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Effects of Calcium Antagonists on Superoxide Generation, NADPH Oxidase Activity and Phagocytic Activity in Activated Neutrophils

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ABSTRACT

NADPH oxidase dependent superoxide generation and phagocytosis in neutrophils stimulated with opsonized zymosan or heat aggregated IgG were coincided with the process of calcium uptake. The responses in activated neutrophils were enhanced with increasing concentrations of extracellular calcium and these effects were significantly inhibited by calcium chelators, EGTA and EDTA. The superoxide generation in activated neutrophils was reduced by dantrolene and chlorpromazine. Calcium antagonists, bepredil, diltiazem, verapamil, nifedipine and nimodipine effectively inhibited the calcium uptake, superoxide generation and phagocytosis in activated neutrophils, and NADPH oxidase activity was also inhibited. The results suggest that calcium antagonists may inhibit the superoxide generation and phagocytosis in activted neurtophils by the inhibition of calcium influx and by the action on intracellular redistribution of calcium and NADPH oxidase system.

Key Words: Ca^{++} antagonists, Superoxide generation, NADPH oxidase, Phagocytosis (Human neutrophil)

INTRODUCTION

In the neutrophil, calcium appears to play an important regulatory role in the expression of chemotaxis, phagocytosis and oxygen radicals generating capacity (Marasco *et al.*, 1980; Snyderman and Goetzl, 1981; Young *et al.*, 1984). A change in the steady state levels of cytosolic calcium has been suggested to be involved in the early triggering of activation of neutrophil's response by either particulate or soluble surface reacting stimuli (Newburger *et al.*, 1980). The calcium ionophore A23187 stimultes degranulation, superoxide generation and aggregation (Estensen *et al.* 1976; Goldstein *et al.*, 1974). Decreasing internal calcium, either by omission from the medium or by addition of EGTA, reduces but does not entirely abolish aggregation, degranulation or generation of superoxide radical in response to various stimuli (Smolen *el al.*, 1981).

It is reported that NADPH oxidase system which is specific to the oxygen burst of phagocytes involves the following ionic and molecular events in the plasma membrane; Na^{+} influx (Korchak and Weissmann, 1980; Showell and Becker, 1976), Ca^{++} mobilization (Bareis et al., 1982; Mottola and Romeo, 1982) and phospholipid turnover (Gil *et al.*, 1982; Hirata *et al.*, 1979). Furthermore, calcium induces enzyme release from specific granules and modulates azurophile granule discharge from PMN leukocytes prepared in calcium free media (Hoffstein and Weissmann, 1978; Naccache *et al.*, 1977). It is also reported that calcium increases neutrophil myosin ATPase activity which has been shown to be

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necessary for microfilament related contractions (Shibata, 1972). Thus, these findings suggest that the rise in $[Ca^{++}]_i$ or the calcium influx may control chemotaxis, phagocytosis, degranulation and generation of oxygen radicals through the stimulation of NADPH oxidase system (Gabig, 1983; McPhail *et al.*, 1976) in activated neutrophils.

Although the mechanism of action of organic calcium antagonists is not completely understood, it is well known that they act as calcium channel blockers and interefere with translocation of calcium ions across the cardiac and smooth muscle cell membrane (Eto *et al.*, 1974). However, calcium antagonists also interfere with other calcium requiring processes than these contractile machinery, such as chemotaxis of neutrophils in the presence of FMLP (Elferink and Deierkauf, 1984), exocytosis of histamine by mast cells (Suzuki *et al.*, 1982) and the aggregation of blood platelets (Ono and Kimura, 1981). Thus, it is suggested that calcium antagonists may interfere with other calcium dependent targets which are located intracellularly. It has been shown that verapamil and other calcium channel antagonists may interfere with neutrophil functions such as chemotaxis (Elferink and Deierkauf, 1984), superoxide generation (Irita *et al.*, 1986; Simchowitz and Spilberg, 1979) and degranulation (Elferink, 1982).

We have tried to confirm the roles of calcium in activated neutrophils responded to complement or immunoglobulin. Effects of calcium antagonists, calcium chelators, chlorpromazine and dantrolene on the calcium transport, oxygen radicals production, NADPH oxidase activity and phagocytosis in stimulated neutrophils were investigated.

MATERIALS AND METHODS

Chemicals

Verapamil, diltiazem, nifedipine, chlorpromazine (CPZ), ethyleneglycol-bis (ß-amino-ethylether), N,N,N'N',-tetraacetic acid (EGTA), ethylene diaminetetraacetic acid (EDTA), zymosan (from saccharomyces cerevisiae), human immunoglobulin G, NADPH, ferricytochrome c and dextran (M.W. 465,000) were obtained from Sigma Chemical Co., Murexide was purchased from J.T. Baker Chemical Co.; $CaCl_2$ from Kanto Chemical Co., Inc. Bepredil and nimodipine were obtained as a gift from Dr. Schwartz, A., the Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine.

Preparation of neutrophils

Neutrophils were isolated from heparinized venous blood of healthy donors by dextran (average molecular weight 460,000) sedimentation of erythrocytes and treatment with 0.85% ammonium chloride as previously described (Trush et al., 1978). The purity of neutrophil suspensions averaged 90% as judged by Wright-Giemsa stain.

Preparation of NADPH oxidase containing granule rich fraction of neutrophils

Neutrophils activated by opsonized zymosan or heat aggregated IgG at 37°C for 15 min, or control neutrophils were centrifuged at 1,500g for 3 min and the pellets were resuspended in 0.25 M sucrose to a concentration of 10^8 cells/ml. The cell suspension was the disrupted by sonication for three 15 sec intervals at 25 watts power with a Branson sonifier cell disruptor (Mod. W185D). Unbroken cells and nuclei were sedimented by centrifugation at 800g for 5 min. Sucrose was then added to the postnuclear supernatant with constant stirring and the final volume adjused to the sucrose concentration to 40%(W/V). The suspension was centrifuged at 48,000g for 1 h in a Beckman L5-50B ultracentrifuge. The supernatant completely removed and the pellets were resuspended in 0.25 M sucrose. The suspensions were centrifuged at 48,000g for 1 h and the pellets (granule rich fraction) were suspended in 25% ethylene glycol with a Teflon glass homogenizer (Gabig *et al.*, 1982; Hohn and Letrer, 1975). The protein concentration was determined by the method of Lowry et al. (1951).

Measurement of calcium uptake by neutrophils

Calcium uptake was measured by the spectrophotometric method using an Aminco-Chance dual wavelength-split beam spectrophotometer. The reaction mixtures contained 10^6 cells/ml of neutrophils, 50 μM murexide and HBSS buffer or 20 mM HEPES-tris, pH 7.4. After preincubation at 37°C for 10 min, the reaction was initiated by addition of 1 mg/ml opsonized zymosan or 0.5 mg/ml heat aggregated IgG concomittent with 1 mM calcium and final volume was a 1.0 ml. The rate and extent of calcium uptake by neutrophils was measured through the absorbance changes of calcium chelating dye, murexide, at 507-540 nm in a 1.0 ml cuvette (Malmström and Carafoli, 1979).

Assay of superoxide radical generation

The superoxide dependent reduction of ferricytochrome c was measured by the method of

Markert et al. (1985). Reaction mixtures in plastic microfuge tubes contained 10^6 neutrophils, 75 μM ferricytochrome c, HBSS buffer (or saline) and 2 mg/ml of opsonized zymosan or 0.5 mg/ml of heat aggregated IgG in a total volume of 500 μl. The reactions were performed in a 37°C shaking water bath for the stated times. The reactions were then stopped by placing the tubes in melting ice and the cells were rapidly pelleted by centrifuging at 1,500g for 5 min at 4°C. The supertants were taken and the amount of reduced cytochrome c was measured at 550 nm in a Gilford 260 U.V.-spectrophotometer. The amount of reduced cytochrome c was calculated by using an extinction coefficient of $1.85 \times 10^4 M^{-1}cm^{-1}$ at 550 nm.

Assay of NADPH oxidase activity

The activity of NADPH oxidase was measured as reduction of ferricytochrome c by superoxide radicals produced from oxidation of NADPH by NADPH oxidase. Reaction mixture consisted of 0.1 mg/ml granule rich fraction, 100μM NADPH, 75μM ferricytochrome c and 50 mM Tris-HCl, pH 7.4 in a total volume of 500 μl. The reaction mixture was preincubated for 10 min at 37°C and the reaction was initiated by adding NADPH. Reduction rate of ferricytochrome c was measured at 550 nm.

Assay for phagocytosis

The phagocytic activity of neutrophils was determined by the method of Ishibashi and Yamashita, (1982). A neutrophil suspension (10^7 cells/ml) in HBSS or saline was treated with 1 mg/ml of opsonized zymosan or 0.5 mg/ml of heat aggregated IgG. After incubation for the stated time at 37°C, neutrophils were stained with Wright-Giemsa, and the phagocytosis and attachment were determined microscopically. The phagocytic activity of neutrophils was also assayed with a hemocyanometer.

RESULTS

Calcium influx, superoxide generation, NADPH oxidase activity and phagocytosis

A relative requirement for calcium has been

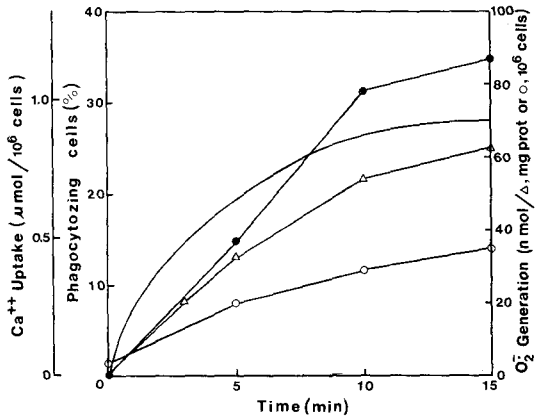

Fig. 1. Time course of Ca^{++} uptake, NADPH oxidase activity, O_2^- generation and phagocytosis in opsonized zymosan activated human neutrophils. The reaction mixture contained 10^6 neutrophils or 1 mg protein of granule rich fraction in HBSS. After 10 min of preincubation at 37°C, the reactions were started by addition of opsonized zymosan or NADPH. Ca^{++} uptake was measured at 507-540 nm by dual wavelength spectrophotometer. Measurement of O_2^- production and NADPH oxidase activity was done at 550 nm. Phagocytic activity was assayed microscopically. The points represents on average of 5 experiments. —, Ca^{++} uptake; ●, phagocytosis; △, NADPH oxidase; O, O_2^- production.

Table 1. Effects of EGTA, verapamil and bepredil on Ca^{++} uptake by activated neutrophils

Additions		Opsonized zymosan treated cells	Heat agg. IgG treated cells
		μmol Ca^{++} uptake/10^6 cells/5 min	
None		0.649 ± 0.018	0.885 ± 0.095
EGTA	1 mM	0.498 ± 0.027	0.265 ± 0.041
Verapamil	0.1 mM	0.528 ± 0.052	0.443 ± 0.022
Bepredil	0.1 mM	0.476 ± 0.034	0.351 ± 0.016

Ca^{++} uptake by resting neutrophils was 0.068 μmol/10^6 cells/5 min.
Neutrophils were preincubated with or without EGTA or calcium antagonists for 10 min at 37°C and Ca^{++} uptake was initiated by addition of opsonized zymosan or heat aggregated IgG. Ca^{++} uptake was measured by dual wavelength spectrophotometer at 507-540 nm. The value represents Mean ± S.E. of 5 experiments.

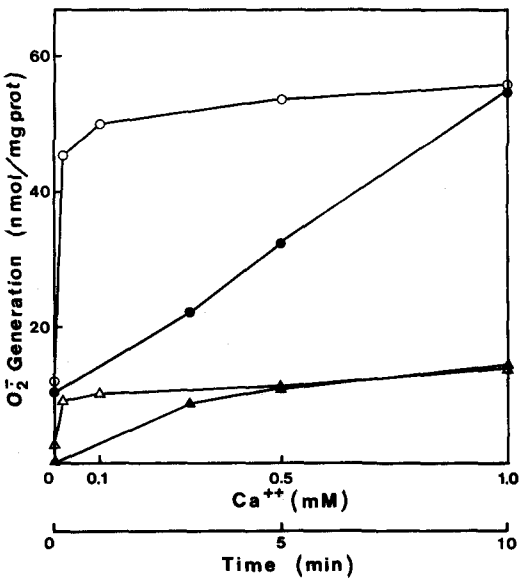

Fig. 2. Effect of Ca^{++} on NADPH oxidase in neutrophils preincubated with opsonized zymosan or not. NADPH oxidase activity was measured as the reduction of ferricytochrome c by NADPH oxidation and the reaction mixture contained 1 mg/ml of granule rich fraction, varying concentrations of calcium in saline. The points represent an average of 4 experiments. The absorbance change, O, with Ca^{++} concentration; ●, with reaction time in NADPH oxidase of cells pretreated with opsonized zymosan and, △, with Ca^{++} concentration; ▲, with reaction time in NADPH oxidase of cells preincubated with unopsonized zymosan.

demonstrated in many of neutrophil's functions including chemotaxis, phagocytosis and superoxide generation. Therefore, we investigate the changes of NADPH oxidase activity, superoxide generation and phagocytosis during calcium uptake in activated neutrophils (Fig. 1). When neutrophils in saline were incubated with calcium and opsonized zymosan *concomittently, fast calcium uptake* was attained at 5 min of reaction time and then calcium uptake was gradually increased as far as 15 min. At 5 min of incubation time, the amount of uptaked calcium was 0.649 μmoles/10^6 neutrophils. In this reaction mix-

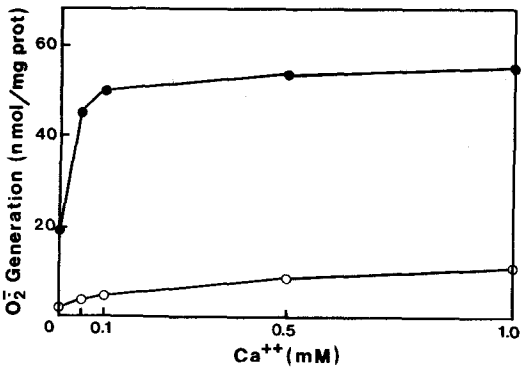

Fig. 3. Effect of Ca^{++} on NADPH oxidase in neutrophils preincubated with opsonized zymosan or opsonized zymosan plus EGTA. Experimental conditions were the same as described in Fig. 2. The points represent an average of 4 experiments. The absorbance change, ●, with 1 mg/ml opsonized zymosan and O, with 1mg/ml opsonized zymosan plus 10mM EGTA.

Table 2. Effects of EGTA, verapamil and bepredil on activated NADPH oxidase by opsonized zymosan or heat aggregated IgG

Additions		Opsonized zymosan treated cells	Heat agg. IgG treated cells
		superoxide nmol/mg protein/10 min	
None		55.67 ± 2.13	29.20 ± 1.12
EGTA	10 μM	54.59 ± 1.74	16.76 ± 1.35
	100 μM	26.48 ± 2.46	7.02 ± 1.02
Verapami	1 mM	47.02 ± 2.21	19.98 ± 1.50
Bepredil	0.1 mM	48.10 ± 1.97	22.16 ± 2.49

Activity of NADPH oxidase from resting neutrophils was 5.41 μmol/mg protein/10 min.
Granule rich fraction was preincubated with agents for 10 min at 37°C and the reaction was initiated by addition of NADPH. Reduction of ferricytochrome c by interaction of NADPH and NADPH oxidase was measured at 550 nm. The value represents Mean ± S.E. of 5 experiments.

tures, superoxide generation and phagocytic activity were coincided with calcium uptake process. Activity of prepared NADPH oxidase which located in intracellular plasma membrane was also enhanced with increasing reaction time.

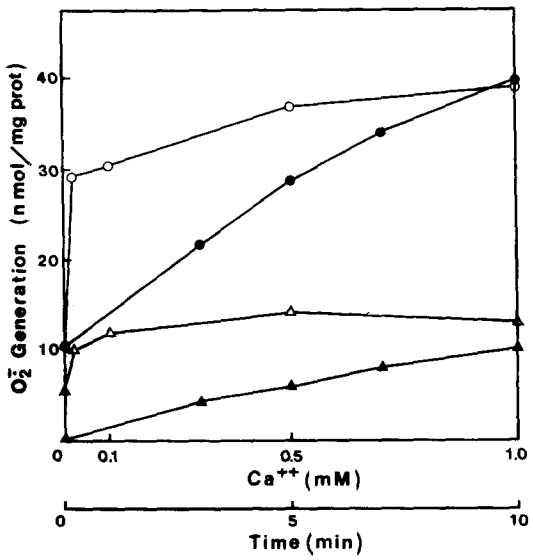

Fig. 4. Effect of Ca^{++} on NADPH oxidase in neutrophils preincubated with heat aggregated IgG or not. Experimental conditions were the same as described in Fig. 2 except containing heat aggregated IgG. The points represent an average of 4 experiments. The absorbance chage, O, with Ca^{++} concentration; ●, with reaction time in NADPH oxidase of neutrophils pretreated with heat aggregated IgG and △, with reaction time in NADPH oxidase of cells pretreated with IgG.

Effects of EGTA and verapamil on calcium uptake in activated neutrophils

It is suggested that a calcium influx plays an important in the superoxide generating capacity of the neutrophil. In the present study, both calcium chelators and calcium channel blockers inhibited the superoxide generation in activated neutrophils. Thus,

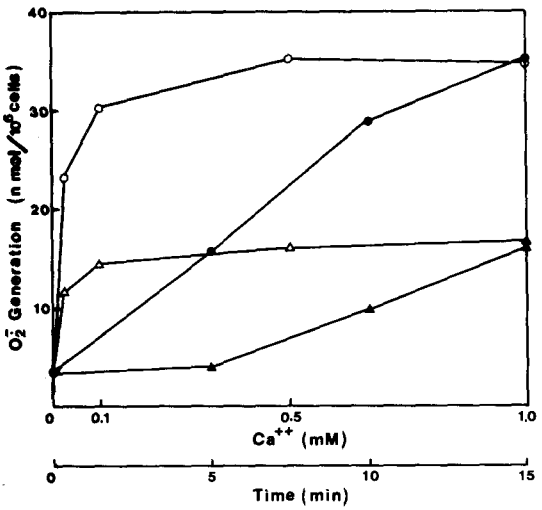

Fig. 5. Effect of Ca^{++} on opsonized zymosan or heat aggregated IgG induced superoxide generation by neutrophils. Incubation performed at 37°C for state times in the presence of 10^6 neutrophils and varying concentration of Ca^{++}. The points represent an average of 10 experiments. The absorbance change, O, with Ca^{++} concentration; ●, with reaction time in opsonized zymosan activated neutrophils and △, with reaction time in heat aggregated IgG activated neutrophils.

Table 3. Effects of EDTA and EGTA on opsonized zymosan or heat aggregated IgG induced O_2^- generation by neutrophils

Additions		Opsonized zymosan treated cells	Heat agg. IgG treated cells
		superoxide nmol/10^6 cells/15 min	
None		32.23 ± 0.49	15.91 ± 0.43
EDTA	1 mM	20.07 ± 0.21	8.70 ± 0.37
	10 mM	3.36 + 0.25	4.24 ± 0.23
EGTA	1 mM	17.83 ± 0.67	9.46 ± 0.17
	10 mM	3.84 ± 0.37	4.65 ± 0.27

Superoxide generation by resting neutrophils was 3.49 μmol/10^6 cells/15 min.
Neutrophils were preincubated with or without metal chelators for 10 min at 37°C. Measurement of O_2^- generation by neutrophils was done as described in Materials and Methods. Each value represents Mean ± S.E. of 4 experiments.

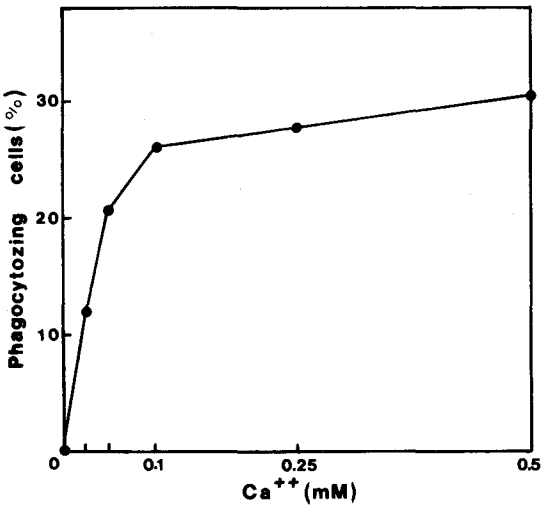

Fig. 6. Effect of Ca^{++} on phagocytosis of opsonized zymosan. Neutrophils were preincubated with the indicated concentration of Ca^{++} for 10 min at 37°C. Then opsonized zymosan was added and incubation was carried out for 15 min at 37°C. Subsequently zymosan uptake was determined microscopically.

effects of these agents on calcium uptake of neutrophils were investigated (Table 1).

Neutrophils stimulated by opsonized zymosan or heat aggregated IgG in HBSS at 37°C showed a calcium uptake of 0.649 or 0.885 μmoles/10^6 neutrophils at 5 min of reaction time, respectively. However, inactivated neutrophils which treated with unopsonized zymosan or no treated IgG, also uptaked calcium of 0.252~0.293 μmoles/10^6 cells at 5 min of reaction time. Calcium uptake in activated neutrophils was effectively inhibited by EGTA, verapamil and bepredil.

Role of calcium in NADPH oxidase activity

Since calcium may require in NADPH oxidase system which produce oxygen radicals (Irita et al., 1986; Ochs and Reed, 1981), role of calcium on activity of NADPH oxidase in neutrophils which were preincubated with opsonized zymosan was much more greater than that in neutrophils preincubated with unopsonized zymosan (Fig. 2). NADPH oxidase activity in neutrophils treated with either opsonized zymosan or unopsonized zymosan was increased with increasing both the incubation time and the concentrations of calcium. In the experimental conditions containing 0.5 mM calcium at 37°C for 10 min of incubation time, the amounts of reduced cytochrome c by NADPH-NADPH oxidase system in neutrophils preincubated with opsonized zymosan or unopsonized zymosan were 54.6 or 14.6 nmoles/mg protein/10 min, respectively. On the other hand, NADPH oxidase activities in neutrophils preincubated with opsonized zymosan or opsonized plus EGTA were also dependent on the concentrations of calcium (Fig. 3).

Similar findings were also observed in NADPH oxidase system of neutrophils pretreated with heat aggregated IgG or no treated IgG (Fig. 4). In the above described experimental conditions, the amounts of reduced cytochrome c by NADPH-NADPH oxidase interaction in cells preincubated with heat aggregated IgG or no treated IgG were 29.2 and 10.3 nmoles/mg protein/10 min, respectively.

Effects of EGTA and calcium channel blockers on NADPH oxidase activity

NADPH oxidase activity in neutrophils preincubated with either opsonized zymosan or heat aggregated IgG was almost completely inhibited by 100 μM EGTA. It is reported that calcium antagonists may act at intracellular components (Elferink, 1982). Thus, we tested effects of calcium antagonists on the activity of NADPH oxidase (Table 2). Reduction of cytochrome c by NADPH oxidase in activated neutrophils was slightly inhibited by 1 mM verapamil and 0.1 mM bepredil as ranging from 13.6% to 31.5%. In this reaction systems, NADPH oxidase in neutrophils preincubated with IgG was more sensitively responsed to calcium antagonists than in neutrophils stimulated with opsonized zymosan.

Role of calcium in superoxide generation by neutrophils

Superoxide generation in isolated human peripheral neutrophils was observed with respect to both the incubation time and the concentrations of calcium (Fig. 5). The amounts of superoxide radical generated in PMN leukocytes stimulated with 2 mg/ml of opsonized zymosan or 0.5 mg/ml of heat aggregated IgG in HBSS were increased with increasing incubation time and at 15 min, the amounts of reduced cytochrome c were 33.02, 15.90 nmoles/10^6 neutrophils/min, respectively. Even though extracellular calcium was removed by either omission from the reaction mixtures or addition of EGTA, superoxide generation in activated neutrophils was occured (Fig. 5, Table 3). However, generation of superoxide radical in neutrophils stimulated with opsonized zymosan was enhanced by calcium in a dose

dependent manner. Capacity of superoxide generation in neutrophils was maximum at a calcium concentration of 0.5 mM and fell off as this calcium concentration was slightly decreased.

These findings were also investigated in heat aggregated IgG stimulated neutrophils.

Effects of calcium chelators and dantrolene on superoxide generation in activated neutrophils

Since superoxide generation in activated neutrophils was affected by intracellular calcium distribution and activation of neutrophils have been shown to enhance uptake of calcium from the extracellular medium, effects of calcium chelators and dantrolene which is known to inhibit release of calcium from intracellular calcium storage sites (Van Winkle, 1976) on superoxide generation in activated neutrophils were investigated.

EDTA and EGTA effectively inhibited superoxide production by opsonized zymosan in HBSS and calcium dependent O_2^- production was almost completely abolished at 10 mM of calcium chelators.

As shown in Table 3, enhanced generation of superoxide radical by heat aggregated IgG was also significantly inhibited by EDTA and EGTA.

Dantrolene reduced superoxide generation and its

Table 4. Effects of dantrolene, EGTA and chlorpromazine on opsonized zymosan or heat aggregated IgG induced O_2^- generation by neutrophils

Additions	No activated cells	Opsonized zymosan treated cells	Heat agg. IgG treated cells
	superoxide nmol/10^6 cells/5 min		
None	2.54 ± 0.24	33.64 ± 0.41	15.75 ± 0.12
Dantrolene 0.1 mM	1.12 ± 0.06	27.79 ± 0.11	10.23 ± 0.28
EGTA 1 mM	2.35 ± 0.17	18.47 ± 0.59	9.31 ± 0.14
Dantrolene 0.1 mM + EGTA 1 mM	1.41 ± 0.16	16.22 ± 0.28	8.45 ± 0.47
CPZ 0.1 mM	2.21 ± 0.13	16.10 ± 0.15	—

Neutrophils were preincubated with or without agents for 10 min at 37°C. Each value represents Mean ± S.E. of 6 experiments.

Table 5. Effects of calcium antagonists on opsonized zymosan or heat aggregated IgG induced O_2^- generation by neutrophils

Additions		Opsonized zymosan treated cells	Heat agg. IgG treated cells
		superoxide nmol/10^6 cells/15 min	
None		33.02 ± 0.37	15.90 ± 0.33
Diltiazem	0.1 mM	30.17 ± 0.18	—
	1 mM	6.19 ± 0.48	5.52 ± 0.24
Verapamil	0.1 mM	28.27 ± 0.52	—
	1 mM	4.73 ± 0.53	4.84 ± 0.21
Nifedipine	0.1 mM	26.53 ± 0.36	11.87 ± 0.34
	1 mM	14.46 ± 0.42	—
Nimodipine	0.1 mM	22.09 ± 0.35	11.08 ± 0.11
	1 mM	11.56 ± 0.12	—
Bepredil	1 μM	27.08 ± 0.27	—
	10 μM	24.92 ± 0.49	—
	0.1 mM	5.85 ± 0.24	4.62 ± 0.16

Neutrophils were preincubated with or without calcium antagonists for 10 min at 37°C. Each value represents Mean ± S.E. of 4-6 experiments.

Table 6. Effects of calcium chelators and calcium antagonists on phagocytosis of opsonized zymosan

Additions		Normal cells	Attached cells	Phagocytozing cells
		Relative percentage for total cells		
None		60.1	7.7	32.2
EGTA	1 mM	88.3	3.4	8.3
	10 mM	95.8	2.6	3.8
Verapamil	1 mM	82.9	2.7	14.4
Diltiazem	1 mM	84.8	3.7	11.5
Bepredil	0.1mM	82.4	3.4	14.2

Neutrophils were preincubated with agents for 10 min at 37°C and the reaction was initiated by addition of opsonized zymosan. After 15 min of reaction time, zymosan uptake was determined microscopically. The value represents an average percentage of phagocytozing cells for control in 6 experiments.

effect on IgG stimulated neutrophils was greater than zymosan stimulated neutrophils (Table 4). On the other hand, when stimulated neutrophils were concurrently treated with dantrolene plus EGTA, superoxide generation was reduced as numerical summation.

Chlorpromazine, known as protein kinase c inhibitor and calmodulin inhibitor also significantly inhibited superoxide generation in opsonized zymosan activated neutrophils (Table 4).

Thus, the results suggest that changes of extracellular calcium concentration and intracellular redistribution of calcium apparently affect the ability of activated neutrophils which produce oxygen radicals.

Effects of calcium channel blockers on superoxide generation in activated neutrophils

The present study suggests that existance of extracellular calcium or uptake of calcium from the extracellular medium may be necessary for superoxide generation in activated neutropils.

All the calcium channel blockers tested were shown to inhibit superoxide generation in neutrophils activated by opsonized zymosan (Table 5). In the presence of calcium channel blockers at concentration of 10^{-4}M, reduction of ferricytochrome c by neutrophils stimulated with opsonized zymosan was inhibited as ranging from 8.6% in the presence of diltiazem to 82.3% in the case of bepredil. Superoxide generation in zymosan activated neutrophils was markedly inhibited by bepredil in a dose dependent manner.

Similar effects were seen when neutrophils were stimulated with heat aggregated IgG and the most effective inhibition was again achieved in the presence of bepredil (Table 5).

The reduction of ferricytochrome c by zymosan or IgG-stimulated cells was effectively inhibited by superoxide dismutase (data not shown).

Generation of H_2O_2 in activated netrophils was also effectively inhibited by EGTA and calcium antagonists (data not shown).

Effects of EGTA and calcium antagonists on phagocytic activity of neutrophils

Phagocytic activity of neutrophils stimulated with opsonized zymosan was dependent upon existance of extracellular calcium and activity was increased by calcium concentrations.

Because phagocytic activity of neutrophils was affected by extracellular calcium concentrations, the effects of EGTA and calcium antagonists on phagocytic function of neutrophils were investigated. Phagocytic activity of neutrophils stimulated by opsonized zymosan in HBSS was markedly inhibited by EGTA, verapamil, diltiazem and bepredil (Table 6).

DISCUSSION

The trigger mechanism for stimulating the respiratory burst in neutrophils may consist of a rapid shift of divalent cations from the environment and plasmalemma binding sites toward special zone of the cytoplasm (Gallin and Rosenthal, 1974; Goldstein *et al.*, 1975). A series of recent studies indicate that an increase of free cytoplasmic calcium is involved in the early triggering of the responses of neutrophils to surface stimulation by both particulate and soluble agents (Lew *et al.*, 1984; Young *et al.*, 1984). It is also suggested that fMet-Leu-Phe promotes influx of calcium ions across the plasma membrane of neutrophils by opening of receptor dependent calcium channels (Andersson *et al.*, 1986).

The present results indicate that extracellular calcium is required for the responses of activated neutrophils such as enhanced phagocytosis and activation of NADPH oxidase (Fig. 2,4,6). As can be seen in Fig. 1, activity of NADPH oxidase, superoxide generation and phagocytic activity in neutrophils stimulated by opsonized zymosan or heat aggregated IgG were correspond with the process of calcium uptake in same conditions of neutrophils.

On the other hand, it is demonstrated that some degrees of superoxide generation (Fig. 5, Table 3) and phagocytosis in activated neutrophils were occured independently in the existance of calcium in the reaction medium (Lew *et al.*, 1985). However, this findings do not mean that calcium is not required for above functions because that neutrophils can perform a number of calcium dependent cell functions by mobilizing calcium from intracellular stores. In addition, these findings are supported by that NADPH oxidase dependent superoxide generation and phagocytic capacity in activated neutrophils were markedly inhibited by calcium chelators (Table 3).

Since a calcium influx may play an important role in the responses of neutrophils and the results show that extracellular calcium is required for the neutrophil functions, we investigated the effects of calcium channel blockers on calcium uptake, NADPH oxidase dependent superoxide generation and phagocytosis in neutrophils. Calcium antagonists have been shown to selectively block the calcium channel, an entry pathway of calcium influx, in contraction of cardiac muscle (Fleckenstein, 1971), in depolarization of neural axons (Janis, 1981), and in hormonal release by neurohypophyseal and pancreatic islet cells (Malaisse and Sener, 1981; Russell and Thorn, 1974). Verapamil and diltiazem have been shown to have no effect on the sodium channel and either to have no effect on or to inhibit calcium efflux (Trriggle, 1981; Wollheim *et al.*, 1978). In the present study, calcium antagonists over the concentration of 10^{-4}M effectively inhibited calcium uptake (Table 1), superoxide generation (Table 5) and phagocytosis (Table 6) in neutrophils stimulated with opsonized zymosan or heat aggregated IgG. On the other hand, calcium antagonists at the concentration under 10^{-5}M showed a little effect (Table 5). However, it is well known that the potency of calcium antagonists to block calcium channels in leukocytes and neurons seems to be considerably less than in cardiac cells (Romey and Lazdunski, 1982). At high concentrations such as $10^{-4}\sim10^{-3}$M, verapamil and other calcium antagonists exhibited an effective inhibitory effects on the degranulation and locomotion in neutrophils (Elferink, 1984). Thus, these results indicated that calcium antagonists selectively blocked calcium influx through calcium channel, they inhibited superoxide generation in activated neutrophils, and suggested that the two processes of calcium influx and superoxide generation may be related.

The production of superoxide by neutrophils is a consequence of two separate precesses: activation (trigger) and enzyme activity. Some drugs may stimulate NADPH oxidase dependent superoxide generation through effect on the activation systems located in the plasma membrane (Ochs and Reed, 1981). It has been suggested that two possible intracellular mediators of activation of the respiratory burst, namely calmodulin (Takeshige and Minakami, 1981) and protein kinase c (Virgilio *et al.*, 1986), both of which are calcium ion dependent. On the other hand, since calcium antagonists strongly inhibit chemotaxis and locomotion in the absence of extracellular calcium (Irita *et al.*, 1986), it seems therefore likely that calcium antagonists have an intracellular site of action. This view is supported by the fact that the lipophilic characteristics of these drugs permit an easy penetration into the cells. Although the nature of the intracellular structure on which calcium antagonists act is unknown, there are a number of potential targets. One of the possibilities is that calcium antagonists interfere with calcium translocation across membranes of intracellular organelles (Zoster and Church, 1983). Another possibility with which calcium antagonists may interfere, is calmodulin (Schlondorff and Satriano, 1981). Chlorpromazine, known as a calmodulin inhibitor significantly inhibited superoxide generation in activated neutrophils (Table 4). Thus, it is suggested that calcium antagonists inhibited superoxide generation in activated neutrophils by inhibition of calcium influx.

We investigated role of calcium and effects of calcium antgonists on the superoxide generating activity of preparative NADPH oxidase system. Superoxide generation by NADPH oxidase obtained from activated neutrophils was greater than that from untreated neutrophils. NADPH oxidase activity in neutrophils, whether stimulated by opsonized zymosan and heat aggregated IgG or not, was enhanced with the concentrations of calcium (fig. 2,4). Meanwhile, the changing pattern of NADPH oxidase activity for calcium in unopsonized zymosan treated was similar to that in neutrophils which treated with opsonized zymosan plus EGTA (Fig. 3). Superoxide generating activity of NADPH oxidase was remarkably inhibited by EGTA (Table 1). Superoxide generation in NADPH oxidase system

was also reduced by dantrolene (Table 4) which is known to inhibit the release of calcium from intracellular calcium storaging organelles such as sarcoplasmic reticulum (Van Winkle, 1976). Thus, results suggest that calcium move from intracellular calcium storaging sites to the functional systems containing NADPH oxidase activator and calcium is necessary for the activation of NADPH oxidase. In these reaction, calcium antagonists at the concentrations of 10^{-4}M slightly inhibited superoxide generation in NADPH oxidase system (Table 2). The findings suggest that calcium antagonists may act directly at the NADPH oxidase system.

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=국문초록=

칼슘 길항제가 활성화된 호중구에서의 O_2^-의 생성, NADPH oxidase활성도 및 탐식작용에 미치는 영향

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면역보체가 결합되어 있는 zymosan 또는 열로 응집된 IgG에 의하여 활성화된 호중구에서의 NADPH oxidase 의존적인 O_2^-의 생성과 탐식작용은 칼슘의 흡수과정과 일치하였다. 활성화된 백혈구의 반응은 세포외 칼슘 농도에 따라 항진되었으며, 이는 칼슘의 킬레이트제인 EGTA나 EDTA에 의하여 유의하게 억제되었다. 활성화된 백혈구로부터 O_2^-의 생성은 dantrolene과 chlorpromazine에 의하여 억제되었다.

칼슘 길항제인 bepredil, diltiazem, verapamil, nifedipine, nimodipine은 효과적으로 활성화된 백혈구의 칼슘흡수, O_2^-생성 그리고 탐식 작용을 억제하였고 NADPH oxidase 활성도 또한 억제하였다.

그러므로, 칼슘 길항제는 칼슘 유입을 억제하거나 칼슘의 세포내 재분포 및 NADPH oxidase 반응계에 작용하여 활성화된 백혈구에서의 O_2^-의 생성과 백혈구의 탐식작용을 억제할 것으로 시사되었다.