Vol. 115, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1983 Pages 261-267

Essential Requirement of Magnesium Ion for Optimal Activity of the NADPH Oxidase of Guinea Pig Polymorphonuclear Leukocytes

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Received July 13, 1983

SUMMARY: The NADPH-dependent O_2^-(H_2O_2)-forming oxidase-rich plasma membranes were purified from myristate(MA)-activated polymorphonuclear leukocytes using a Percoll-density gradient method. The specific activity of the enzyme in the plasma membrane fraction was twelve times higher than that in the cells. Studies on the effect of divalent cations and chelators on the O_2^- and H_2O_2 generating activity of the oxidase showed that Mg^{2+}, but not Ca^{2+}, enhanced the activity significantly. Zn^{2+}, on the other hand, was slightly inhibitory to the oxidase activity. EDTA markedly inhibited the oxidase activity whereas EGTA enhanced it. The optimal oxidase activity was seen in the presence of μmolar concentrations of Mg^{2+} and reached a maximum at Mg^{2+} concentrations of 40-50 μM. The addition of Mg^{2+} resulted in a decrease in the apparent Km of the oxidase for NADPH from 40 μM to 25 μM and an increase in apparent Vmax by 1.5 times. These results suggest that Mg^{2+} enhances both NADPH binding and catalytic activities of the oxidase.

When exposed to bacteria or soluble stimulants, polymorphonuclear leukocytes(PMN) exhibit a respiratory burst which is characterized by an increase in cyanide-insensitive respiration(1-3). An NADPH oxidase, that generates O_2^- and H_2O_2 by coupled oxidation of NADPH, is responsible for the repiratory burst, because this activity appears only when PMN are exposed to those stimulants(4,5). In view of recent reports(6-8), it is reasonable to consider that the oxidase is embedded in the plasma membrane and its NADPH binding site is located on the inner surface of the membrane(9). Previous reports(10-12) using the cellular system showed that Ca^{2+} and Mg^{2+} are essential for the optimal activation of the oxidative metabolism of PMN. However, some reports using the subcellular system showed inconsistent results. When an NADPH oxidase fraction containing various organelles(12) and a filtrate of the solubilized oxidase(13) were used, Ca^{2+} and Mg^{2+} had no significant effect on the oxidase activity,

Abbreviations: PMN, polymorphonuclear leukocytes; MA, myristate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N, N, N', N',-tetraacetic acid; CCP, cytochrome c peroxidase.

0006-291X/83 $1.50

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while a detergent-treated paticulate fraction(14) was used, the oxidase required both Ca^{2+} and Mg^{2+} for its maximum activity. The discrepancy appears to be attributed to the difference in the method of enzyme preparation. To clarify this problem one needs to use more purified NADPH oxidase fraction.

As reported recently(8), we found that the Percoll-density gradient method was superior to the sucrose-density gradient method for the purification of the plasma membrane NADPH oxidase. In this report, such a fraction was used to study the effect of divalent cations and their chelators on the plasma membrane-bound NADPH oxidase. The present data shows that the NADPH oxidase requires Mg^{2+} for optimal activity.

MATERIALS AND METHODS

Cell preparation Guinea pig PMN were isolated from the peritoneal cavity as described previously(1) except for the use of 6% casein solution instead of 12% casein solution. The collected cells were suspended in 17 mM N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid buffer saline(pH 7.4) containing 5 mM KCl and 1.2 mM $MgCl_2$(HBS).

Preparation of the plasma membrane fraction from MA-activated PMN The plasma membrane fraction was prepared from MA-activated PMN as reported elsewhere(8) with a modification. The cells in HBS containing 5 mM glucose were activated with MA(110 nmol/10^7 cells) for 30 sec at 37°C. Soon after the incubation, the cell suspension was loaded on a chilled silicone oil layer and centrifuged immediately at 1400 x g for 3 min at 4°C. Sedimented cells were suspended in 0.34 M sucrose containing 10 mM Tris-HCl(pH 7.4) and homogenized in an ice bath with a Teflon pestle homogenizer at 1000 rpm(total of 60 strokes). The homogenate was centrifuged at 400 x g for 10 min at 4°C. The resulting supernatant was loaded on a 0 to 50% Percoll density gradient in 0.34 M sucrose containing 10 mM Tris-HCl(pH 7.4) cushioned by 50% sucrose. The density gradient centrifugation was performed with a Beckman 14 Ti Zonal rotor at 20,000 rpm for 60 min. Fractions of which the 5'-nucleotidase activity was highest, were centrifuged at 105,000 x g for 60 min. The resulting pellet was washed 3 times with 0.34 M sucrose containing 10 mM Tris-HCl(pH 7.4) by ultra-centrifugation.

The assay of O_2^- and H_2O_2 generating activity The O_2^- generating activity was measured by following the reduction of acetylated cytochrome c as described previously(15). The basal assay mixture contained 15 μM 63% acetylated cytochrome c, 5 μg/ml catalase, 0.1 mM NADPH, and an aliqout of the plasma membranes in 65 mM sodium pottasium phosphate buffer/0.17 M sucrose(pH 7.0). The rate of H_2O_2 generation was measured according to the cytochrome c peroxidase method(5). The basal assay medium contained 5 μM cytochrome c peroxidase(CCP), 0.1 mM NADPH, and an aliqout of the plasma membranes in the same sucrose buffer as described for the O_2^- assay.

Enzymatic activities 5'-Nucleotidase activity was measured as a plasma membrane marker as reported previously(16). The phosphorus released was estimated according to the method of Youngburg and Youngburg(17). NADPH cytochrome c reductase and lysozyme activities were assayed as described previously as microsomal and granule markers, respectively(18,19).

Protein Protein was determined by the method of Lowry et al.(20) with bovine serum albumin as standard. Interferance by Tris or other components of reaction mixtures was avoided by using samples precipitated with 5% trichloroacetic acid.

Materials Cytochrome c(TypeIV, from horse heart), catalase, NADPH and AMP were purchased from Boehringer Co., Mannheim. Superoxide dismutase and bovine serum albumin were obtained from Sigma Co., St. Louis. Micrococcus lysodeikticus (Millipore Co., N.J.) and Percoll(Pharmacia Fine Chem. Co., London) were purchased. EDTA and ethylene glycol bis(β-aminoethyl ether)-N, N, N', N',-tetra-acetic acid(EGTA), and myristate was obtained from Wako Pure Chem. Co., Tokyo. Acetylated cytochrome c was prepared as reported previously(15). Cytochrome c peroxidase was purified from baker's yeast according to the method of Yonetani(21). All other reagents were of analytical grade. Deionized and double-distilled water was used for the buffers throughout this study.

RESULTS

Partial purification of plasma membranes from MA-activated PMN: Table I compares activities of various marker enzymes in the plasma membrane fractions and those in whole cells. 5'-Nucleotidase, NADPH cytochrome c reductase, and lysozyme activities were measured as markers to represent plasma membranes, microsomes and granules, respectively. The specific activity of O_2^- generation by the NADPH oxidase in the plasma membrane fraction was 12.4 fold higher than that in whole cells. The specific activity of 5'-nucleotidase in the plasma membrane fraction increased 12.3 fold, corresponding to the increment ratio of the NADPH oxidase activity. In contrast, the plasma membrane fraction showed only 2.3 times higher NADPH cytochrome c reductase activity and one-tenth lower lysozyme activity than those in whole cells. These data indicate that the plasma membrane fraction prepared by the present procedures contained a high NADPH oxidase activity with little contamination by other organelles.

The effect of divalent cations and its chelators on the NADPH oxidase in the plasma membrane fraction: Table II summerized the effect of divalent cations

Table I Specific activities of marker enzymes in the plasma membrane fraction and in whole cells

	O_2^- generation (nmol/min/mg protein)	5'-nucleotidase (μmol/15 min/mg protein)	NADPH-cytochrome c reductase (nmol/min/mg protein)	lysozyme (unit/mg protein)
Whole cells	2.45	0.9	1.07	31.9
Plasma membrane fraction	30.2	11.2	2.50	3.49

Cells were activated with myristate and fractionated by Percoll density-gradient centrifugation. For the assay of O_2^- generating activity, the assay mixture contained 15 μM acetylated cytochrome c, 5 μg/ml catalase, 0.1 mM NADPH and an aliquot of sample in 0.17 M sucrose/65 mM Na-K-phosphate buffer(pH 7.0). For the other enzyme assays see MATERIALS AND METHODS.

Table II. The effect of divalent cations and its chelators on the NADPH oxidase in the plasma membrane fraction

		H_2O_2 generating activity (nmol/min)	(percent of control)
Control		1.42	100
$MgCl_2$	0.15 mM	2.45	173
	1.50 mM	2.45	173
$CaCl_2$	0.15 mM	1.38	97
	1.50 mM	1.34	95
$ZnCl_2$	0.15 mM	0.82	58
	1.50 mM	0.67	47
EDTA	0.15 mM	0.32	23
	1.50 mM	0.40	28
EGTA	0.15 mM	2.15	151
	1.50 mM	1.96	138

The reaction mixture contained 51 µg/ml protein of plasma membranes, 5 µM CCP and 0.1 mM NADPH in the same sucrose buffer as described for the O_2^- assay.

and their chelators on the NADPH oxidase. Mg^{2+} enhanced the rate of H_2O_2 generation by the oxidase, whereas Ca^{2+} had no effect. Zn^{2+} inhibited the NADPH oxidase activity. EDTA markedly inhibited the H_2O_2 generation by the oxidase while EGTA was stimulatory. Similar results were obtained for its O_2^- generating activity(data not shown). Figure 1 shows the effect of various concentration of Mg^{2+} on the NADPH oxidase in terms of O_2^- generation. The

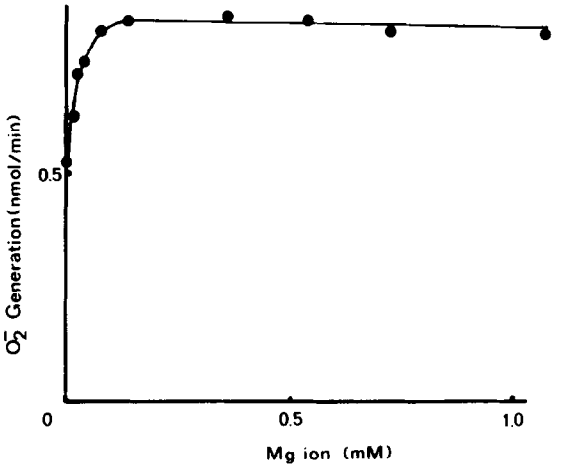

Figure 1. The NADPH-dependent O_2^- generation by the plasma membrane-bound oxidase under various Mg^{2+} concentrations. The assay mixture was as for Table I, except that 28.8 mg/ml protein of plasma membranes was used.

maximum activity was observed with 40-50 μM Mg^{2+}. A similar result was obtained for H_2O_2 production with this membrane-bound oxidase fraction.

Kinetics of the effect of Mg^{2+} on the NADPH oxidase in the plasma membarne: A kinetic study of the NADPH oxidase was performed to analyze the stimulatory effect of Mg^{2+}. Figure 2 shows Lineweaver-Burk plots of the NADPH oxidase activity as a function of NADPH concentration in the presence and absence of 0.25 mM Mg^{2+}. The apparent K_m of the oxidase for NADPH was 40 μM in the absence of Mg^{2+} and 25 μM in the presence of Mg^{2+}, respectively. The apparent V_{max} of the NADPH-dependent H_2O_2 generating activity in the presence of Mg^{2+} was 1.5 times greater than that in the absence of Mg^{2+}. Thus, Mg^{2+} enhances both NADPH binding and catalytic activities of the oxidase(22).

DISCUSSION

Mg^{2+} was found to enhance the NADPH oxidase activity of partially purified plasma membranes, in which both 5'-nucleotidase and NADPH oxidase activities were about 12 fold higher than those in cells. The results suggest that the NADPH oxidase in plasma membranes of activated PMN can exhibit the optimal activity to form $O_2^-(-H_2O_2)$ at intracellular concentrations of Mg^{2+}. The apparent

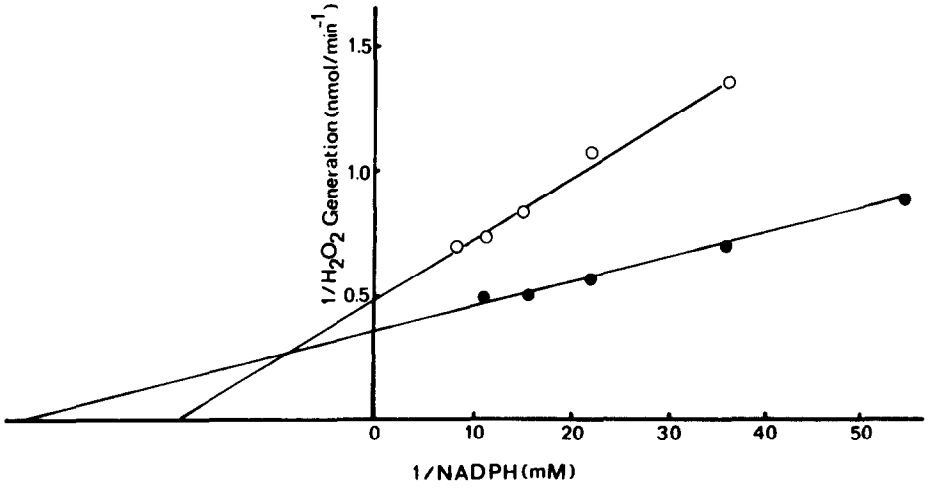

Figure 2. Lineweaver-Burk plots of H_2O_2 generation by the plasma membrane-bound oxidase as a function of NADPH concentration in the presence(closed circle) and absence(open circle) of Mg^{2+}. The assay mixture contained 51 μg/ml protein of plasma membranes, 5 μM CCP and various amounts of NADPH in sucrose buffer with or without 0.25 mM Mg^{2+}.

K_m for NADPH of the oxidase in the presence of Mg^{2+} was similar to that obtained previously by using a 20,000 x g pellet that contained the NADPH oxidase and other organelles(23). The 20,000 x g pellet may contain over 40 μM Mg^{2+}. Recently, Green et al.(14) reported that both Ca^{2+} and Mg^{2+} were requied for the optimal oxidase activity. Our present data showed that Ca^{2+} had no effect on the NADPH oxidase(Table II). The stimulatory effect of Mg^{2+} on the NADPH oxidase was not influenced by the addition of Ca^{2+}(data not shown). In addition, EGTA, a potent chelator of Ca^{2+}, was found to enhance the NADPH oxidase activity(Table II). From the present data, we may conclude that the NADPH oxidase located in the plasma membrane requires Mg^{2+} but not Ca^{2+} for its optimal activity. The apparent stimulatory effect of Ca^{2+} on the oxidase activity, as reported by Green et al.(14), who employed a Triton X-100-treated paticulate fraction, may be attributed to the interaction of the detergent with Ca^{2+}.

Zn^{2+} inhibited the H_2O_2 generation by the NADPH oxidase. In the present experiments, the membrane-bound NADPH oxidase generated a quantity of H_2O_2 which amounted to the level of oxidized NADPH in the presence of EGTA(data not shown). Therefore, the effect of EGTA was not attributed to the inhibition of the cuprizinc superoxide dismutase. The effect of EGTA on the NADPH oxidase is similar to its effect on the Na-K-ATPase. The activity of this enzyme was increased by the addition of EGTA, probably due to the chelation of Zn^{2+} but not Ca^{2+}(24,25). Although, the mechanism of the enhancement of the NADPH oxidase by EGTA is unknown, its stimulatory effect could occur by a similar mechanism as in the case of Na-K-ATPase(25).

ACKNOWLEDGEMENTS

This work was partially supported a grant from the Ministry of Education, Science and Culture of Japan. We are very grateful to Professor Toshio Asakura for critically reading this manuscript.

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