using a value of $1.85 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for the absorption coefficient of the reduced-minus-oxidized forms of cytochrome C.

The stability of the superoxide generating activity in our plasma membrane preparations was also investigated. PMA-stimulated neutrophil membranes prepared from discontinuous sucrose gradients as described above were assayed immediately for SOD inhibitable superoxide generating activity on the Perkin-Elmer Lambda 4C dual beam spectrophotometer using the conventional method as described above. Aliquots of the membranes were then stored at 4°C and −70°C and measured for superoxide generating activity at specified times. For time points of 0, 1, 4, and 7 days, the superoxide generating activity was measured for each of the two storage conditions. The membranes stored at 4°C had specific activities of 45.4 ± 2.1, 33.1 ± 2.0, 20.6 ± 5.8, and 16.8 ± 4.6 nmol/min per mg, respectively (expressed mean ± S.D.; $n = 3$), and those membranes stored at −70°C had activities of 45.0 ± 1.6, 42.7 ± 1.7, 42.5 ± 1.0, and 41.7 ± 1.0 nmol/min per mg, respectively (mean ± S.D.; $n = 3$). Thus, there was marked instability of the oxidase system if the samples were stored at 4°C, but the activity could be stabilized by storing them at −70°C if immediate assay was not possible.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out at room temperature using 9% polyacrylamide slab gels or 7–18% gradient gels containing 0.1% (w/v) SDS [36]. Fraction samples were mixed with an equal volume of sample buffer (1 part 10% SDS (w/v) in H_2O, 1 part 0.5 M Tris base (pH 6.8), 1 part glycerol, 0.03% Bromophenol blue, and 500 mM 2-mercaptoethanol) and then applied to the gel. The electrophoretic mobility of the samples was compared with the mobility of prestained standard proteins. Proteins were visualized on the gels by first staining for 30 min with 0.1% Coomassie blue G in 50% methanol and 10% acetic acid. Gels were then destainecd in 25% isopropanol/10% acetic acid and hydrated in H_2O for two days. Hydrated gels were then silver stained under basic conditions as described by Wray et al. [37].

Western blotting experiments

Electrophoretic transfer of proteins from SDS-polyacrylamide gels onto Zeta-Probe blotting membranes was performed according to Bio-Rad instructions. Briefly, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine) for approximately 20 min. At the same time, the zeta-probe membrane, filter paper, and fiber pads were also soaked in transfer buffer. The transfer cassette was then assembled and transferred for approx. 200 volt-h with cooling (15°C) in a Bio-Rad Trans-Blot cell. After transfer, the Zeta-Probe membranes were first incubated for at least 1 h in blocking buffer consisting of 10% goat serum and 3% BSA in 0.5 M NaCl and 10 mM Hepes (pH 7.4). The transfers were then incubated for 3 h with 1 μg/ml dilutions of rabbit IgG (anti-actin or anti-fodrin) or 1/1000 dilutions of mouse IgM (monoclonal anti-actin) in DPBS (−) plus 3% goat serum, 1% BSA, and 0.2% Tween 20. After rinsing the Zeta-Probe five times with wash buffer (0.25 M NaCl, 10 mM Hepes, 0.2% Tween 20 (pH 7.4)), it was then incubated for 1 h at 20°C with 1 μg/mol of peroxidase conjugated goat anti-rabbit IgG (or anti-mouse IgM) in DPBS (−) plus 3% goat serum, 1% BSA, and 0.2% Tween 20. After rinsing five times with wash buffer, the transfers were color developed for 5–30 min in a solution of developer consisting of 30% methanol, 0.5 mg/ml peroxidase color developer (4-chloro-1-naphthol), and 5 mM H_2O_2 in 0.25 M NaCl, 10 mM Hepes (pH 7.4). The reaction was terminated by the transfer of the Zeta-Probe membrane to distilled H_2O.

Results

Subcellular distribution of the components of superoxide generation in PMA-stimulated human neutrophils

To study the molecular organization of the superoxide generating system in the plasma membrane of stimulated human neutrophils, highly purified plasma membranes were prepared by N_2 cavitation and sucrose density gradient sedimentation. The subcellular distribution of organelles was analyzed by assaying for the presence of distinct organelle markers in the sucrose density gradient fractions. The peak activities of the plasma membrane markers, alkaline phosphatase and ^{125}I-wheat germ agglutinin surface label; the specific granule marker, lactoferrin; and the azurophil granule marker, myeloperoxidase, sedimented to approximately the same density as has been previously reported by our laboratory for unstimulated human neutrophils [38]. The peak Golgi markers sedimented at 26–28% sucrose for the light Golgi and at 41–43% sucrose for the heavy Golgi which was coincident with the specific granule/

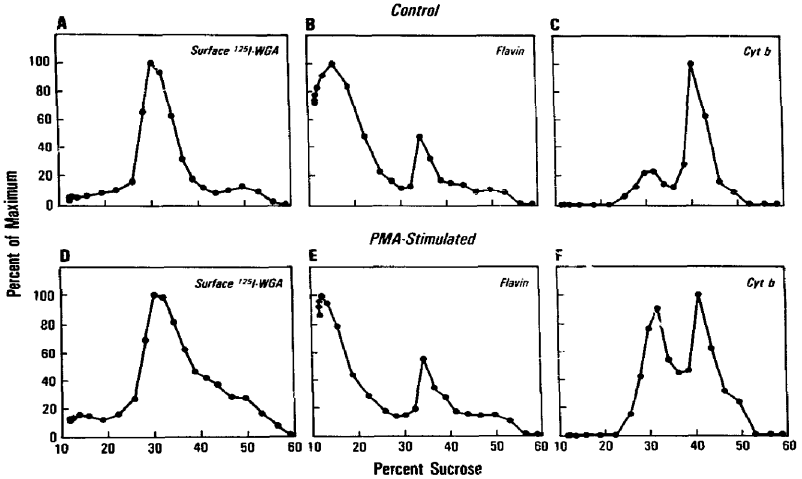

Fig. 1. Subcellular distribution of surface ^{125}I-wheat germ agglutinin (^{125}I-WGA) and NADPH oxidase components (flavin and *b*-cytochrome) from control and PMA-stimulated neutrophils on isopycnic sucrose density gradients. $5 \cdot 10^8$ neutrophils prelabeled with $3 \cdot 10^5$ cpm (15 ng) ^{125}I-WGA were washed and divided into two equal aliquots. Control cells (top row) were warmed to 37°C with no stimulation. The PMA-stimulated cells (bottom row) were warmed to 37°C and stimulated with 1 μg/ml PMA for 5 min. The cell suspensions were cooled to 4°C, washed, and disrupted by N_2 cavitation. Fractionation of the membranes was performed as described under Materials and Methods. ^{125}I-WGA (panels A and D), flavin (panels B and E), and *b*-cytochrome (panels C and F) were measured in the fractions as described under Materials and Methods. Percent of maximal activity recovered from the gradient is plotted as a function of percent sucrose (w/w). 100% activity levels represent: panel A, 3575 cpm; panel B, 76.4 pmoles; panel C, 237.0 pmoles; panel D, 1367 cpm; panel E, 62.2 pmoles; and panel F, 160.3 pmoles. 75–110% of the activity applied to the gradients was recovered. One of three experiments.

cytochrome *b* peak activities (not shown). As noted previously [38], the distributions of alkaline phosphatase and surface marker ^{125}I-WGA were essentially coincident (not shown).

Closer inspection of the plasma membrane distribution and comparison to that obtained from unstimulated cells revealed significant and reproducible differences. In unstimulated cells, the ^{125}I-WGA surface marker completely sedimented into the gradient in a unimodal distribution (Fig. 1). The location of the peak ^{125}I-WGA occurred at essentially the same density for PMA-stimulated cells. However, in the latter case a shoulder became evident in the denser fractions (34–44% sucrose) possibly suggesting a reorganization of plasma membrane glycoproteins to a denser subpopulation of plasma membrane vesicles analogous to those recently reported in our laboratory [27]. In addition, this redistribution was observed over a wide range of PMA concentrations membrane (0.1–5 μg/ml).

Analysis of the content of cytochrome *b*-599 in the sucrose gradient fractions suggested a dual localization (Fig. 1) with one peak cosedimenting with the plasma membrane markers (30–32% sucrose) and the other in the region containing the specific granule/Golgi markers (41–43% sucrose). Approximately 20–30% of the total *b*-cytochrome coisolated with the plasma membrane in control cells, while there was a significant increase in *b*-cytochrome cosedimenting with the plasma membrane after stimulation of the cells with PMA. A corresponding decrease in *b*-cytochrome associated with the specific granule/Golgi fraction was observed. These results are in agreement with those of Borregaard et al. [39] and Borregaard and Tauber [40] who previously reported a PMA induced redistribution of *b*-cytochrome. Because the membrane- and specific granule/Golgi-derived cytochrome profiles overlap significantly in the region of the dense plasma membrane fractions, it is possible only to give a rough estimate of the maximum amount of cytochrome *b*-559 available in those fractions (approx. 25% of the total cellular cytochrome or approx. 39% of the total plasma membrane-bound cytochrome).

Analysis of the neutrophil flavin also resulted in a bimodal distribution with the majority of flavin cosedimenting with cytosolic fractions (Fig. 1). Approximately 8–10% of the flavin sedimented at a density of 1.15–1.16 (approx. 34–36% sucrose) which was slightly higher than the density where the peak activity of the plasma membrane markers were located. This membrane associated flavin activity sedimented at the same location (34–36% sucrose) in both control and PMA-stimulated cells and matched the density observed for the new shoulder of ^{125}I-WGA. This result confirms the previous work of Parkos et al. [38] and indicates that the flavin cosediments more closely with elements found in the dense plasma membrane fraction.

Subcellular localization of superoxide production

The sedimentation patterns of the two putative components of superoxide generation appear to have slightly differing distributions on the sucrose gradients. Thus, it was essential to determine where superoxide generating activity was localized. Purified human neutrophils were stimulated with 1 μg/ml PMA and fractionated on isopycnic sucrose density gradients as described under Materials and Methods. On typical sucrose gradients in which superoxide was measured, the average recovery of superoxide generating activity was 12.9 ± 1.5 and 25.3 ± 1.8 nmol/min per mg (mean ± S.D., $n = 2$) for the 1KS homogenate and peak gradient fraction, respectively. Given that the membrane activity was not measured in the presence of detergent, which increases activity by approximately a factor of two for all the gradient or crude fractions [21], the reported values conform to those previously reported for membranes isolated from PMA-stimulated neutrophils.

The distribution of superoxide generating activity on isopycnic sucrose gradients is shown in Fig. 2 (panel B). The peak activity occurred at a density of 1.15–1.16 (approx. 34–36% sucrose). This peak activity cosedimented with the neutrophil flavin but only exhibited alignment with a shoulder of the *b*-cytochrome. Since PMA-stimulated superoxide generating activity has been previously shown to be surface in origin [38], the distribution reported here confirms that this activity is indeed located to a distinct population of plasma membrane vesicles sedimenting at a higher density than the bulk plasma membrane. This result also suggests that superoxide generation is restricted to a population of plasma membrane vesicles which either contain both flavin and *b*-cytochrome or contain a mixture of flavin-bearing and cytochrome-bearing vesicles.

Distribution of actin and fodrin

Recent studies by Jesaitis et al. [27] have demonstrated the presence of actin/fodrin-rich plasma membrane microdomains in subcellular fractions prepared from human neutrophils. The presence of a denser

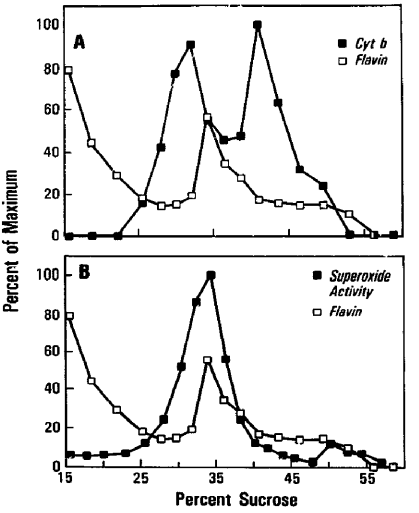

Fig. 2. Subcellular localization of NADPH oxidase components and superoxide generating activity from PMA-stimulated neutrophils. $1 \cdot 10^8$ neutrophils were stimulated and fractionated on isopycnic sucrose density gradients as described in Fig. 1 and under Materials and Methods. Flavin (panels A and B), *b*-cytochrome (panel A), and superoxide activity (panel B) were measured in fractions as described under Materials and Methods. Percent of maximal activity recovered from the gradients is plotted as a function of percent sucrose (w/w). 100% activity levels represent 62.2 pmoles flavin, 160.3 pmoles cytochrome *b*, and 95 pmoles $O_2^{\cdot-}$. The specific activity of the peak fraction was 27 nmol $O_2^{\cdot-}$/min per mg protein. 85–110% of the activity applied to the gradients was recovered. One of three experiments.

subpopulation of plasma membrane vesicles in the present studies suggested that these vesicles may also be enriched in cytoskeletal proteins such as actin and/or fodrin. Therefore, the sucrose gradient fractions were analyzed for actin and fodrin using SDS-PAGE and Western blotting techniques as described under Materials and Methods. The top panel of Fig. 3 shows a silver-stained polyacrylamide gel of the gradient fractions. The locations of the peaks of the various organelle markers and superoxide generating activity are indicated. Western blots using anti-fish skeletal muscle actin antibody [41] and anti-human brain fodrin antibody [42] are also shown in Fig. 3 (middle and lower panels, respectively). As shown, the peak actin (34–36% sucrose) and peak fodrin (34–36% sucrose) bearing particles sedimented at approximately the same location in the

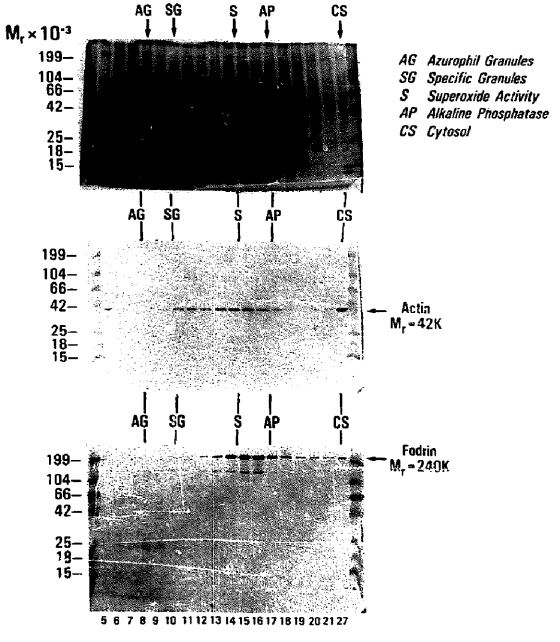

Fig. 3. Cosedimentation of actin and fodrin with NADPH oxidase activity on isopycnic sucrose density gradients. $1 \cdot 10^8$ neutrophils were stimulated with 1 μg/ml PMA and fractionated as described in Fig. 1 and under Materials and Methods. Fractions were subjected to SDS-polyacrylamide gel electrophoresis on 9% gels. The gels were fixed and silver stained as described under Materials and Methods. The upper panel shows the silver-stained profile. Fractions were also analyzed for actin (middle panel) and fodrin (lower panel) content using Western blotting techniques with anti-fish skeletal muscle actin antibody and anti-human brain fodrin antibody, respectively, as described under Materials and Methods. The positions of the peak activities of the subcellular organelle markers and superoxide activity are shown above the gel and between the blots: Azurophil granules (AG), specific granules (SG), superoxide activity (S), alkaline phosphatase activity (AP), and cytosol (CS). Fraction number is indicated at the bottom of the figure with fraction 1 representing 59% sucrose (w/w) and fraction 27 representing 15% sucrose (w/w). One of two experiments.

sucrose density gradient as the peak flavin and peak superoxide producing fractions.

Detergent insolubility of the superoxide generating complex

The distribution of flavin and superoxide generating activity appears restricted to an actin/fodrin enriched subpopulation of vesicles, suggesting that the membrane skeleton might be participating in organizing these components on the cell surface. Such participation may result from the direct or indirect interaction of superoxide generating components with components of the membrane skeleton. To test this hypothesis, purified plasma membranes were prepared on step gradients as described in Materials and Methods. These membranes were then exposed to several different detergent cocktails previously used to prepare cytoskeletons from intact cells [35]. After incubation for 30–60 min at 4°C, these extracts were sedimented at 180000 × *g* for 2 h at 4°C in an ultracentrifuge. The recoveries of flavin, cytochrome *b*, and superoxide generating activity in the pellet and supernatants are shown in Table I. Ap-

TABLE I

Coisolation of superoxide generating activity and oxidase components from stimulated neutrophils with the detergent-insoluble membrane skeletal matrix

Sample	Pelleted superoxide generating activity		Distribution of flavin (pmole)		Distribution of cytochrome (pmole)		Pellet flavin/cytochrome ratio	Pelleted protein (μg)
	nmol/min per mg	% total	pellet	supt.	pellet	supt.		
Control	20.3±3.4	(100)	9.6±4.3	2.9±1.7	26.0±8.1	0±0	1:2.9±0.5	30.2±6.4
0.1% Lubrol	20.7±1.4	96.5±8.5	3.8±1.0	4.4±1.0	21.5±0.7	0±0	1:6.0±1.4	23.5±4.5
0.1% Renex	19.6±4.9	95.3±8.6	4.1±2.3	9.2±3.5	16.4±0.8	13.5±2.0	1:5.0±3.7	21.7±4.7
0.5% Triton	7.2±1.6	35.0±2.2	3.5±2.0	7.2±3.1	13.6±4.3	13.0±5.0	1:5.5±3.6	20.7±4.4

Human neutrophils ($1 \cdot 10^8$/ml) stimulated with 5 μg/ml PMA as described under Materials and Methods were disrupted by N_2 cavitation and fractionated using a discontinuous sucrose density gradient. Similar results were obtained using 1 μg/ml and 100 ng/ml PMA (not shown). Purified plasma membrane fractions were collected and treated for 30 min at 4°C with detergents as indicated, and the mixture was then centrifuged at 180000 × *g* for 2 h at 4°C. Control membranes were treated identically but without detergents. The supernatants and pellets were collected and assayed for SOD-inhibitable O_2^- activity, flavin, cytochrome *b*, and protein as described under Materials and Methods. Negligible O_2^- activity was recovered in the supernatants for all samples. 71–98% of applied flavin and 80–100% of applied cytochrome and protein was recovered in the samples. The results are expressed as mean ± standard deviation, $n = 3$.

proximately 85–105% of the superoxide generating activity was recovered in the pellet fraction of Renex extracts suggesting that the activity was coupled to a large sedimentable matrix generally interpreted as the membrane skeleton. Extraction with Triton X-100 resulted in lower recovery of superoxide generation, possibly because the integrity of the membrane skeletal matrix was being destroyed. Indeed, shorter extraction times (15 min) with Triton X-100 resulted in a greater recovery of activity (approx. 50% recovery). A recent abstract by Babior and co-workers [43] reporting the association of oxidase activity, cytochrome, and members of the 48 kDa phosphoprotein family with the Triton X-100 insoluble pellet from PMA-stimulated neutrophils supports these findings. No activity was recovered after deoxycholate treatment.

In the Renex treated samples, approximately 70–80% of the membrane-bound flavin was not sedimentable, suggesting that only 20–30% was relevant to superoxide production. Since the solubilization was performed on fractions obtained from bulk plasma membrane preparations obtained from discontinuous sucrose gradients (see Materials and Methods), much of the *b*-cytochrome was derived from the light plasma membrane fraction. Thus, its solubilization would be consistent with the lack of cytoskeletal interactions in those fractions. Indeed, a higher percentage of cytochrome *b* is solubilized from the light fractions than from the heavy membrane fractions (data not shown). Although much of the cytochrome from the heavy membrane fractions remains insoluble under conditions which maintain the integrity of the superoxide generating system, it can be completely solubilized under appropriate conditions, i.e., when cytoskeletal matrix proteins are removed by 1 M NaCl wash of the membranes [44].

Approximately 30% of the total protein was solubilized by our extraction procedure, as judged by protein determination and SDS-PAGE of the pellet and supernatant fractions of the detergent extracts retaining superoxide activity, indicating that much of the protein remained associated with the cytoskeletal matrix under these conditions. Fig. 4 (upper panel), which shows the protein composition of pellets and supernatants (at 10-fold higher dilution), demonstrates the extraction of a number of major protein species (e.g., at $M_r = 180000$, 85000, 70000, 42000, 22000, 18000). One of the most abundant bands in the pellet fractions which appears enriched in the Renex and Triton pellets is of $M_r = 42000$, the molecular weight of actin. Western blot analysis of such fractions using monoclonal anti-actin antibody indicated that this band did indeed contain actin, as is shown in Fig. 4 (lower panel).

Sedimentation of superoxide generating activity in detergent-containing sucrose gradients

In order to gain a rough estimate of the size of the superoxide generating particles, the pellets of the above Renex extract were resuspended in the extraction buffer, layered onto 0.1% Renex-containing 10–40% sucrose gradients and sedimented for 0.6 h at 180000 × *g* in a Dupont-Sorvall TV865 vertical rotor. The fractions were then analyzed for superoxide generating activity, flavin, and cytochrome *b*. Fig. 5 (upper panel) shows the distribution of these activities normalized to maximal activity. Clearly, the flavin, cytochrome, and superoxide generating activity peaks cosedimented, and the peak fraction had specific activity of approximately 49 nmol/min per mg which was over twice that of the original detergent insoluble pellet (approx. 22 nmol/min per mg). The flavin to cytochrome molar ratio in the peak fraction was 1:3.5. From the sedimentation rate, an approximate apparent sedimentation coefficient of 80S was estimated, suggesting that the oxidase components were part of a high molecular weight complex of

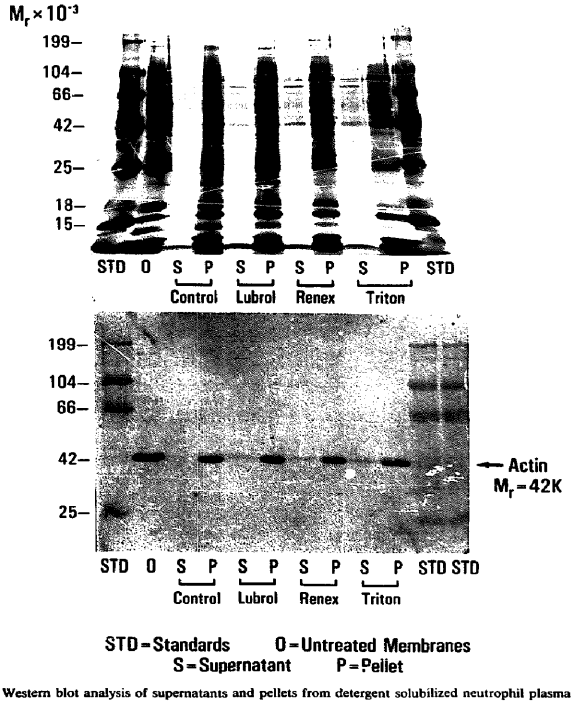

Fig. 4. SDS-PAGE and Western blot analysis of supernatants and pellets from detergent solubilized neutrophil plasma membranes. PMA-stimulated neutrophil membranes isolated by discontinuous sucrose density gradients (see Methods) were solubilized for 30 min at 4°C in solubilization buffer (2 μM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Hepes (pH 7.4)) containing 0.1% Lubrol WX, 0.1% Renex 30, or 0.5% Triton X-100. The mixture was centrifuged at 180000 × g for 2 h (4°C) and supernatant and pellet fractions were collected. Control membranes were treated identically but without detergents. In every case, 80–90% of the total applied protein was recovered. The samples were then subjected to SDS-PAGE on 7–18% gradient gels (upper panel) or 9% slab gels (lower panel) as described under Materials and Methods. The upper panel shows the silver-stained profile of the supernatants (S) and pellets (P) from each of the separate conditions as well as the profile of the original, untreated membranes (O). The lower panel shows the same fractions analyzed for actin content using Western blotting techniques with monoclonal anti-actin antibody as described under Materials and Methods. One of two experiments.

proteins roughly the size of ribosomes. SDS-PAGE analysis of the gradient fractions showed that a number of additional proteins did not sediment with the main peak suggesting that they had been extracted and were, therefore, irrelevant to superoxide generating activity in the detergent extracts. Actin distribution was analyzed by densitometric scanning of the 42 kDa actin band in the SDS-polyacrylamide gels. As shown in Fig. 5 (middle panel), a large part of the actin did not sediment, part was pelleted, and the rest (approx. 17% of the total) sedimented with the superoxide generating activity. Western blots of the gradient fractions using anti-fish skeletal muscle actin antibody [41] (see Methods) confirmed the actin distribution shown in Fig. 5 (data not shown). Such a distribution suggests that some of the actin was associated with even larger complexes that did not include the above superoxide components and that some selectivity in structure was retained. The distribution of protein on the gradient (Fig. 5, lower panel) also indicated a wide range of particle sizes distributed across

the gradient; however, only a small fraction of the total protein was associated with the active peak.

Discussion

Previous studies on neutrophils and other cell systems provide evidence that the plasma membranes of unpolarized cells are regionalized and laterally differentiated. These include early studies of patching and capping [45], localization of specific receptor and cytoskeletal proteins to focal contacts [46], restriction of surface proteins to specific regions of sperm cells [47], and studies of platelet membrane organization [48]. In the neutrophil, Tsunawaki [49] observed membrane heterogeneities, suggesting the possible existence of different populations of plasma membrane fragments in cell homogenates of activated neutrophils. Our recent studies [27] suggested a lateral segregation of occupied chemotactic peptide receptors to plasma membrane microdomains enriched in cytoskeletal proteins but depleted of transducing proteins. This segregation appeared functionally relevant since it correlated with the homologous desensitization of the cell. Consequently, the investigation reported here was initiated to explore whether an analogous organization of plasma membrane components of superoxide production existed and whether it could possibly be relevant to the function of the respiratory burst.

The results of our studies demonstrate the presence of two distinct subpopulations of plasma membrane vesicles formed upon nitrogen cavitation of phorbol myristate acetate-stimulated human neutrophils. The light population (30–32% sucrose) contains the majority of alkaline phosphatase activity, ^{125}I-WGA surface label, and cytochrome *b*-599. The heavy population (34–36% sucrose) contains most membrane bound flavin, actin, and fodrin. It also contains a small fraction of the plasma membrane markers and cytochrome *b*, but the limits of resolution on the gradients precluded precise quantitation of the relative amounts belonging to each vesicle population. The plasma membrane wheat germ agglutinin binding sites and alkaline phosphatase activities appear to increase in this heavy fraction upon activation with phorbol ester, but flavin content is invariant. In addition, NADPH-dependent superoxide generating activity is associated only with the heavy

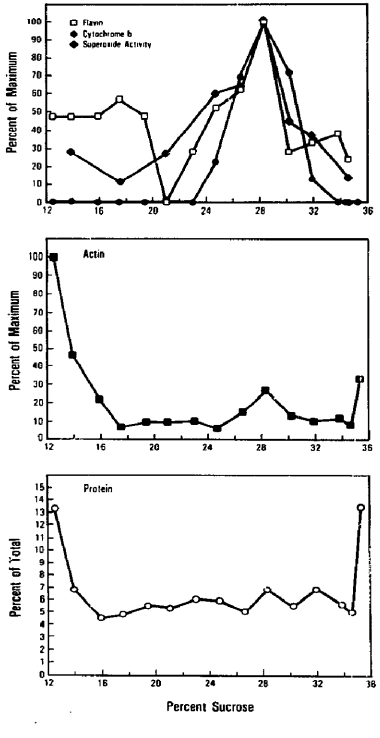

Fig. 5. Cosedimentation of superoxide generating activity, cytochrome *b*, and flavin in detergent-containing sucrose gradients. Membranes (200 μl) prepared from discontinuous sucrose gradients as described under Materials and Methods were extracted with a detergent cocktail containing 0.1% Renex 30 and sedimented at 180000× *g* (r_{max}) for 2 h at 4°C. The detergent insoluble pellets were resuspended in extraction buffer by sonication and further fractionated in rate-zonal, detergent-containing sucrose gradients as described under Materials and Methods. The fractions were analyzed for superoxide generating activity, cytochrome *b*, and flavin as described under Materials and Methods (upper panel). Percent of maximal activity recovered from the gradients is plotted as a function of percent sucrose (w/w). 100% activity levels represent 0.75 pmole flavin, 2.6 pmoles cytochrome *b*, and 87.9 pmoles O_2^-/min. The specific activity of the peak fraction was 49 nmole O_2^-/min per mg protein. 87% of the flavin and 90% of the cytochrome *b* applied to the gradients was recovered. Fractions were subjected to SDS-polyacrylamide gel electrophoresis on 7–18% gradient gels. The gels were fixed and silver stained as described under Materials and Methods. The gels were then photographed and the negative was scanned using a Zeineh laser densitometer (Biomed Instruments, Inc., Chicago, IL). The middle panel shows the distribution of the 42 kDa actin band as determined densitometrically. The lower panel shows the protein profile plotted as percent of total protein recovered in the gradient as a function of percent sucrose (w/w). 26.4 μg protein was added per gradient and approximately 90–95% of the added protein was recovered. One of two experiments.

fraction. Recent studies by Borregaard et al. [50] suggested that a novel intracellular vesicular pool of alkaline phosphatase activity was localized to fractions slightly more dense than the plasma membrane; however, this population of vesicles was fused to the plasma membrane after PMA-stimulation and cosedimented with surface alkaline phosphatase activity. In contrast, the heavy plasma membrane vesicles we observe are present even after stimulation and are, therefore, distinct from those reported by Borregaard and co-workers [50].

The localization of NADPH oxidase activity to a plasma membrane population containing all membrane-bound flavin but only a minor fraction of the cytochrome *b*-559 implies that only a small fraction of the cytochrome is involved in superoxide production. This sub-fraction probably complexes with the flavin bearing component in a larger, rapidly sedimenting detergent stable complex. Furthermore, since NADPH-dependent superoxide production is absent in the lighter plasma membrane population (which contains a significant amount of *b*-cytochrome), we conclude that an organizational framework may be necessary to promote and control interaction of the components of superoxide generation. Further support for the role of an organizational requirement for superoxide generation in intact cells is suggested by studies in which superoxide production was reconstituted in specific granule fractions [51] and in light plasma membrane fractions [26,52,53] using cytosol and membrane perturbing concentrations of SDS and arachidonate (approx. 100 μM).

Our results further strengthen the argument that there is a direct participation of neutrophil cytochrome *b* in superoxide generation and electron transport. Since a relatively small percentage of the cytochrome is necessary for superoxide production in membranes, arguments employing low cytochrome recoveries in purified NADPH oxidase preparations are weakened. In addition, our studies add an organizational dimension to this discussion. This organizational aspect may be of critical importance in the intact cell when it is considered that superoxide production should be vectorially targeted to microbial surfaces in contact with the neutrophil. The cytochrome, being a transmembrane heme protein [16,43], would be the logical electron transferase [54].

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