Respiratory Burst Enzyme in Human Neutrophils

EVIDENCE FOR MULTIPLE MECHANISMS OF ACTIVATION

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ABSTRACT Alteration of the surface of human neutrophils with the nonpenetrating, protein-inactivating agent *p*-diazobenzenesulfonic acid (DASA) was found to prevent activation of the respiratory burst by some stimuli, but not others. Production of superoxide anion (O_2^-) stimulated by concanavalin A or the chemotactic peptide formyl-methionyl-leucyl-phenylalanine FMLP was inhibited by DASA pretreatment, whereas O_2^- production stimulated by phorbol myristate acetate (PMA), sodium fluoride, or the ionophore A23187 was not inhibited by DASA. Pretreatment with DASA inhibited oxygen uptake stimulated by FMLP, but not oxygen uptake stimulated by PMA. DASA reproducibly inhibited activities of two known surface enzymes, Mg^{++}-ATPase and alkaline phosphatase, by 45–55% and 60–70%, respectively. The inhibition by DASA of O_2^- production did not appear to be caused by interference with binding of the affected stimuli, since pretreatment with DASA did not inhibit release of the lysosomal enzymes lysozyme and myeloperoxidase induced by concanavalin A or FMLP. Membrane-rich particulate fractions from neutrophils have been shown to contain NADPH-dependent oxidative activity that is presumably responsible for the phagocytosis-associated respiratory burst of intact cells. The PMA-activated enzyme was susceptible to inhibition if directly exposed to DASA in this particulate fraction. These findings suggest that more than one mechanism exists for activation of the respiratory burst oxidase in human neutrophils, and that the neutrophil possesses at least one oxidase that is not an ectoenzyme.

INTRODUCTION

Phagocytosis by human neutrophils induces a dramatic stimulation of their oxidative metabolism, during which oxygen is converted to superoxide anion (O_2^-), hydrogen peroxide, and hydroxyl radical (1). These oxygen metabolites are essential to the microbicidal function of the neutrophil (1, 2), and the biochemical basis of their generation is of great interest. The enzyme generally believed to initiate this respiratory burst is an oxidase which utilizes as electron donor NADPH, NADH, or both (1, 3). It has not yet been purified or well characterized, and its location in the neutrophil is still controversial (3, 4).

Activation of the respiratory burst enzyme is initiated by perturbation of the plasma membrane and does not require phagocytosis. A number of soluble compounds are effective stimuli, including chemotactic factors, detergents, lectins, certain lipids, and ionophores (5). Activation of the respiratory burst appears to involve calcium ion with some of these stimuli but not with others (6, 7), raising the possibility that different stimuli activate the oxidase by different mechanisms. One possible approach to this question would be to test the effects of chemically altering the plasma membrane on the ability of various stimuli to activate oxidative metabolism.

One chemical agent previously used to inhibit surface membrane proteins is *p*-diazobenzenesulfonic acid (DASA)[1] (8). Pretreatment of neutrophils by DASA was reported to inhibit the ability of the lectin concanavalin A (Con A) to stimulate O_2^- production (9). This was widely interpreted as evidence that the O_2^- forming oxidase was an ectoenzyme, but the experiments also raised the possibility that another step in the pathway of activation used by that stimulus was affected. We found that DASA pretreatment did not inhibit stimulation of O_2^- production by the surface-active lipid phorbol myristate acetate (PMA). This prompted us to survey the influence of DASA pretreatment on activa-

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[1] *Abbreviations used in this paper*: Con A, concanavalin A; DASA, *p*-diazobenzenesulfonic acid; DMSO, dimethyl sulfoxide; FMLP, formyl-methionyl-leucyl-phenylalanine; KRP-D, Krebs-Ringer phosphate buffer with dextrose; LDH, lactate dehydrogenase; PBS-D, phosphate-buffered saline with dextrose; PMA, phorbol myristate acetate.

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tion of the respiratory burst by a variety of soluble stimuli. The results, described below, suggest that more than one mechanism for activating the respiratory burst exists in human neutrophils.

METHODS

Isolation of neutrophils and particulate fractions. Human neutrophils were isolated from heparinized venous blood by dextran sedimentation and Ficoll-Hypaque centrifugation (2). Cells were counted in a hemocytometer and suspended at the appropriate concentration in Krebs-Ringer phosphate buffer, pH 7.34, containing 0.2% dextrose (KRP-D). Preparations routinely contained 97–99% granulocytes.

Particulate fractions were isolated as described (10, 11). Briefly, neutrophil suspensions at 5×10^7/ml were incubated for 3 min at 37°C with 1 mM KCN or 1 mM NaN_3 (12) and PMA (Consolidated Midland Corp., Brewster, N. Y.) dissolved in dimethylsulfoxide (DMSO) at a final concentration of 100 ng/ml, or with an equivalent amount of DMSO. In some cases (as noted in Results), particulate fractions were prepared from cells incubated in a similar manner for 3 min with opsonized zymosan (10), at a final zymosan concentration of 11 mg/ml but in the absence of KCN or NaN_3. Treated cell suspensions were diluted with an equal volume of cold KRP-D and were centrifuged in the cold for 8 min at 180 *g*. Cells were resuspended in cold 0.34 M sucrose to 2.5×10^7/ml and sonicated immediately in an ice-water bath for 1.5 min at a setting of 60 W for 30-s intervals alternating with cooling (Branson Sonifier, Model W185; Branson Sonic Power Co., Danbury, Conn.). Sonicates were centrifuged at 500 *g* for 10 min; supernates were then centrifuged at 27,000 *g* for 15 min. The pellets from this final centrifugation (particulate fractions) were resuspended in 0.34 M sucrose to a protein concentration of 1–2 mg/ml (13), and stored at −70°C.

Treatment of intact cells and particulate fractions with DASA. DASA was prepared just before use by the method of Berg (14) and dissolved in KRP-D to an estimated concentration of 5 mM. Neutrophil suspensions at 5×10^7/ml were incubated with an equal volume of this DASA solution for 10 min at 37°C. The reaction was terminated by the addition of an equal volume of 20% heat-inactivated (30 min, 56°C) fetal calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) in 0.9% NaCl, and samples were centrifuged at 180 *g* for 8 min at 4°C. Cells were washed once with the 20% fetal calf serum and once with KRP-D by centrifuging at 180 *g* for 8 min at 4°C, then resuspended in KRP-D to the desired concentration.

Particulate fractions were treated with DASA in a similar manner, except that incubation was at room temperature for 15 min to prevent inactivation of control samples at 37°C. Final DASA concentration was 2.5 mM, and final protein concentration was ~1.0 mg/ml. After incubation, the reaction was terminated with 20% heat-inactivated fetal calf serum as described above, and fractions were washed once with 0.34 M sucrose by centrifugation at 27,000 g for 30 min at 4°C. Fractions were resuspended in 0.34 M sucrose to the original concentration. Control fractions were treated with KRP-D in exactly the same manner as with DASA.

The effects of treatment with DASA at 37°C or room temperature were compared, and no differences in DASA effects on intact cell O_2^- production were observed with formyl-methionyl-leucyl-phenylalanine (FMLP) or PMA as stimuli.

Measurement of superoxide anion release. The production of O_2^- by intact cells was measured by the reduction of cytochrome *c* as previously described (2). Incubation conditions varied somewhat depending on the stimulus used. The assay buffer was KRP-D in experiments with the stimuli PMA, Con A (Sigma Chemical Co., St. Louis, Mo., Lot 16C-1790; Pharmacia Fine Chemicals, Uppsala, Sweden, Lot DB-5333), and the ionophore A23187 (kindly supplied by Dr. Robert L. Hamill of Eli Lilly, Inc., Indianapolis, Ind.). Con A was dissolved in KRP-D before use. Ionophore A23187 was stored at −70°C as a 0.1 mM stock solution in DMSO. Equivalent amounts of O_2^- were produced by normal cells stimulated with either Sigma or Pharmacia Con A; experiments with DASA were performed using Sigma Con A. Assays with the stimulus FMLP (Vega-Fox Biochemicals, Div. Newbery Energy Corp., Tucson, Ariz.) used phosphate-buffered saline containing 0.2% dextrose (PBS-D), and were performed in the presence of 1 mM $CaCl_2$. FMLP was used from a 0.1 mM stock in distilled water kept at −70°C. The use of NaF (Fisher Scientific Co., Fairlawn, N. J.) as a stimulus required a special buffer based on Dulbecco's phosphate-buffered saline, but containing only 0.117 M NaCl and 0.31 mM $CaCl_2$ (15) to correct for the electrolytes added with the NaF and to prevent the precipitation of CaF_2. In assays where less than 20 mM NaF was used, NaCl was added to balance electrolytes. All assays with NaF were performed in plastic tubes to prevent adsorption of the stimulus onto glass. NaF was used from a freshly made 0.6 M stock in distilled water.

NADPH-dependent O_2^- production by isolated particulate fractions was measured as reduction of cytochrome *c* (11). The assay mixture consisted of 0.05 M potassium phosphate buffer, pH 7.0, 0.2 mM NADPH, 0.08 mM cytochrome *c*, and 0.1–0.3 mg/ml particulate fraction. Half of the mixture was placed in the sample cuvet and half in the reference cuvet, which also contained 50 μg/ml superoxide dismutase. The assay was performed at ambient temperature and was followed continuously on a Cary 219 double-beam spectrophotometer (Varian Associates, Industrial Equipment Group, Palo Alto, Calif.). Initial slopes were used for calculations, and background in the absence of particulate fraction was subtracted.

Measurement of oxygen consumption. The consumption of oxygen by intact cells was measured with a Clark oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) (6). Assays were performed at 37°C with 1×10^7 cells in a final volume of 3 ml PBS-D, containing 1 mM $CaCl_2$. Initial rates after addition of stimuli were used for calculations.

Chemiluminescence of particulate fractions. The assay for chemiluminescence was performed as described (11). Final volume was 2.0 ml, containing 0.2 mM NADPH, 0.1 M potassium phosphate buffer, pH 7.0, and 0.1–0.2 mg/ml particulate fraction. Activity was recorded as peak counts per minute (11).

Measurements of enzyme release. In a final volume of 1.0 ml, reaction mixtures contained 4×10^6 neutrophils in KRP-D, cytochalasin B (Sigma Chemical Co., St. Louis, Mo.) at 1.0 μg/ml if present, and KRP-D or the appropriate stimulus at the following concentrations: PMA, 10 ng/ml; FMLP, 0.1 μM; Con A, 100 μg/ml; the ionophore A23187, 0.5 μM. Neutrophils were preincubated for 5 min at 37°C with or without cytochalasin B, followed by addition of buffer or stimulus. After further incubation for 20 min, samples were iced and centrifuged at 180 *g* for 8 min at 4°C. Supernates and total cell mixtures that were uncentrifuged were treated with 0.1% Triton X-100 and assayed for myeloperoxide, lysozyme, and lactate dehydrogenase (LDH) as described previously (16).

Other enzyme assays: ATPase. Mg^{++}-ATPase was assayed according to the procedure of Harlan et al. (17), using $AT^{32}P$ (New England Nuclear Corp., Boston, Mass.) purified over a column of Dowex 1-X8 (J. T. Baker Chemical Co., Phillipsburg, N. J.) before use (17). Activity was expressed as the percentage

of AT^{32}P hydrolyzed, measured by the amount of inorganic ^{32}P released.

Alkaline phosphatase. The assay for alkaline phosphatase was modified from that of DeChatelet et al. (18). The assay mix included 0.03 M 2-amino-2-methyl-1-propanol, pH 10.0, *p*-nitrophenylphosphate, 0.067 mg/ml, and 0.05–0.10 mg cell protein in a final volume of 0.6 ml. Samples were incubated for 60 min at 37°C, 3 ml of 0.1 N NaOH was added, and the absorbance was read at 405 nm.

Lactic dehydrogenase. The release of LDH was used as a measure of viability (16). Duplicate aliquots from treated neutrophils were removed; one-half of each was centrifuged at 180 *g* for 8 min at 4°C; and supernates were assayed for LDH. The other half was used to determine total cellular and extracellular LDH after treatment with 0.1% Triton X-100, then centrifugation. Supernatant LDH was expressed as a percentage of this total. In several experiments, trypan blue exclusion was also used to measure viability. In every instance, the percentage of cells excluding trypan blue paralleled the extent of cell lysis estimated by LDH release.

RESULTS

In initial experiments, we studied the effect of pretreatment of neutrophils with DASA on the ability of either PMA or Con A to trigger the respiratory burst. Cells were exposed to either DASA or buffer, then O_2^- production in the presence of the desired stimulus was measured. Results are shown in Fig. 1 as a function of time of incubation with stimulus. Both PMA and Con A stimulated control neutrophils to produce O_2^-, although PMA-stimulated release was much faster and greater. Pretreatment of the neutrophils with DASA almost completely inhibited O_2^- production in the presence of Con A, in confirmation of Goldstein et al. (9), but caused no inhibition when the stimulus was PMA.

Results obtained with all of the stimuli examined are shown in Fig. 2, in which O_2^- release is expressed as a function of stimulus concentration. The tripeptide FMLP was a more effective stimulus for O_2^- release than Con A, but its stimulatory activity, like that of Con A, was significantly inhibited by DASA pretreatment of the cells. On the other hand, DASA pretreatment did not prevent activation of oxidase function to normal levels by the stimuli PMA, NaF, or A23187 at any concentration of stimulus examined (Fig. 2). Thus, the ability of DASA to inhibit the respiratory burst in intact cells was dependent on the stimulus used.

The opposing effects by DASA pretreatment on FMLP- and PMA-mediated stimulation of the respiratory burst were confirmed by measurements of oxygen consumption, as shown in Table I. DASA pretreatment of neutrophils markedly inhibited FMLP-stimulated consumption of oxygen, but had no effect on oxygen consumption stimulated by PMA. DASA pretreatment enhanced PMA-mediated O_2^- release, but did not affect PMA-mediated oxygen consumption.

The inhibition by DASA of FMLP- and Con A-mediated O_2^- production could be caused by interference with binding of these stimuli to the neutrophil. If so, then another function of stimulus binding, the release of lysosomal enzymes, should also be inhibited by DASA pretreatment. This was not the case for release of either lysozyme or myeloperoxidase, as shown in Fig. 3 for lysozyme. Studies were performed in the presence and absence of cytochalasin B because only low levels of enzyme release were observed with some stimuli in the absence of this agent. With or without cytochalasin B, however, spontaneous release of lysozyme and release induced by any of the stimuli were increased after DASA treatment of the cells. Myeloperoxidase release was measured on the same supernates and cells, and results were almost identical to those obtained with lysozyme, except that little release occurred with Con A, PMA, or FMLP in the absence of cytochalasin B. Thus, DASA treatment did not reduce the exocytosis of granule lysozyme or myeloperoxidase that was induced by FMLP or Con A.

The release of cytoplasmic LDH was also measured on the same supernates and cells. With either untreated or DASA-treated cells, the presence of cytochalasin B or stimulus had no effect on LDH release compared with release from unstimulated cells. Pre-

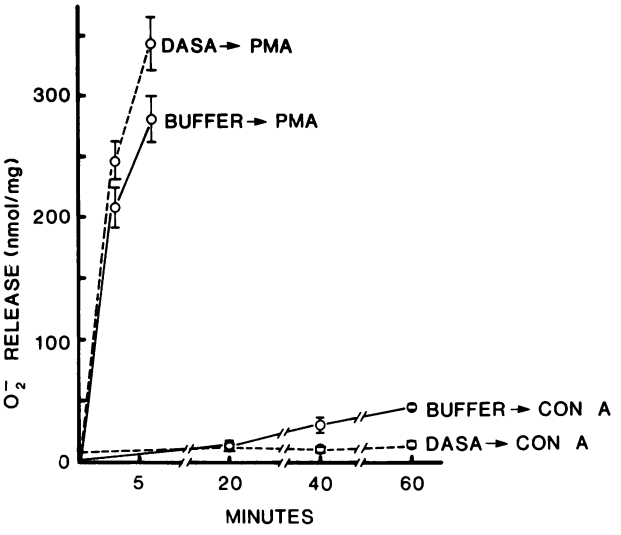

FIGURE 1 Effect of DASA pretreatment on O_2^- release by neutrophils stimulated with PMA or Con A, plotted as a function of time. Neutrophils were exposed to DASA in buffer (KRP-D) or to buffer alone, washed, then assayed for O_2^- production in the presence of either PMA (10 ng/ml) or Con A (100 μg/ml). The values plotted for each stimulus are the means; the bars represent the SEM. The means of three experiments performed in triplicate with Con A as stimulus are shown; the number of experiments (in triplicate) in the presence of PMA was four at 0 min, eleven at 3 min, and 9 at 6 min. Inhibition by DASA with Con A as stimulus was significant at 40 min ($P = 0.025$) and 60 min ($P = 0.006$), using paired values in Student's *t* test.

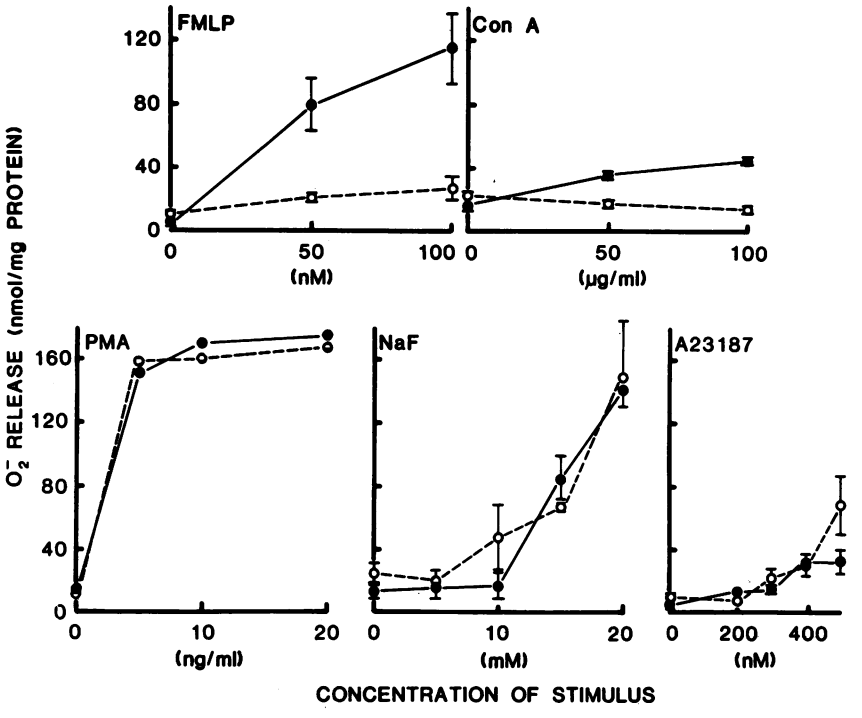

FIGURE 2 Effect of DASA pretreatment on O_2^- production by neutrophils exposed to various agents, plotted as a function of stimulus concentration. Values plotted are the means of three experiments with FMLP, Con A, and A23187, and two experiments with NaF, except that only one experiment was performed at 200 nM A23187. The bars represent the SEM. All experiments were performed in triplicate. For PMA, one representative experiment of three performed is shown because a large day-to-day variation was observed at these low PMA concentrations. In nine experiments with 10 ng/ml PMA, untreated cells released 279±19 nmol O_2^-/mg protein and DASA-treated cells released 343±22 nmol O_2^-/mg protein (mean±SEM). Time of incubation of neutrophils with stimulus and cytochrome *c* (established in preliminary experiments) was 5 min with FMLP, 60 min with Con A, 6 min with PMA, 30 min with NaF, and 5 min with A23187. Inhibition by DASA pretreatment was statistically significant by Student's *t* test with FMLP or Con A as stimulus (50 nM FMLP, $P = 0.028$; 100 nM FMLP, $P = 0.037$; 50 μg/ml Con A, $P = 0.002$; 100 μg/ml Con A, $P = 0.006$). ——, normal (untreated) cells; -----, DASA-treated cells.

treatment of the neutrophils with DASA was the only factor affecting release of LDH; therefore, the data for either untreated or DASA-treated cells in the presence and absence of stimuli were combined. Expressed as mean±SEM ($n = 38$) of percent release, with range given in parentheses, the data were as follows: untreated cells, 3.6±0.2 (0.4–5.9); DASA-treated cells, 11.1±0.7 (2.5–19.2). This increase in LDH release by DASA-treated cells correlates with the increased release of the lysosomal enzymes from DASA-treated cells in the absence of a stimulus. Total LDH, lysozyme, and myeloperoxidase activities were the same in normal and DASA-treated cells, indicating DASA was not entering the cells and inhibiting intracellular enzymes (9).

An NADPH-dependent oxidase that is activated by stimulation of intact neutrophils with PMA or opsonized zymosan has been localized by us and others to a particulate fraction of the cell (10, 19–22). We used this fraction as a source of the respiratory burst enzyme for two purposes: (*a*) to show that the enzyme was actually susceptible to DASA inhibition if directly exposed to this agent in the isolated fraction, and (*b*) to explore the effect of DASA treatment of the intact cell on function of the enzyme in the cell-free system.

Particulate fractions from either resting or stimulated cells exhibited greatly reduced NADPH oxidase activity after direct exposure to DASA. As measured by NADPH-dependent O_2^- production, particulate fractions from resting cells had a control activity of 0.52 ±0.17 nmol O_2^-/min per mg and an activity of 0.15 ±0.09 nmol/min per mg when treated with DASA (mean±SEM, $n = 4$). Fractions from neutrophils that had phagocytized opsonized zymosan (in the absence of KCN) had a mean activity of 1.55±0.49 nmol/min per mg and only 0.36±0.10 nmol/min per mg after DASA treatment ($n = 5$). This represents inhibition of 71 and 77% in fractions from resting and phagocytizing cells,

TABLE I

Effect of DASA Pretreatment on O_2^- Production and Oxygen Consumption by Neutrophils Stimulated with FMLP or PMA

Pretreatment*	Stimulus	Oxidative measurement‡	
		O_2^- release	Oxygen consumption
		nmol/5 min/mg	*nmol/min/mg*
Buffer	FMLP	117±34 (4)	23.8±6.6 (4)
DASA	FMLP	54±16 (4)	5.1±2.6 (4)
Buffer	PMA	326±61 (5)	46.2±2.7 (5)
DASA	PMA	415±47 (5)	43.9±2.7 (5)

* Neutrophils were pretreated with KRP-D or DASA as described in Methods.

‡ Resuspended cells were divided and assayed for either O_2^- release in the presence of cytochrome *c* or oxygen consumption using an oxygen electrode. Stimulus concentration was 100 nM FMLP or 10 ng/ml PMA. Incubation was carried out for 5 min for O_2^- release. Oxygen consumption was recorded continuously, and initial slopes were used for calculations. Values represent mean±SEM; the number of experiments is given in parentheses. Each experiment was performed in triplicate. O_2^- release and oxygen consumption by cells pretreated with DASA and stimulated with FMLP were significantly reduced compared with values obtained with buffer controls ($P < 0.04$ for both, paired *t* test).

respectively. Fractions from cells stimulated with 100 ng/ml PMA in the presence of KCN had a mean activity of 10.6±3.6 nmol/min per mg ($n = 3$); this was inhibited 98% by treatment with DASA (to 0.2±0.4). We conclude that the oxidase, before or after activation by phagocytosis or PMA, could be inhibited by DASA if the agent could gain access to the enzyme.

We attempted to isolate an NAD(P)H-dependent oxidase system from FMLP-stimulated neutrophils. Using identical conditions, whole cell sonicates and particulate fractions from neutrophils that showed three- to four-fold stimulation of intact cell O_2^- release by FMLP demonstrated no NADPH-dependent activity in six attempts. NADH was also an ineffective electron donor in this system ($n = 3$).

Particulate fractions were also examined for oxidase activity after DASA pretreatment and PMA stimulation of intact cells. Results are summarized in Table II. Particulate fractions from neutrophils stimulated with PMA showed markedly increased NADPH-dependent chemiluminescence and O_2^- production compared with fractions from unstimulated cells. Treatment of neutrophils with DASA before exposure to PMA did not inhibit, and in fact somewhat increased the enhancement of these activities by PMA.

We explored the possibility that the enhancement of oxidative activity in fractions from cells that were pretreated with DASA before stimulation with PMA might be due to inhibition by DASA of a normal mechanism for inactivating the oxidase. Such a mechanism has been described in neutrophils stimulated by opsonized zymosan (12). Fractions were obtained from buffer- or DASA-pretreated cells stimulated with PMA for either 3 or 5 min in the presence of 1 mM NaN_3. In four experiments, oxidase activity in fractions from

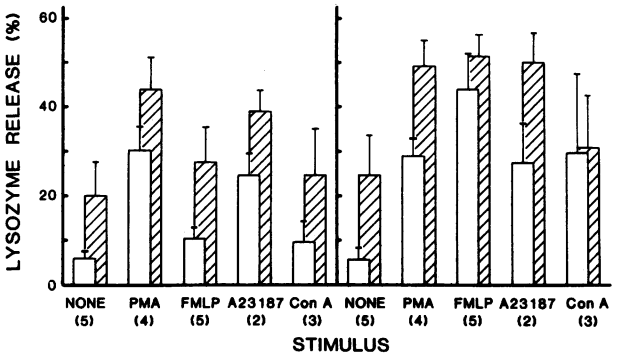

FIGURE 3 Release of lysozyme by normal (□) and DASA-treated (▨) neutrophils in response to various stimuli. Experiments were performed as described in Methods. The heights of the columns indicate means, and the number of experiments, each performed in duplicate or triplicate, are shown below the name of the stimulus. The bars indicate the SEM. Left: in the absence of cytochalasin B. Right: in the presence of 1.0 μg/ml cytochalasin B. The spontaneous (unstimulated) release was not subtracted from the stimulus-mediated release, since spontaneous release would not be expected to occur to the same extent during stimulus-coupled release.

TABLE II

Effect of DASA Pretreatment on NADPH-dependent Oxidative Activities of Particulate Fractions from PMA-stimulated Neutrophils

Treatment of cells*	Activities of particulate fractions‡	
	Chemiluminescence	O_2^- release
	cpm/mg	*nmol/min/mg*
Buffer, DMSO	4,758±920 (6)	0.3±0.1 (4)
DASA, DMSO	6,001±439 (5)	0.3±0.1 (4)
Buffer, PMA	31,876±3,117 (6)	13.4±2.5 (7)
DASA, PMA	39,305±4,122 (5)	21.5±3.7 (7)

* Neutrophils were preincubated in DASA or buffer, then washed and resuspended in buffer, as described in Methods. Resuspended cells were stimulated with PMA, 100 ng/ml, or with DMSO as control. Particulate fractions were made from some of the cells, and others were studied for ATPase and alkaline phosphatase activity or LDH release. Fractions assayed for O_2^- release were from cells stimulated in the presence of 1 mM KCN or NaN_3.

‡ Values are expressed as mean±SEM; the number of experiments performed is shown in parentheses. Each experimental value is the average of duplicate or triplicate determinations.

buffer-treated cells did not decline from 3 to 5 min, possibly because of the presence of NaN_3 (12) (at 3 min, 15.1±3.4 nmol O_2^-/min per mg; at 5 min, 15.8±4.9; mean±SEM). Fractions from DASA-treated cells demonstrated higher activity at the two time points and also showed no evidence of decline (at 3 min, 22.0±4.8; at 5 min, 23.1±4.9). Thus, inactivation of the oxidase in normal cells and prevention of such in DASA-treated cells appears unlikely in the presence of NaN_3.

In all the experiments in which DASA was employed, inhibition of the surface enzymes Mg^{++}-ATPase and alkaline phosphatase was used to monitor the effectiveness of DASA pretreatment. In eight experiments, Mg^{++}-ATPase was inhibited 50% in cells exposed to DASA, compared with cells exposed to buffer (control, 9.2±1.0; DASA-treated, 4.6±0.5% hydrolysis per mg, mean±SEM, $P < 0.001$). Alkaline phosphatase activity in cells exposed to DASA was decreased 66% compared with buffer-treated cells (control, 0.079±0.009; DASA-treated, 0.030±0.004ΔOD/min per mg, mean±SEM, $n = 23, P < 0.001$). Release of LDH, as a monitor of cell viability, was <10% in all of these experiments.

DISCUSSION

The plasma membrane must play a crucial role in activation of the enzyme system responsible for the respiratory burst in human neutrophils. A variety of different soluble stimuli can trigger the neutrophil's oxidative metabolism, and it is possible that different stimuli act through different mechanisms (5–7). We have presented evidence here for the existence of at least two mechanisms of activation. Modification of the neutrophil's plasma membrane by the nonpenetrating chemical agent DASA resulted in a cell that could respond to certain stimuli (PMA, NaF, A23187) with normal O_2^- production, but was unable to respond normally to others (FMLP, Con A).

These results were confirmed for PMA and FMLP using oxygen consumption as another measure of oxidative metabolism. However, oxygen consumption did not show the same enhancement in DASA-pretreated cells stimulated with PMA that was seen with O_2^- production. One possible explanation is that DASA, by its alteration of the surface membrane, has increased the accessibility of the generated O_2^- to the detection protein cytochrome *c*. The lack of enhanced oxygen consumption suggests that the PMA-activated oxidase system has not been directly affected by DASA.

It appears unlikely that the DASA-sensitive site for FMLP and Con A activation is binding of the stimulus. Goldstein et al. (9) reported that DASA treatment of neutrophils did not alter their ability to bind radiolabeled Con A. The exocytosis of lysozyme, another function of the cell induced by binding of stimulus, was also unchanged (9). We found that DASA treatment under our conditions did not impair the release of lysozyme and myeloperoxidase mediated by FMLP and Con A, suggesting that effective binding of these stimuli was normal. Although unstimulated release was enhanced by DASA, this could reflect the slightly decreased viability of DASA-treated neutrophils.

Particulate fractions from phagocytosis- or PMA-stimulated neutrophils have been shown previously by us and others to contain NADPH oxidase activity and to produce O_2^- and hydrogen peroxide in the presence of NADPH (10, 11, 19–23). We used this fraction to demonstrate that oxidase activity was indeed susceptible to DASA inhibition. However, fractions from neutrophils that were pretreated with DASA, washed, and then stimulated with PMA demonstrated no inhibition of NADPH-dependent oxidase activity. These observations favor the possibility that PMA activates an oxidase system whose DASA-sensitive site is not on the surface of the neutrophil. The actual location of this system has not been determined. Reports from other laboratories using sucrose density gradient centrifugation to separate cell fractions have yielded conflicting results. Oxidase activity has been found to sediment either deep in the gradient near the azurophil granule (24–26) or with the plasma membrane at the top of the gradient (27), depending on the assay and separation technique used. Our data would be compatible with either of these locations, but suggest that if the enzyme is associated with the plasma membrane, it is not situated as an ectoenzyme.

The enhancement of oxidase activity in fractions from cells pretreated with DASA and then stimulated with PMA is difficult to explain. One possibility is that DASA has prevented a normal inactivation system from operating. However, this is unlikely in view of a previous report providing evidence that an early phase of inactivation does not occur when stimulation of the cell is performed in the presence of KCN or NaN_3 (12). We found no evidence of inactivation of oxidase activity at 5 min in normal cells under these same conditions. Also, oxygen consumption by PMA-stimulated cells was unchanged by DASA pretreatment, suggesting that the level of oxidase activity had not been altered. Another possible explanation is that modification of the plasma membrane by DASA has allowed better recovery of the oxidase from the intact cell. Although our data suggest that DASA has not directly affected the PMA-activated oxidase, this evidence is not conclusive.

The lack of apparent oxidase activity in broken cell preparations from FMLP-treated neutrophils is puzzling. However, this finding further supports our contention that FMLP and PMA act through separate mechanisms to activate the respiratory burst.

Taken together, these results clearly suggest that more than one mechanism exists for activating the oxidative metabolism of neutrophils. This concept is supported by reports of patients whose neutrophils re-

spond normally to some stimuli but do not respond to others (28, 29). Although it has been widely accepted that the oxidase is a single enzyme that resides entirely on the outside of the plasma membrane, our data clearly say that the situation is more complex.

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