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ROLE OF Mg^{2+} IN ACTIVATION OF NADPH OXIDASE OF HUMAN NEUTROPHILS: EVIDENCE THAT Mg^{2+} ACTS THROUGH G-PROTEIN

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The membrane fraction and three cytosolic proteins of neutrophils, p47-phox, p67-phox and a G-protein, are involved in the cell-free activation of the O_2^--generating NADPH oxidase in the presence of SDS, though it has been controversial whether the G-protein is required or just enhancing the activity. We have used the three cytosolic factors, the solubilized membrane fraction, GTPγS and SDS, and found that both G-protein and GTPγS are essential for the activation of the NADPH oxidase. The effect of GTPγS is modified by Mg^{2+}: the cations enhance the O_2^- generation at low concentrations of GTPγS, whereas they attenuate the activity at higher concentrations of GTPγS. In the presence of 10 μM GTPγS, the maximal activity is observed at 0.1 μM Mg^{2+}, which is several-fold higher than that at 1 mM Mg^{2+}. The omission of Mg^{2+} followed by the chelation with EDTA results in loss of the activation, which is completely restored by the addition of Mg^{2+}. Thus, Mg^{2+} seems to modulate the activation of the NADPH oxidase at the level of the G-protein.

Neutrophils generate superoxide anions (O_2^-) in response to various stimuli by means of an NADPH-dependent oxidase, which is an electron transport enzymatic complex [1]. The molecular approach to the mechanism of the oxidase activation has advanced by the development of a cell-free activation system consisting of both membrane and cytosolic fractions of neutrophils in the presence of SDS [2]. Essential factors for the activation in the membrane fraction are cytochrome b_{558} and phospholipids [3, 4] and those in the cytosolic fraction are the proteins (with the indicated molecular mass in kDa) p47-phox and p67-phox [5,6]. In addition to p47-phox and p67-phox, a third cytosolic component, a low molecular weight G-protein, participates in the cell-free activation of the NADPH oxidase [7-10]. The participation of the G protein is initially suggested by the enhancing effect of GTP and GTPγS on the oxidase activation [11-16], and is confirmed by the purification of the protein followed by the determination of its partial amino acid sequences [7-9]. In addition, it has been reported that

Abbreviations used: GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); GDPβS, guanosine 5'-*O*-(2-thiodiphosphate).

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recombinant small G-proteins can enhance the activation of the oxidase [8,10]. It is, however, still controversial whether the G-protein is obligatory for the activation or just stimulating the activation, and whether GTP is essential or not. To address these questions, we have partially purified the G-protein and reconstituted the activation system. During the course of this study, we have noticed an effect of Mg^{2+} on the cell-free activation of the NADPH oxidase. It is well known that Mg^{2+} is not only essential for the binding of guanine nucleotides to small G-proteins but also controls the activation of G-proteins by affecting their protein conformations: the rate of GTP/GDP exchange in submicromolar concentrations of Mg^{2+} is much higher than that in millimolar concentrations of Mg^{2+} [17-21]. Although Gabig et al. have shown that Mg^{2+} is required for the cell-free activation of the NADPH oxidase [13], little is known about the role of Mg^{2+} in the activation process.

The present study shows that both the G-protein and GTPγS are essential for the activation of the NADPH oxidase and that Mg^{2+} modulates the activation at the level of the G-protein.

MATERIALS AND METHODS

Materials—GTPγS and GDPβS were purchased from Sigma Chemical., St. Louis; n-Octyl β-glucopyranoside (octyl glucoside) and (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (p-APMSF) from Wako Pure Chemical Co., Osaka; 2',5'-ADP-Sepharose CL-6B and DEAE-Sepharose CL-6B from Pharmacia Fine Chemicals, Uppsala, Sweden. Other reagents were of analytical grade. Recombinant p47-phox and p67-phox were prepared by an expression system using recombinant baculoviruses as described by Letto et al. [22].

Preparation of subcellular fractions of human neutrophils—Human neutrophils were isolated from healthy volunteers by dextran sedimentation, hypotonic lysis and the Conray-Ficoll centrifugation as described previously [23]. After several washes, the cells were suspended at 2 x 10^8 cells/ml in a phosphate-buffered saline composed of 131 mM NaCl, 20 μg/ml chymostatin, 20 μM p-APMSF, 15 μg/ml leupeptin, 2 μg/ml pepstain, 1 mM EGTA and 10 mM potassium phosphate, pH 7.0. The cells were disrupted by sonication using a Branson sonifier (model 185) at 0 °C for three 15-s bursts. The membrane and cytosolic fractions were prepared by sequential centrifugations as described previously [24]. The membrane fraction at a final concentration of 8 x 10^7 cell equivalents/ml was solubilized with 0.75 % octyl glucoside as described by Nozaki et al. [4].

Chromatographic procedures—The cytosolic fraction (26.4 mg of protein) was applied to a 2',5'-ADP-Sepharose CL-6B column (1 x 5 cm) equilibrated with 131 mM NaCl in buffer A composed of 1 mM EGTA, 2 mM NaN_3, 0.1 mM dithiothreitol, 10 μM p-APMSF and 10 mM potassium phosphate, pH 7.0. After several washes with the buffered saline until absorbance at 280 nm is decreased below 0.05, elution was performed with the buffer A containing 0.5 M NaCl. The flow-through fraction on the column (fraction A1) and the fraction which was eluted with the 0.5 M NaCl solution (fraction A2) were obtained. Fraction A1 was dialyzed for 12 hours against 10 mM potassium phosphate, pH 7.2. The dialyzed sample was applied onto a DEAE-Sepharose CL-6B column (1 x 5 cm) equilibrated with buffer B composed of 1 mM EGTA, 2 mM NaN_3, 0.1 mM dithiothreitol, 10 μM p-APMSF and 10 mM potassium phosphate, pH 7.2. After washes, the elution was performed with 0.2 M NaCl in the buffer B and then with 0.6 M NaCl in the same buffer. The flow-through fraction, the fraction eluted with 0.2 M NaCl and the fraction eluted with 0.6 M NaCl were termed as fractions D1, D2 and D3, respectively. All chromatographic procedures were performed at 4 °C.

Assay of the NADPH oxidase activity—The NADPH-dependent O_2^--generating activity was measured by determining the rate of superoxide dismutase-inhibitable cytochrome *c* reduction at 550-540 nm using a Hitachi 557 dual-wavelength spectrophotometer. The rates are calculated using a molar absorption coefficient of 19.1 $mM^{-1} \cdot cm^{-1}$. The assay mixture was composed of 75 μM cytochrome *c*, 10 μM FAD, 1 mM NaN_3, 1 mM EGTA and 50 mM potassium phosphate, pH 7.0, with the indicated concentrations of $MgCl_2$ and GTPγS. Free Mg^{2+} concentrations in the mixture were calculated as described by Sunyer et al. [25]. After the membrane and cytosolic components were preincubated for 3 min at 25°C with 110 μM SDS, NADPH was added to the

reaction mixture at the final concentration of 250 μM. Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin as standard.

Immunoblot analyses—Monoclonal antibodies, MoAb3 and MoAb19, were prepared using recombinant human p47-phox and p67-phox produced in E. coli, respectively [26,27]. Immunoblotting of proteins from SDS-PAGE (10% acrylamide) gels were performed and the blots were probed with MoAb3 and MoAb19 (1:1500) and then with secondary antibody/peroxidase conjugates (1:500) according to the method described by Leto et al. [22].

RESULTS AND DISCUSSION

Pick and co-workers have demonstrated that the cytosolic components of guinea pig macrophages are separated on a 2',5'-Sepharose column into two fractions, the flow-through and the bound ones, both of which are necessary for the activation of the NADPH oxidase [28]. In addition, they have recently purified the G-protein, a low molecular weight G-protein, from the former fraction and shown that the latter fraction is composed of p47-phox and p67-phox [7]. We used the same column to fractionate the cytosolic factors of human neutrophils as shown in Fig. 1(*a*). The flow-through and bound fractions of the 2',5'-ADP-Sepharose column, designated as A1 and A2, respectively, were tested in the activation system of the NADPH oxidase reconstituted from the solubilized membrane fraction and SDS. The addition of both the fractions A1 and A2, instead of the unfractionated cytosolic fraction, resulted in the activation of the oxidase, whereas the addition of either fraction alone was ineffective (Fig. 1(*a*)), a finding consistent with that by Pick's group [28]. The immunoblot analyses with monoclonal antibodies to human p47-phox and p67-phox demonstrated that both proteins exist in the fraction A2 but not in the fraction A1 (Fig. 1(*a*), inset). The combination of the recombinant p47-phox and p67-phox completely replaced the fraction A2 in activating the oxidase (data not shown). These findings indicate that the active components in the fraction A2 are p47-phox and p67-phox, and the fraction A1 contains the third cytosolic factor, the G-protein [7,8,28].

As shown in Fig. 1(*b*), the active component in the fraction A1 was further purified on a DEAE-Sepharose column, which has been used by Pick's group for the purification of the G-protein [7]. The partially purified factor in the fraction D2 which eluted at 0.2 M NaCl activated the NADPH oxidase as an essential component in a dose-dependent manner (Fig. 1(*b*), inset), whereas the other fractions D1 and D3 possessed negligible activities. In addition to p47-phox and p67-phox, thus, the third cytosolic factor, the G-protein, is also absolutely required for the activation of the oxidase.

Using the fractions A2 and D2, we reconstituted the activation system of the NADPH oxidase together with the membrane fraction and SDS, and tested the effect of GTPγS to clarify the role of the G-protein. As shown in Table I, GTPγS was required for activating the oxidase. The effect of GTPγS was observed even without Mg^{2+} added, but still seemed to be dependent on the cations. The omission of Mg^{2+} followed by the addition of EDTA resulted in loss of the activation, which was completely restored when Mg^{2+} (6 mM) in molar excess of EDTA (5 mM) was present in the activation mixture, in comparison with the activity in the presence of 1 mM $MgCl_2$ added(Table I). Although the requirement for GTPγS was clearly shown without Mg^{2+} added, we observed that Mg^{2+} activated the oxidase even without GTPγS added (Table I). This activation, however, may also be a GTP-requiring process, because the Mg^{2+}-dependent

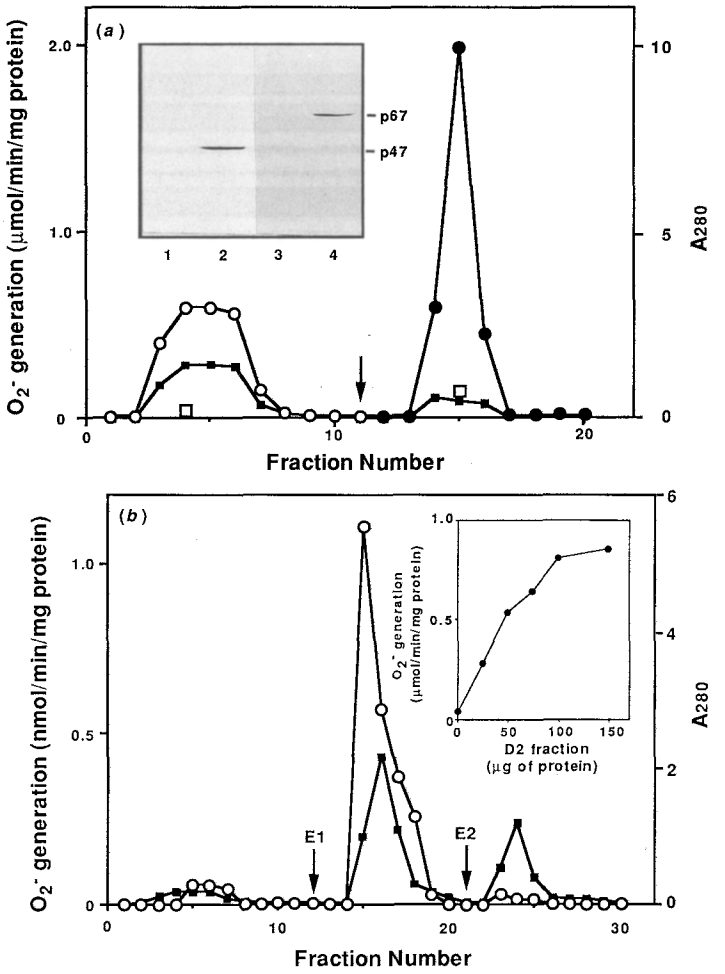

Fig.1. Chromatographic fractionation of the neutrophil cytosolic fraction
(*a*) 2',5'-ADP-Sepharose Chromatography of the cytosolic fraction. The cytosolic fraction was applied to a 2',5'-ADP-Sepharose column. The arrow represents the start of elution with 0.5 M NaCl. Tracings record absorbance at 280 nm (■) and the O_2^--generating activity upon combining with 40 μl of fraction 4 (●), 40 μl of fraction 14 (○) or none (□) from the 2',5'-ADP-Sepharose column in the presence of the solubilized membrane fraction (6.6 μg) as described under Materials and Methods. (*Inset*) Immunoblot analysis of the 2',5'-ADP-Sepharose fractions. The flow-through fraction, termed A1 (123.2 μg: lanes 1 and 3) and the bound fraction, termed A2 (37.8 μg: lanes 2 and 4) were analyzed by SDS-PAGE on 10 % acrylamide and immunoblotted with monoclonal antibodies to p47-phox (lanes 1 and 2) and to p67-phox (lanes 3 and 4) as described under Materials and Methods.
(*b*) DEAE-Sepharose chromatography of the A1 fraction. After dialysis, the fraction A1 was applied to a DEAE-Sepharose CL-6B column. Arrows E1 and E2 indicate the start positions of elution with 0.2 M NaCl and with 0.6 M NaCl, respectively. Tracings record absorbance at 280 nm (■) and the O_2^--generating activity upon combining with the fraction A2 (25.2 μg) in the presence of 6.6 μg of the solubilized membrane fraction (○) as described under Materials and Methods. (*Inset*) Dose dependence of the NADPH oxidase activation on the fraction which eluted at 0.2 M NaCl (fraction D2). The indicated amounts of the fraction D2 were used for the O_2^--generating activity upon combining with the fraction A2 (25.2 μg) in the presence of 6.6 μg of the solubilized membrane fraction as described under Materials and Methods.

activation was inhibited by GDPβS (data not shown). The effect of Mg^{2+} in the absence of GTPγS added could be explained by residual endogenous GTP of the partially purified G-proten. Thus, both GTPγS and Mg^{2+} are required for the activation of the NADPH oxidase.

Table I. Effects of GTPγS and Mg^{2+} on the cell-free activation system of the NADPH oxidase

Combination	O_2^- generation (μmol/min/mg protein)
none	0.14
GTPγS	2.18
GTPγS + EDTA (5 mM)	0.16
GTPγS + EDTA (5 mM) + $MgCl_2$ (6 mM)	1.76
GTPγS + $MgCl_2$ (1 mM)	1.71
$MgCl_2$ (1 mM)	1.14

The NADPH oxidase was activated using the fractions A2 (37.8 μg), the fraction D2 (123.2 μg), the solubilized membrane fraction (6.6 μg) and 110 μM SDS in the presence or absence of 10 μM GTPγS with or without the indicated concentrations (given in parentheses) of $MgCl_2$ and EDTA. The fractions A2 and D2 were prepared as described under Materials and Methods. The O_2^--generating activity was determined as described under Materials and Methods and expressed as μmol/min per mg of membrane protein.

The mode of the Mg^{2+} action appears to be slightly complicated. The O_2^- generation without Mg^{2+} added was enhanced by 1 mM Mg^{2+} in the presence of less than 1μM GTPγS, but was attenuated by the cations at higher concentrations of GTPγS (Table II), suggesting that Mg^{2+} modulates the activation at the level where GTPγS acts, *i.e.* the G-protein. It is well known that Mg^{2+} is not only essential for the binding of guanine nucleotides to small G-proteins but also controls the activation of G-proteins by affecting their protein conformations: the rate of GTP/GDP exchange in submicromolar concentrations of Mg^{2+} is much higher than that in millimolar concentrations of Mg^{2+} [17-21]. The NADPH oxidase activation was strongly

Table II. Effect of the Mg^{2+} on the activation of the NADPH oxidase in various concentrations of GTPγS

[GTPγS]	O_2^- generation (μmol/min/mg protein)		fold-increase by Mg^{2+}
	no $MgCl_2$ added	1mM $MgCl_2$	
10^{-8} M	0.14	0.83	5.93
10^{-7} M	0.65	1.09	1.68
10^{-6} M	1.35	1.39	1.03
10^{-5} M	1.69	1.01	0.60
10^{-4} M	1.53	0.79	0.52

The NADPH oxidase was activated using the fractions A2 (25.2 μg), the fraction D2 (123.2 μg), the solubilized membrane fraction (6.6 μg) and 110 μM SDS with or without 1 mM $MgCl_2$ in the presence of various concentrations of GTPγS. The O_2^--generating activity was determined as described under Materials and Methods and expressed as μmol/min per mg of membrane protein.

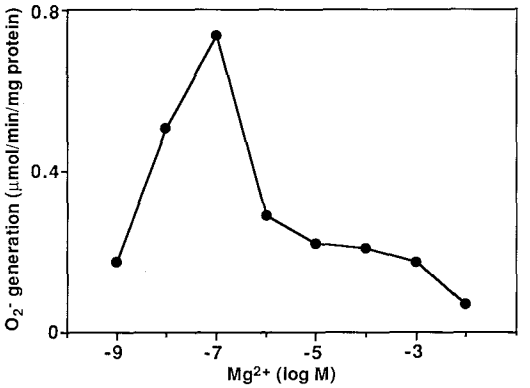

Fig.2. Effect of the Mg^{2+} concentrations on the activation of NADPH oxidase
The NADPH oxidase was activated using the solubilized membrane fraction (6.6 μg), the fractions A2 (25.2 μg) and D2 (61.6 μg), and 10 μM GTPγS in the presence of various concentrations of Mg^{2+}. Free Mg^{2+} concentrations were calculated as described previously [25]. The O_2^--generating activity was determined as described under Materials and Methods and expressed as μmol/min per mg of membrane protein.

dependent on Mg^{2+} concentrations with the maximal activity being observed at 0.1 μM Mg^{2+}, which was several-fold higher than that at 1 mM Mg^{2+} (Fig. 2). The dependence is the same as those on Mg^{2+} concentrations of the GTP-binding rate to small G-proteins [18-20], suggesting that low concentrations of Mg^{2+} may facilitate the binding of GTPγS to the G-protein, leading to the activation of the oxidase.

In the present study, we showed that the third cytosolic factor, the G-protein, is essential for the activation of the NADPH oxidase, in addition to p47-phox and p67-phox. A GTP-bound form of the G-protein is considered to be active, since GTPγS activates the oxidase as an essential factor but GDPβS inhibits the GTP-dependent activation. Mg^{2+} is not only required for the GTP-binding, but also may change the rate of the binding to the G-protein to modulate the activation as suggested by the present study. The G-protein, thus, plays a central role in the NADPH oxidase activation.

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