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THE O_2^- GENERATING OXIDOREDUCTASE OF HUMAN NEUTROPHILS: EVIDENCE OF AN OBLIGATORY REQUIREMENT FOR CALCIUM AND MAGNESIUM FOR EXPRESSION OF CATALYTIC ACTIVITY

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SUMMARY: NADPH-dependent O_2^- generating oxidoreductase activity recovered from cell lysates of phorbol myristate acetate-stimulated human neutrophils exhibits dependence on Ca^{+2} and Mg^{+2} for full expression of its catalytic activity. O_2^- generating activity was completely abolished by exposure of the oxidoreductase to EDTA, then reconstituted by exposure of the enzyme to Ca^{+2} and Mg^{+2} in excess of the EDTA concentration used to block catalytic activity. The oxidoreductase responded maximally to either 0.25 mM Ca^{+2} or 0.80 mM Mg^{+2}. The pH optimum of the oxidoreductase exposed to Ca^{+2} and Mg^{+2} is between pH 7.0 and 7.6. The molar ratio of NADPH oxidation to O_2^- production determined at pH 7.6 in the presence of Ca^{+2} and Mg^{+2} is 0.49, indicating 1 mole of NADPH oxidized per 2 moles of O_2^- formed. Particulate fractions recovered from cell lysates of resting neutrophils exhibited no oxidoreductase activity under the same conditions.

The NADPH dependent O_2^- generating oxidoreductase of human neutrophils plays a central role in effecting oxidative killing of bacteria through its capacity to generate O_2^- from NADPH and molecular O_2 (1,2). Several indirect lines of evidence suggest that it is a multienzyme complex regulating the production of O_2^- through a series of linked redox reactions (3-8). Enzyme activity is not seen in unstimulated (resting) cells, but it can be detected and recovered in the subcellular particulate fraction derived from cell lysates of stimulated cells (6-11). Ca^{+2} and Mg^{+2} are essential cations required by the neutrophil for chemotaxis, phagocytosis and degranulation (2). In particular, these two metal ions are required for activation of resting cells wherein expression of latent O_2^- generating activity ensues (2,12,13).

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In this paper we present evidence that the O_2^- generating oxidoreductase of human neutrophils is markedly dependent upon Ca^{+2} and Mg^{+2} for expression of its catalytic activity.

MATERIALS AND METHODS: Ferricytochrome c, superoxide dismutase, NADPH, phorbol myristate acetate (PMA)[2], dimethyl sulfoxide, xanthine, xanthine oxidase and triton X-100 were all obtained from Sigma Chemical Co., St. Louis, MO. Sodium diatrizoate-ficoll was purchased from Bionetics Laboratory Products, Kensington, MD. All other chemicals were of the best grade available.

Purified neutrophils were prepared from whole blood by collection of the buffy coat and subsequent centrifugation on diatrizoate-ficoll density gradients as previously described (14). Oxidoreductase-rich particulate fractions were prepared by resuspending purified neutrophils in 5 ml Hank's buffered saline solution, pH 7.4, to which was added PMA to a final concentration of 10 ug/ml (stock PMA, 1 mg/ml in dimethyl sulfoxide). Following a 5 min centrifugation at 400 x $\bar{g}$, the PMA-stimulated cell pellet was resuspended in 8 to 10 ml of ice-cold 1 mM Tris-HCl, pH 7.0, and lysed by sonication. NADPH-dependent O_2^- generating oxidoreductase activity was recovered in the 27,000 x $\bar{g}$ particulate fraction following 30 min centrifugation of the cell lysate. This fraction contained greater than 90% of the total oxidoreductase activity present in cell lysates and was stored in ice-cold 1 mM Tris-HCl, 15% glycerol, pH 8.6, at a final protein concentration of approximately 1 mg/ml protein. Aliquots of this enzyme suspension were diluted and assayed as described below.

All enzyme assays were made on a Cary 219 double beam spectrophotometer at room temperature. O_2^- generating activity was assayed by the cytochrome c assay essentially as previously described (7), except the total reaction volume was reduced to 1.25 ml, and the buffer employed in the final assay mixture was varied as indicated in the text. Ca^{+2} and Mg^{+2} solutions were prepared from $CaCl_2$ and $MgSO_4$, respectively. Variations in the trapping efficiency of the cytochrome c assay for O_2^- through additions of metals to the assay buffers were assessed by the xanthine-xanthine oxidase O_2^- generating couple (15).

NADPH oxidase activity was also similarly assayed, but at 340 nm with exclusion of cytochrome c and superoxide dismutase from the reaction mixture. Calculation of the NADPH oxidation rate was made by using an absorbtivity coefficient of 6.22 $mM^{-1}cm^{-1}$ (16).

Immediately before assaying for oxidoreductase activity enzyme aliquots were diluted 10% by volume in 2% triton X-100 made up in 1 mM Tris-HCl, 15% glycerol, pH 8.6. One milliunit (mU) of oxidoreductase activity corresponds to the production of 1 nmole/min of O_2^-.

Protein was determined by the method of Lowry et al. (17).

RESULTS AND DISCUSSION: Curve A of Figure 1 shows NADPH-dependent O_2^- generating activity in the presence of 0.32 mM Ca^{+2} and 0.20 mM Mg^{+2} (Ca/Mg ratio = 1.6). Omission of Ca^{+2} and Mg^{+2} from the assay buffer resulted in approximately half as much activity (curve B), whereas exposure of the enzyme to 0.80 mM EDTA resulted in almost complete inhibition (curve C). Particulate fractions derived from unstimulated cells, even with metal additions, exhibited no NADPH-dependent O_2^- generating activity (curve D). Phosphate had no apparent effect on the Ca/Mg response at the concentrations employed since the same effect was also seen with

[2]Abbreviations: O_2^-, superoxide; PMA, phorbol myristate acetate.

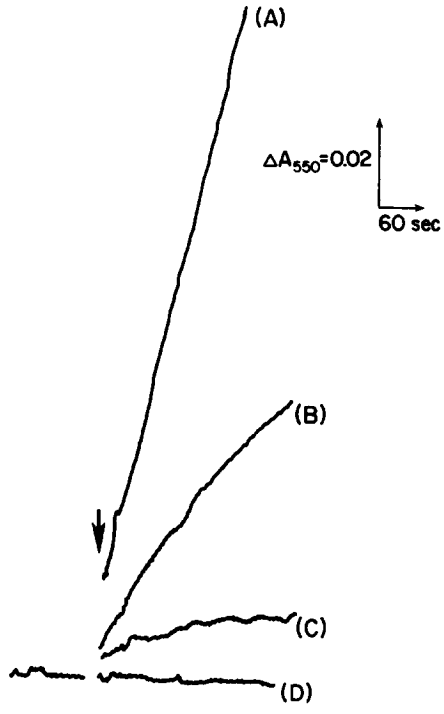


Figure 1. Effect of metal additions and EDTA chelation on NADPH-dependent O_2^- generating activity. 30 ul aliquots of the 27,000 x $\bar{g}$ particulate fraction derived from PMA-stimulated neutrophils were assayed as described in Materials and Methods in: (A), 10 mM potassium phosphate buffer, pH 7.6, supplemented with 0.32 mM $CaCl_2$ and 0.20 mM $MgSO_4$; (B), buffer without metal additions; (C), buffer supplemented with 0.80 mM EDTA; (D),27,000 x $\bar{g}$ particulate fraction recovered from cell lysates of unstimulated neutrophils assayed as in (A). Arrow indicates addition of enzyme. Omission of enzyme or NADPH from the final reaction mixture resulted in a flat tracing illustrated by the tracing preceeding enzyme addition.

Ca^{+2} and Mg^{+2} suspended in 10 mM Tris-HCl. The effect of Ca^{+2}, Mg^{+2} and EDTA in these experiments was not an artifact of the cytochrome c trapping assay for O_2^- created by alterations in the trapping efficiency of cytochrome c for O_2^-. Control experiments with xanthine oxidase as a means of generating O_2^- revealed no difference in the rate of O_2^- production as measured by the cytochrome c assay whether Ca^{+2}, Mg^{+2} or EDTA was present or absent from the assay buffer.

As shown in Table I the presence of both Ca^{+2} and Mg^{+2} were required for maximal catalytic activity. Absolute exogenous concentrations of Ca^{+2} and Mg^{+2} as low as 60 and 50 uM, respectively, were effective in enhancing catalytic activity. Maximum activity was achieved upon exposure of the oxidoreductase to concentrations of Ca^{+2} and Mg^{+2} on the order of 0.26 and 0.20 mM, respectively

Table I. Effect of EDTA, Ca^{+2} and Mg^{+2} on O_2^- Generating Oxidoreductase Activity.[a]

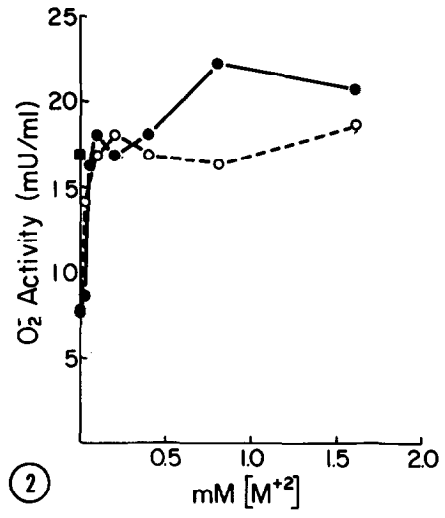
	mM Ca^{+2}	mM Mg^{+2}	mM EDTA	Ca/Mg Ratio	mU/ml
Experiment I.	-	-	-	-	3.8
Effect of Ca^{+2} and Mg^{+2} alone.	1.0	-	-	-	7.9
	-	1.0	-	-	7.6
Experiment II.	0.06	0.05	-	1.25	9.2
Effect of Ca^{+2} and Mg^{+2} together.	0.26	0.20	-	"	11.8
	1.0	0.80	-	"	12.2
Experiment III.	1.0	0.40	1.6	2.5	0
Effect of EDTA chelation	2.0	0.8	"	"	6.5
and reconstitution with Ca/Mg additions.	2.0	0.8	-	"	10.6

[a] All enzyme assays were conducted in 1 mM potassium phosphate buffer with additions and deletions as shown at a final pH of 7.6. The pH was confirmed by direct measurement of the reaction mixture with a standard pH electrode placed in the assay cuvette. Experiments I, II and III were all conducted in series on the same lot of enzyme as described in Materials and Methods.

(cf., Table I). Complete inhibition was achieved upon exposure of the enzyme suspension to 1.6 mM EDTA, whereas inclusion of Ca^{+2} and Mg^{+2} in the assay buffer at concentrations exceeding that of the EDTA partially restored catalytic activity (Table I).

Catalytic activity was also enhanced upon addition of either Ca^{+2} or Mg^{+2} alone to the assay buffer. Under these circumstances peak activity was achieved in the presence of 0.25 mM Ca^{+2} or, alternatively, 0.80 mM Mg^{+2} (Figure 2). No shift in the pH optimum of the oxidoreductase was evident with respect to total O_2^- generating activity whether Ca^{+2} and Mg^{+2} additions to the assay buffer were made or omitted. Optimal activity occurred at pH values ranging from approximately 7.0 to 7.6 (Figure 3).

The specific activity of the oxidoreductase assayed at pH 7.6 in the presence of 0.32 mM Ca^{+2} and 0.20 mM Mg^{+2} averaged 50 mU mg^{-1} protein. The molar ratio of NADPH oxidation to O_2^- production under the same assay conditions was 0.49 ± 0.05 (n=6; $\pm$ SD). This latter value is in excellent agreement with the



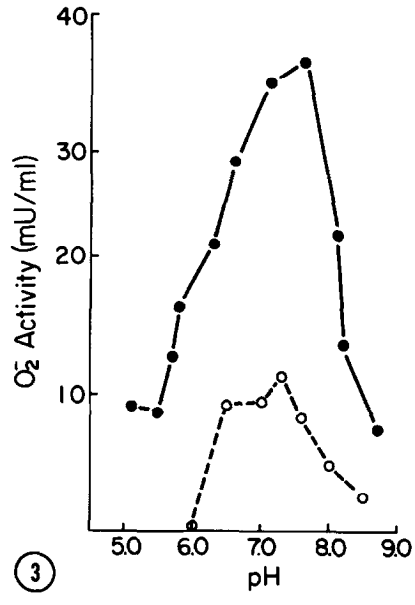


Figure 2. Effect of Ca^{+2} and Mg^{+2} additions on oxidoreductase activity following 30 min exposure to 0.2% triton X-100 in 1 mM Tris-HCl, 15% glycerol, pH 8.6. (○), enzyme activity at varying concentrations of Ca^{+2}; (●), enzyme activity at varying concentrations of Mg^{+2}; (■), enzyme activity in the presence of 0.32 mM Ca^{+2} and 0.20 mM Mg^{+2}.

Figure 3. pH optimum of NADPH-dependent O_2^- generating activity in the presence of 0.32 mM Ca^{+2} and 0.20 mM Mg^{+2} (●), and in the absence of exogenous metal ion additions (○). All assays were conducted as described in Materials and Methods in 10 mM potassium phosphate buffer at varying pH values as indicated.

theoretical value of 0.50, assuming one mole of NADPH is oxidized for every two moles of O_2^- formed (6,10,11).

At lower pH values near 5.5 Mn^{+2}-dependent oxidoreductase activity has been found in association with the particulate fraction derived from stimulated neutrophils (18). The low pH optimum seen in the presence of Mn^{+2} has been attributed to artifactual nonenzymatic O_2^- dependent free radical reactions between Mn^{+2}, NADPH and O_2^- (2,19). An important observation in these studies, nevertheless, is that the oxidoreductase is essential in initiating these reactions. Thus there is already precedence for the participation of a divalent metal ion in the catalysis of O_2^- production mediated by the oxidoreductase. In the present study we have identified yet another form of metal ion dependency whereby the oxidoreductase exhibits optimal catalytic activity at physiological

pH. The mechanism by which Ca^{+2} and Mg^{+2} effect O_2^- production through the action of the oxidoreductase on NADPH remains to be elucidated.

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