Immunology 1981 **44** 847

Polymorphonuclear leucocyte membrane-stimulated oxidative metabolic activity—the effect of divalent cations and cytochalasins

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Accepted for publication 30 June 1981

Summary. The effect of divalent cations and cytochalasins on the oxidative metabolic response of polymorphonuclear leucocytes (PMNL) following membrane stimulation by opsonized zymosan, phorbol myristate acetate (PMA), digitonin and n-formyl-methionyl-leucyl-phenylalanine (FMLP) was investigated using several techniques. For optimal ferricytochrome C reduction, oxygen consumption, luminol- and lucigenin-dependent chemiluminescence by PMNL on exposure to opsonized zymosan, extracellular magnesium was necessary. In contrast FMLP- and digitonin-induced PMNL metabolic activities were less dependent on extracellular magnesium than on calcium. Phorbol myristate acetate-induced PMNL oxidative metabolic activity occurred independently of extracellular magnesium and calcium concentration. Incubation of PMNL with cytochalasins B and E had little effect on PMA-induced metabolic activity, enhanced FMLP-induced metabolic activity but inhibited digitonin-induced metabolic activity. Investigation of zymosan-induced PMNL metabolic activity after incubation with cytochalasins demonstrated a dissociation between the different parameters of metabolic activity measured.

The results described in this study support the suggestion that PMNL oxidative metabolism can be activated by at least two different mechanisms depending on the stimulus used, and that these mechanisms can in part be differentiated by their dependence on extracellular divalent cations.

INTRODUCTION

Following membrane stimulation, phagocytic cells undergo a burst of respiratory activity with increased consumption of oxygen (O_2; Baldridge & Gerard, 1933) and the production of reactive substances including the superoxide anion ($O_2^{\cdot-}$; Babior, Kipnis & Curnutte, 1973), hydrogen peroxide (H_2O_2; Iyer, Islam & Quastel, 1961) and the hydroxyl radical ($OH^{\cdot}$; Tauber & Babior, 1977) which play an important role in the microbicidal activity of the cell (Babior, 1978). This oxidative metabolic activity can be stimulated by soluble agents as well as by opsonized particles (Repine, White, Clawson & Holmes, 1974; Simchowitz & Spilberg, 1979; Goldstein & Weissmann, 1979).

The mechanisms involved in the initiation of this respiratory burst activity are the subject of considerable debate. The majority opinion proposes that activation of a membrane oxidase catalyses the NADPH-dependent reduction of O_2 to O_2^-. (Babior, 1978). As a result of investigation of the metabolic activity of polymorphonuclear leucocytes (PMNL) from certain patients (Weening, Roos, Weemaes, Homan-Müller & Van Schaik, 1976; Harvath &

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0019-2805/1200-0847$02.00
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Anderson, 1979) as well as further *in vitro* studies using normal PMNL exposed to several different stimulating agents (Lehmeyer, Snyderman & Johnston, 1979) it has been suggested that there are at least two separate mechanisms that mediate the activation of this PMNL $O_2^{\overline{\cdot}}$-forming enzyme (English, Roloff & Lukens, 1981).

To monitor phagocytic cell metabolic activity a number of techniques are available. As well as measurements of oxygen consumption (Weening, Roos & Loos, 1974), and assays of $O_2^{\overline{\cdot}}$ release (Babior *et al.*, 1973), H_2O_2 release (Chance, Sies & Boveris, 1979) and $OH^{\cdot}$ release (Tauber & Babior, 1977; Weiss, Rustagi & Lo Buglio, 1978; Green, Hill, Okolow-Zubkowska & Segal, 1979), the stimulation of enhanced chemiluminescence following phagocytic membrane stimulation has been described using several different substrates including organic particles (Allen, Stjernholm & Steele, 1972; Rosen & Klebanoff, 1976), luminol (Allen & Loose, 1976) and lucigenin (Williams & Cole, 1981a). By monitoring these different parameters of metabolic activity it has been shown that the various components of the respiratory burst may be dissociated from one another and that stimulation of one component need not necessarily imply stimulation of another. An example of this is the dissociation between $O_2^{\overline{\cdot}}$ release as measured by ferricytochrome C reduction and O_2 consumption following membrane stimulation by opsonized zymosan in PMNL that have been exposed to cytochalasin B (Roos, Homan-Müller & Weening, 1976).

In an attempt to delineate further the relationship between different phagocytic cellular metabolic events, we have investigated in this study the stimulation of PMNL cell membranes by particulate and soluble agents using the techniques of luminol- and lucigenin-dependent chemiluminescence. We have assessed the effect of extracellular cation concentration and cytochalasins on the response, drawing comparison with their effects on O_2 consumption and ferricytochrome C reduction.

MATERIALS AND METHODS

Medium and chemicals

Medium 199 and Hanks's balanced salt solution containing calcium and magnesium but without phenol red were obtained from Flow Laboratories. Hanks's balanced salt solution (HBSS) without calcium and magnesium was obtained from Gibco (Europe). Zymosan A (From *Saccharomyces cerevisiae*), luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione), lucigenin (dimethyl-biacridylium nitrate), N-formylmethionyl-leucyl-phenylalanine(FMLP), digitonin, cytochalasin B (from *Helminthosporium dematioideum*), cytochalasin E (from *Rosellinia necatrix*), phorbol myristate acetate (PMA), dimethyl sulphoxide (DMSO), ethylene diamine tetraacetic acid (EDTA), ethylene-glycol-bis (β-aminoethylether) N,N'-tetraacetic acid (EGTA), ferricytochrome C (horse heart type VI), superoxide dismutase (SOD), calcium chloride dihydrate, sodium dithionite and HEPES (n-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) were obtained from Sigma Laboratories. Magnesium sulphate ($MgSO_47H_2O$) and magnesium chloride ($MgCl_2.6H_2O$) were obtained from BDH Laboratories. Verapamil was obtained from Abbott Laboratories.

Cytochalasin B (1 mg/ml), cytochalasin E (1 mg/ml), FMLP (5 mg/ml) and PMA (10 mg/ml) were dissolved in DMSO and then diluted to the required concentration with HBSS. Zymosan, SOD, digitonin and ferricytochrome C were dissolved or suspended in phosphate buffered saline (PBS). Calcium chloride (25 mg/ml), magnesium chloride (15 mg/ml) and magnesium sulphate (15 mg/ml) were dissolved in deionised water and then pH adjusted to 7·4. Luminol (10^{-2}M) was dissolved in DMSO and then diluted to the required concentration HEPES-buffered HBSS. Lucigenin was dissolved in HEPES-buffered HBSS. In experiments that were not concerned with cation concentration luminol and lucigenin were diluted in HEPES-buffered Hanks's balanced salt solution with calcium and magnesium.

Cell preparation, opsonization and cell membrane stimulating agents

Polymorphonuclear leucocytes were separated by a modification (Williams, Hastings, Easmon & Cole, 1980) of the method described by Böyum (1968) from the peripheral blood of healthy adult donors. The cells were then washed four times with and suspended in HEPES-buffered HBSS (2×10^6/ml). In experiments not directly concerned with the effects of divalent cations, medium 199 was substituted for HBSS. The cells were kept in siliconized glass containers at 37° until used.

Zymosan was opsonized by incubation with pooled normal human serum at 37° for 30 min. Four hundred microlitres of serum was added to zymosan (20 mg)

suspended in medium (1.9 ml). Unopsonized zymosan was prepared in a similar way but PBS was substituted for serum.

Other PMNL membrane stimulating agents used were PMA (33 ng/ml), digitonin (13 μg/ml) and FMLP (2×10^{-7}M).

Chemiluminescence assay

Chemiluminescence was measured in a photometer (Luminometer 1250; LKB Wallac), the details of which have been previously described (Easmon, Cole. Williams & Hastings, 1980) using 2 ml cylindrical polystyrene cuvettes (Sterilin). The reaction mixture consisting of luminol (10^{-5}M; 900 μl) or lucigenin (10^{-4}M; 900 μl) with cell suspension (500 μl) and cytochalasin as indicated was transferred to the reaction carousel (37°) before the addition of stimulating agent. In experiments concerned with the effects of divalent cations or cytochalasins, the relevant volume of magnesium (50% magnesium sulphate, 50% magnesium chloride) solution, calcium solution, MgEGTA, EDTA, verapamil or cytochalasin (3.3 μg/ml) was added to the reaction vial before the addition of cell suspension.

The light output after the addition of an aliquot (100 μl) of stimulating agent was recorded graphically on a chart recorder. In experiments with zymosan or PMA an estimate of the integrated response (12 min) was calculated and used for data analysis. In experiments with FMLP or digitonin the peak response following their addition was used for analysis.

In the absence of either stimulating agent or PMNL there was no response when using luminol. With lucigenin, in the absence of cytochalasins, there was a low level response due to reaction between PMNL and the vial (Williams & Cole, 1981a). It had reached its peak before the addition of stimulating agents and was totally suppressed in the presence of cytochalasins. Control experiments with appropriate amounts of DMSO were performed and were not found to affect the results although DMSO at higher concentrations (>0·5%) inhibits luminol-dependent chemiluminescence.

The results (mean; SEM) are expressed as a percentage of the control value for each individual experiment. In experiments with divalent cations, the results obtained with a calcium concentration of $2{\cdot}5 \times 10^{-3}$M and a magnesium concentration of $0{\cdot}89 \times 10^{-3}$M were used as controls. In experiments with cation chelators, the results obtained in the absence of extracellular calcium and magnesium were used as controls. In experiments with cytochalasins, the results obtained in the absence of cytochalasin were used as controls.

Ferricytochrome C reduction

Superoxide release was determined by the SOD inhibitable reduction of ferricytochrome C (Babior *et al.*, 1973). Polymorphonuclear leucocytes (10^6/ml) in HBSS or medium 199 were incubated for 15 min at 37° with ferricytochrome C (46 μM) in the presence or absence of SOD (66 μg/ml) after the addition of an aliquot (100 μl) of stimulating agent. The amount of reduced cytochrome C in the cell free supernatants was determined spectrophotometrically (Unicam SP 1700; Pye) at 550 nm and the $O_2^{\cdot-}$ release calculated using the extinction coefficient of 21×10^3/cm/M (Massey, 1959). To ensure that ferricytochrome C was present in excess a few grains of dithionite were added to the vial with the highest response. Appropriate control tubes demonstrated no spontaneous reduction of ferricytochrome C by metal salts in the absence of PMNL nor did they interfere with the ability of ferricytochrome C to be reduced by sodium dithionite.

Oxygen consumption

Oxygen consumption was measured polarographically using a Clarke-type O_2 electrode (Rank Brothers, Bottisham, Cambridge) at 37°; 2.7 ml of cell suspension (2×10^6/ml) in medium 199 or HBSS with or without cytochalasin E was allowed to equilibrate in the chamber before the addition of stimulating agent (200 μl). Oxygen consumption was then monitored during the next 10 min. Control experiments were performed in the absence of PMNL.

RESULTS

Effect of extracellular calcium and magnesium concentration on chemiluminescence

The kinetics of the chemiluminescent response stimulated by the addition of opsonized particles or soluble agents to PMNL in the presence of luminol or lucigenin varies according to the stimulus used (Williams & Cole, 1981b). While there is a lag-period of about 30 sec before the onset of chemiluminescence following the addition of opsonized zymosan or PMA, it is less than 10 sec following the addition of FMLP. Similarly, the effect of extracellular cation concentration is related to the cell membrane stimulating agent used (Figs 1 and 2). Phorbol myristate acetate stimulates the production of lucigenin- and

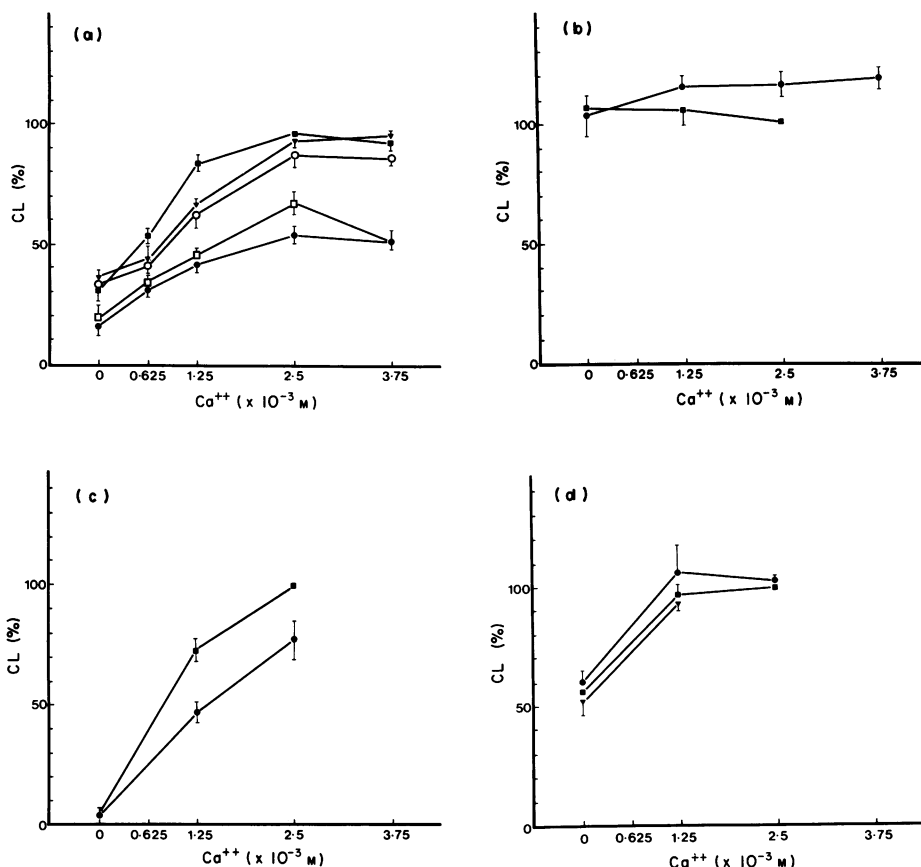

Figure 1. Effect of extracellular divalent cation concentration on luminol-dependent chemiluminescence (CL) following the addition of (a) opsonized zymosan (1.3 mg/ml), (b) phorbol myristate acetate (33 ng/ml), (c) digitonin (13 μg/ml) and (d) n-formyl-methionyl-leucyl phenylalanine (2×10^{-7}M) to a reaction vial (37°) containing luminol (900 μl; 10^{-5}M) and polymorphonuclear leucocyte suspension (500 μl; 2×10^{6}/ml in HBSS without calcium and magnesium). Before the addition of stimulating agent calcium (as on abscissa) and magnesium (● ...... 0; □ ..... 0·22 mM; ○ ...... 0·45 mM; ■ ..... 0·89 mM; ▼ ..... 1·78 mM) were added to give final concentrations as indicated. For CL stimulated by opsonized zymosan or PMA an estimate of the integrated response for the 10 min following the addition of stimulating agent was calculated for data analysis; for FMLP and digitonin-stimulated CL the peak response was used for data analysis. Results (expressed as mean ± SEM of at least three experiments) represent the percentage of the result obtained in the presence of calcium (2·5 mM) and magnesium (0·89 mM).

luminol-dependent chemiluminescence in the absence of extracellular divalent cations. With lucigenin the response was inhibited by high concentrations of calcium but there was no significant effect produced by changes in extracellular magnesium concentration. Incubation with MgEGTA (10^{-3}M) to chelate calcium ions and EDTA (10^{-3}M) to chelate calcium and magnesium ions had no effect on luminol-dependent chemiluminescence but enhanced lucigenin-dependent chemiluminescence (Table 1). It is not clear whether this latter observation represents a genuine change in cellular activity or the results of interaction between EDTA and lucigenin. Measurement of pH before and after the addition of EDTA and PMA showed no significant change. Verapamil (10^{-4}M) a selective antagonist of calcium influx, has only a minimal inhibitory effect on lucigenin-dependent chemiluminescence at the concentration used (Simchowitz & Spilberg, 1979).

With opsonized zymosan the results were very

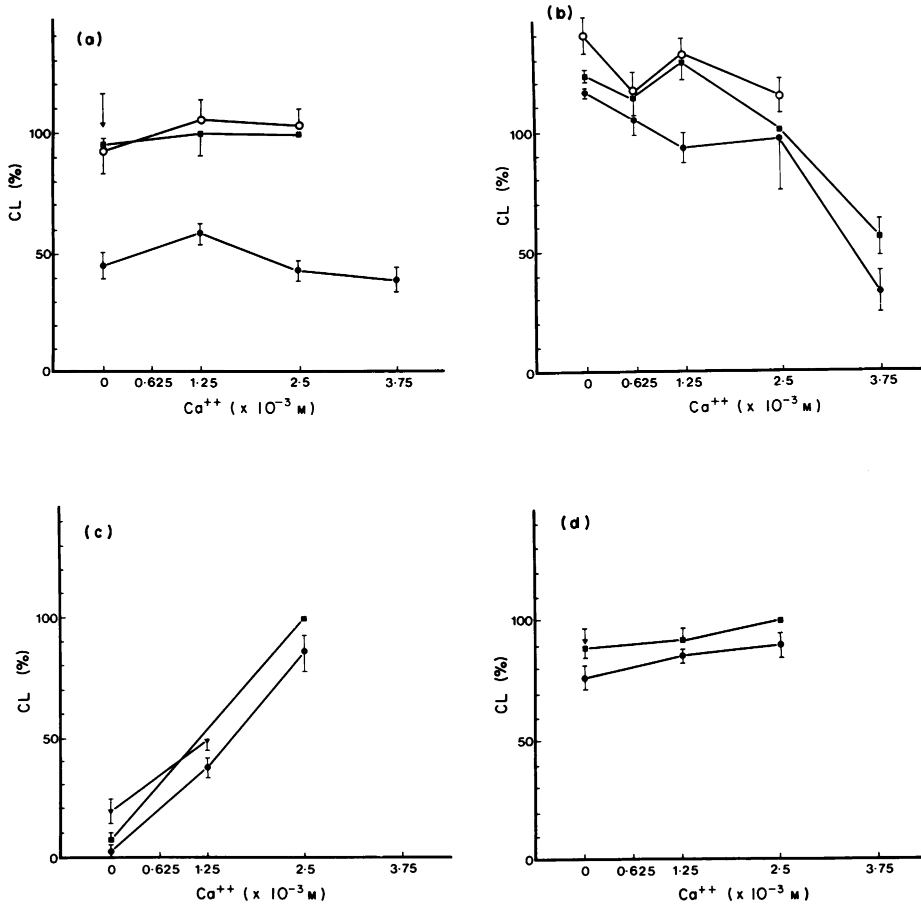

Figure 2. Effect of extracellular divalent cation concentration on lucigenin-dependent chemiluminescence following the addition of (a) opsonized zymosan (1·3 mg/ml), (b) phorbol myristate acetate (33 ng/ml), (c) digitonin (13 μg/ml) and (d) n-formyl-methionyl-leucyl-phenylalanine (2×10^{-7}M) to a reaction vial (37°) containing lucigenin (900 μl; 10^{-4}M) and polymorphonuclear leucocyte suspension (500 μl; 2×10^{6}/ml in HBSS without calcium and magnesium). Before the addition of stimulating agent calcium (as on abscissa) and magnesium (● ...... 0; ○ ...... 0·45 mM; ■ ..... 0·89 mM; ▼ ..... 1·78 mM) were added to give final concentrations as indicated. For CL stimulated by opsonized zymosan and PMA an estimate of the integrated response for the 10 min following the addition of stimulating agent was calculated for data analysis; for FMLP and digitonin-stimulated CL the peak response was used for data analysis. Results (expressed as mean ± SEM of at least three experiments) represent the percentage of the result obtained in the presence of calcium (2·5 mM) and magnesium (0·89 mM).

Table 1. Effect of cation chelators and verapamil on chemiluminescence (CL)

	Lucigenin CL			Luminol CL		
	EDTA 10^{-3}M	MgEGTA 10^{-3}M	Verapamil 10^{-4}M	EDTA 10^{-3}M	MgEGTA 10^{-3}M	Verapamil 10^{-4}M
Opsonized zymosan	5%	79%	55%	0%	ND	ND
PMA	156%	144%	92%	109%	89%	ND
Digitonin	0%	0%	ND	ND	ND	ND
FMLP (*no* Cyto B)	ND	108%	7%	ND	63·5%	0·1%
FMLP (with Cyto B)	200%	68%	ND	80%	30%	ND

Luminol- or lucigenin-dependent chemiluminescence (CL) were assessed following the addition of opsonized zymosan (1.3 mg/ml), phorbol myristate acetate (33 ng/ml), digitonin (13 μg/ml) or formyl-methionyl-leucyl-phenylalanine [(FMLP) with or without cytochalasin B; 3.3 μgm/ml] to reaction vials (37°) containing luminol (900 μl; 10^{-5}M) or lucigenin (900 μl; 10^{-4}M) and cell suspension (500 μl; 2×10^6 PMNL/ml in HBSS without calcium and magnesium).

Cation chelators and verapamil were added before the addition of stimulating agents.

Results (mean of two or three different experiments) for experiments with EDTA and Mg EGTA are expressed as a percentage of those obtained when magnesium (0·89 mM) and calcium (2·5 mM) were added to the medium in the absence of chelator. Results for experiments with verapamil are expressed as a percentage of those obtained in the absence of extracellular calcium, magnesium and verapamil.

different but again depended on the substrate used for the generation of chemiluminescence. Optimal conditions for luminol-dependent chemiluminescence (Fig. 1) required extracellular calcium and magnesium and the effect of the absence of either could not be corrected by excess of the other. Chelation of cations with EDTA further reduced the response (Table 1). In contrast, although zymosan-stimulated lucigenin-dependent chemiluminescence required magnesium (Fig. 2) extracellular calcium was not required in the presence of high magnesium concentrations but increased the response at low magnesium concentrations. Despite only a slight reduction in response as a result of calcium chelation with MgEGTA, in the absence of extracellular cations verapamil produced a further reduction in activity.

The stimulating effect of digitonin for luminol- and lucigenin-dependent chemiluminescence required extracellular calcium and did not occur in its absence regardless of the magnesium concentration. Even in the presence of calcium, magnesium concentration had little effect on the results.

With the chemotactic oligopeptide FMLP the effect of cations on chemiluminescence was substrate dependent. For optimal luminol-dependent chemiluminescence extracellular calcium but not magnesium was required and the response was only 60% maximal in calcium-free medium (Fig. 1). The addition of MgEDTA produced no further reduction but verapamil had a marked inhibitory effect (Table 1). Lucigenin-dependent chemiluminescence was not as sensitive to the extracellular calcium concentration but more so to the magnesium concentration than luminol-dependent chemiluminescence. In the presence of cytochalasin B, EDTA enhanced the response to FMLP but as with PMA the reason for this is not clear.

Effect of extracellular calcium and magnesium concentration on ferricytochrome C reduction and oxygen consumption by membrane stimulated polymorphonuclear leucocytes

The importance of extracellular calcium to

ferricytochrome C reduction by PMNL following membrane stimulation by digitonin or FMLP has been previously demonstrated (Cohen & Chovaniec, 1978; Simchowitz & Spilberg, 1979). The results shown in Table 2 are largely in agreement with these previous observations although the inhibitory effect of the absence of extracellular divalent cations was not as great as that previously reported. Addition of EDTA (10^{-3}M) or MgEGTA (10^{-3}M) further reduced the response with FMLP in the presence of cytochalasin B to 7·5% (13%, 2%) and 11% (18%, 4%), respectively.

Extracellular calcium and magnesium concentration did not affect ferricytochrome C reduction by PMNL stimulated with PMA but it did have a considerable effect on the results obtained with opsonized zymosan (Table 2). For optimal response both calcium and magnesium were necessary although the former was of only minor importance in the presence of magnesium. Despite this, the importance of calcium is demonstrated by the effect of verapamil (10^{-4}M) which in the absence of extracellular divalent cations further reduced the response (44% of that without verapamil). This effect was not seen in the presence of extracellular calcium and magnesium. In the absence of extracellular calcium and magnesium opsonized zymosan stimulated more ferricytochrome C reduction (5·94 ± 1·77 (SD) nmol/10^6cells/15 min; $n = 3$) than non-opsonized (2·79 ± 1·74; $n = 3$; $P < 0·05$) or zymosan that had been incubated with heat treated (56° for 30 min) serum (3·22 ± 1·5; $n = 3$; $P < 0·05$). However the differential between the three was very much greater in the presence of extracellular divalent cations (opsonized zymosan: 23·0 ± 8·17 nmol/10^6·cells/15 min; $n = 3$; non-opsonized zymosan: 2.9 ± 0.8; $n = 3$; $P < 0·01$; zymosan incubated with heat-treated serum: 3·84 ± 1·3; $n = 3$; $P < 0·01$).

Oxygen consumption by PMNL following membrane stimulation by PMA was not affected by extracellular divalent cation concentration but magnesium was necessary for maximal response following exposure to opsonized zymosan (Table 3).

Effects of cytochalasins on polymorphonuclear leucocyte-enhanced chemiluminescence, ferricytochrome C reduction and oxygen consumption

It has previously been shown that treatment of PMNL

Table 2. Effect of extracellular calcium and magnesium concentration on ferricytochrome C reduction by membrane stimulated polymorphonuclear leucocytes

Calcium (mM)	0	1·25	2·5	0	1·25	2·5	0	1·25	2·5
Magnesium (mM)	0	0	0	0·45	0·45	0·45	0·89	0·89	0·89
Opsonized zymosan	33·0 (5·2)	49·5 (9·5)	55·6 (17·9)	83·0 (9·1)	ND	ND	95·0 (8·8)	ND	100
Non-opsonized zymosan	91·5 (14·5)	62·0 (4·2)	ND	62·0 (3·0)	ND	ND	ND	ND	100
PMA	109·0 (3·0)	ND	107·0 (5·6)	ND	ND	ND	103·0 (0·7)	ND	100
Digitonin	28·0 (2·6)	60·0 (4·5)	106·0 (36·0)	62·0 (15·7)	82·0 (1·4)	98·0 (2·1)	47·0 (26·0)	57·0 (20·0)	100
FMLP	60·5 (4·6)	84·0 (3·0)	92·5 (5·3)	ND	ND	ND	56.5 (3·2)	ND	100
FMLP (with cytochalasin B)	20·6 (7·6)	58·5 (1·0)	114·0 (28·0)	ND	ND	ND	22·0 (8·0)	ND	100

Results [mean (SEM) of three or more experiments] represent the ferricytochrome C reduction expressed as a percentage of the result obtained in the presence of calcium (2·5 mM) and magnesium (0·89 mM). For ferricytochrome C reduction, zymosan (1·3 mg/ml), phorbol myristate acetate (33 ng/ml), digitonin (13 μg/ml) or formyl-methionyl leucyl-phenylalanine (FMLP; 2×10^{-7} M; with or without cytochalasin B) were added to a reaction vial containing ferricytochrome C (46 μM), cell suspension (10^6/ml in HBSS without calcium and magnesium), calcium and magnesium as indicated in the presence or absence of superoxide dismutase (66 μg/ml). The vials were incubated for 15 min at 37°.

Table 3. Effect of extracellular calcium and magnesium concentration on O_2 consumption by polymorphonuclear leucocytes when stimulated by opsonized zymosan or phorbol myristate acetate (PMA)

Calcium (mM)	0	1·25	2·5	0	0	2·5
Magnesium (mM)	0	0	0	0·45	0·89	0·89
Opsonized zymosan	24·9 (35·1,14·6)	28·5 (27·5,29·5)	29·3 (29·1,29·5)	61·1 (62·6,59·6)	52·7 (62·7,42·8)	64·1 (65·6,62·6)
PMA	56·4 (62·9,49·9)	ND	ND	ND	ND	48·6 (53·6,43·6)

Results [expressed as mean (individual results)] represent the oxygen consumption (nmol/10^6 cells/10 min) during the 10 min following addition of opsonized zymosan (1·3 mg/ml) or PMA (33 ng/ml) to reaction chamber (37°) containing cell suspension (2×10^6 PMNL/ml in HBSS without calcium and magnesium) with added calcium and magnesium as indicated.

with cytochalasin B before exposure to phagocytic stimuli inhibits phagocytosis (Malawista, Gee & Bensch, 1971; Zigmond & Hirsch, 1972a) and decreases O_2 consumption (Roos *et al.*, 1976) but increases the release of $O_2^{\cdot-}$ (Goldstein, Roos, Kaplan & Weissmann, 1975; Curnutte & Babior, 1975), H_2O_2 (Root & Metcalf, 1977) and lysosomal enzymes (Goldstein *et al.*, 1975) into the extracellular medium. In addition to interfering with microfilament-dependent activities, cytochalasin E in contrast to cytochala-

Table 4. Effect of cytochalasins on ferricytochrome C reduction, chemiluminescence and O_2 consumption by polymorphonuclear leucocytes following membrane stimulation by particulate or soluble agents

	Ferricytochrome C reduction		Luminol CL		Lucigenin CL		Oxygen Consumption
	Cytochalasin E	Cytochalasin B	Cytochalasin E	Cytochalasin B	Cytochalasin E	Cytochalasin B	Cytochalasin E
Opsonized zymosan; (1·3 mg/ml)	67·7 (3·5; 3)	153·5 (20·1; 2)	25·0 (1·4; 2)	2·3 (0·3; 2)	4·3 (1·9; 3)	8·4 (2·9; 3)	9%
PMA (33 ng/ml)	104·0	101·5 (2·47; 2)	137·0 (27·0; 3)	109·0 (11·7; 2)	95·0 (32·0; 3)	23·0	107%
Digitonin 13 μg/ml)	ND	ND	0 ($n=3$)	0 ($n=3$)	13·5 (3·5; 3)	0 ($n=3$)	ND
FMLP (2×10^{-7}M)	455·8 (186·1; 5)	341·0 (85·2; 4)	826·0 (127·0; 8)	3179·0 (1314; 6)	655·0 (272; 4)	849·0 (2·5; 4)	ND

Results [expressed as mean (SEM; *n*)] represent the percentage of the result obtained in the absence of cytochalasin. For ferricytochrome C reduction, stimulating agents were incubated for 15 min at 37° with cell suspension and ferricytochrome C (46 μM). For chemiluminescence (CL) stimulated by opsonized zymosan or phorbol myristate acetate (PMA) an estimate of the integrated response for the 10 min following the addition of stimulating agent was calculated for data analysis; for n-formyl-methionyl-leucyl-phenylalanine (FMLP) and digitonin-stimulated CL the peak response was used for data analysis. For O_2 consumption, the amount of O_2 consumed during the 10 min following addition of PMA or opsonized zymosan was used for analysis.

In all experiments when used, cytochalasins (3·3 μgm/ml in 0·3% DMSO) were added before the stimulating agent. ND, not done.

sin B, induces metabolic changes with stimulation of the release of $O_2^{\overline{\cdot}}$ (Nakagawara & Minakami, 1975).

The effects of incubation of PMNL with either cytochalasin B or cytochalasin E before exposure to various membrane stimulating agents is shown in Table 4. In general, the cytochalasins were similar in their effects on ferricytochrome C reduction and chemiluminescence although cytochalasin B increased while cytochalasin E inhibited ferricytochrome C reduction following exposure of PMNL to opsonized zymosan. This discrepancy might either be due to a deactivation of cell membrane activity on exposure to opsonized zymosan because of prior activity induced by cytochalasin E, or because of direct interference with metabolic pathways by cytochalasin E.

As has previously been demonstrated with cytochalasin B (Roos *et al.*, 1976) cytochalasin E reduced O_2 consumption by PMNL following exposure to opsonized zymosan. As well as this apparent dissociation between the effect of cytochalasin B on O_2 consumption and ferricytochrome C reduction at the time of phagocytosis, it had a considerable inhibitory effect on luminol- and lucigenin-dependent chemiluminescence.

Cytochalasins had no effect on PMA-stimulated ferricytochrome C reduction or O_2 consumption by PMNL. Cytochalasin E increased luminol-dependent chemiluminescence but under the reaction conditions used it had no effect on PMA-stimulated lucigenin-dependent chemiluminescence. In contrast cytochalasin B had no effect on luminol-dependent chemiluminescence but reduced lucigenin-dependent chemiluminescence. Much of the latter was due to a delay in onset of response in the presence of cytochalasin B and when a longer time interval than 12 min was chosen for analysis of the integrated response the difference was reduced (Williams & Cole, 1981b).

Digitonin-stimulated PMNL luminol- and lucigenin-dependent chemiluminescence were both inhibited by prior incubation of PMNL with cytochalasins. Cytochalasin B completely abolished chemiluminescence using either substrate while cytochalasin E abolished luminol-dependent chemiluminescence and markedly reduced lucigenin-dependent chemiluminescence.

In contrast both cytochalasins increased ferricytochrome C reduction and chemiluminescence when PMNL were exposed to FMLP.

DISCUSSION

The reports of selective defects of the initiation of oxidative metabolic activity in PMNL from patients with recurrent infections (Weening *et al.*, 1976; Harvath & Andersen, 1979) raise the possibility that there are several mechanisms for the activation of the phagocytic 'respiratory burst'. Furthermore the dissociation between different parameters of metabolic activity following exposure of PMNL to the fungal metabolite cytochalasin B implies that activation of one component of the respiratory burst does not invariably lead to activation of another (Roos *et al.*, 1976). In this study we have investigated phagocytic oxidative metabolism using four techniques to monitor different aspects of metabolic activity. Superoxide dismutase inhibitable ferricytochrome C reduction is an established assay of $O_2^{\overline{\cdot}}$ release by PMNL (Babior *et al.*, 1973) although doubts have been raised about its validity (Segal & Meshulam, 1979). Evidence from experiments using sodium azide and PMNL from patients with myeloperoxidase deficiency (Stevens, Winston & Van Dyke, 1978) suggests that myeloperoxidase is required for the stimulation of PMNL luminol-dependent chemiluminescence by opsonized particles although this may not be so following stimulation by some soluble agents (Williams & Cole, 1981b). On the other hand lucigenin-dependent chemiluminescence is enhanced by azide but almost completely abolished by SOD (Williams & Cole, 1981b) suggesting that $O_2^{\overline{\cdot}}$ plays some part in its production.

By varying the divalent cation content of the extracellular medium and incubating cells with cytochalasins before exposure to stimulating agents we have attempted to differentiate between different mechanisms of activation of the respiratory burst. The importance of divalent cations for efficient phagocytosis and microbial killing has long been recognized, although their mode of action remains unclear. Not only does the effect of divalent cations vary with different facets of phagocytic cell function but also with the different agents that cause membrane perturbation. Binding of the PMNL to C3b-coated particles requires magnesium, although binding to IgG-coated particles or chemotactically active formylated oligopeptides is independent of the divalent cation concentration (Lay & Nussenzweig, 1969; Simchowitz & Spilberg, 1979). While it has been reported that IgG-coated zymosan is phagocytosed in the presence of the cation chelator EDTA (Roos, de Boer & Weening, 1977) the uptake of non-opsonized particles or particles opsonized with C3b depends on calcium and magnesium ions (Stossell, 1973).

As well as their role in phagocytosis, divalent

cations influence the ability of phagocytic cells to respond to membrane stimulation by different agents with changes in their oxidative metabolism. Whether this is in part due to failure of the stimulus–response sequence in the absence of calcium or solely to a dependence of subsequent metabolic events on divalent cations, again seems to depend on the stimulus involved. While the change in membrane potential that occurs as a result of PMNL adherence to particles coated with IgG is independent of extracellular divalent cations (Korchak & Weissmann, 1978) chelation of calcium ions inhibits the hyperpolarization induced by C5a in macrophages (Gallin & Gallin, 1977).

The findings reported in this study support the view that the role of divalent cations depends on the stimulus used to initiate metabolic activity. When zymosan is incubated with normal human serum it is opsonized primarily by C3b following activation of the alternative complement pathways, although immunoglobulin affects the kinetics and extent of C3 deposition (Schenkein & Ruddy, 1981). Consequently binding of PMNL to opsonized zymosan requires magnesium while uptake requires both calcium and magnesium ions. Because of the former it is not suprising that all four parameters (luminol- and lucigenin- dependent chemiluminescence, ferricytochrome C reduction and O_2 consumption) of PMNL metabolic activity on exposure to opsonized zymosan were inhibited by the absence of extracellular magnesium especially after incubation with EDTA. However with zymosan, the effects of changes in calcium concentration were more complex and showed some dissociation between the different parameters of metabolic activity. The requirement for extracellular calcium by zymosan-stimulated luminol-dependent chemiluminescence might in part reflect a need for phagolysosome formation with degranulation of myeloperoxidase containing granules. The other three parameters of metabolic activity were less dependent on extracellular calcium although as is shown with lucigenin-dependent chemiluminescence (Table 1) the calcium chelator MgEGTA did reduce the response. Furthermore, verapamil a selective blocker of calcium influx further reduced the response possibly by inhibiting fluxes of calcium that are not accessible to MgEGTA because of binding to acidic phospholipids in the plasma membrane (Dawson & Hauser, 1970).

In comparison with the situation with opsonized zymosan, cell membrane stimulation by the chemotactic peptide FMLP and the detergent, digitonin showed more reliance on extracellular calcium than magnesium. Digitonin stimulated chemiluminescence and ferricytochrome C reduction were markedly inhibited by the absence of extracellular calcium and this could only partially be corrected by magnesium. Similar results were obtained with FMLP although the effect on chemiluminescence of incubating cells with cation chelators was both unexpected and currently inexplicable. It may be that EDTA is affecting either some other cellular activity or the reduction of lucigenin itself, rather than the activation of respiratory burst activity by the PMNL. The marked inhibitory effect of verapamil on lucigenin-dependent chemiluminescence would suggest that despite the effect of EDTA, calcium fluxes at the cell membrane are an important aspect of the initiation of metabolic activity.

While opsonized zymosan, FMLP and digitonin showed at least some dependence on both calcium and magnesium for optimal respiratory burst activity this was not the case with PMA. Luminol-dependent chemiluminescence, ferricytochrome C reduction and O_2 consumption were unaffected by the extracellular concentration of divalent cations when PMNL were exposed to PMA and furthermore chelation of cations by incubation with EDTA or MgEGTA had no significant effect on the former. As with FMLP, EDTA increased PMA stimulated lucigenin-dependent chemiluminescence but in addition a similar response was obtained following incubation with MgEGTA, and extracellular calcium had an inhibitory effect at high concentration.

These observations support the suggestion that there are at least two mechanisms of activation of respiratory burst activity in PMNL which can in part be differentiated by their dependence on divalent cations.

The metabolic response following incubation of PMNL with cytochalasins was also dependent on the stimulus used. Measurements of ferricytochrome C reduction and O_2 consumption by cytochalasin-treated PMNL when exposed to opsonized particles were in keeping with previous observations (Roos *et al.*, 1976). It has been suggested that the dissociation of these two parameters of metabolic activity arises because of decreased cell association and thus O_2 consumption in combination with increased release of $O_2^{\cdot-}$ into the extracellular medium rather than into the phagosome thus allowing access to ferricytochrome C. An alternative explanation for the effect of cytochalasins on O_2 consumption is that there is a generalized reduction in cytoplasmic metabolic processes possibly

because of decreased uptake of metabolizable substrates from the medium (Zigmond & Hirsch, 1972b). The inhibitory effect on zymosan-stimulated luminol-dependent chemiluminescence was not unexpected in view of the previous results with divalent cations but the reduction in lucigenin-dependent chemiluminescence was more surprising and emphasizes that this technique, despite its inhibition by SOD is not interchangeable with ferricytochrome C reduction. Partially by delaying the response, cytochalasin B produced a reduction in PMA-stimulated lucigenin-dependent chemiluminescence, while other parameters of metabolic activity were unaffected. It has previously been suggested that cytochalasin B inhibits an active process that limits the duration of ferricytochrome C reduction as a result of the oxidative response of PMNL to FMLP (English *et al.*, 1981). It may be that a similar process occurs with regard to luminol- and lucigenin-dependent chemiluminescence although the time course of the former is not altered to any marked extent (Williams & Cole, 1981b). Finally, PMNL chemiluminescence in response to the addition of digitonin was almost completely abolished by prior incubation of cells with cytochalasins. The mechanisms involved in producing this effect are not understood and it is conceivable that it arises as a result of interaction between cytochalasin and digitonin rather than from combined effects on the cell membrane.

The findings of this study provide supportive evidence for the suggestion that PMNL oxidative metabolism can be activated by at least two different mechanisms which are in part differentiated by dependence on divalent cations. Furthermore it is apparent that activation of the respiratory burst need not be an 'all or nothing' phenomenon and under certain circumstances different parameters of metabolic activity may be dissociated from one another.

ACKNOWLEDGMENTS

AJW was supported by a grant from Abbott Laboratories. This work was supported by the Wellcome Trust. We thank LKB for provision of a Luminometer 1250.

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