Protein kinase C phosphorylates a component of NADPH oxidase of neutrophils

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A protein of 31.5 kDa belonging to the NADPH oxidase of neutrophils was phosphorylated following stimulation of the cells with phorbol myristate acetate. The same protein was phosphorylated in vitro in the presence of cytosol and of Ca^{2+} and phosphatidylserine. The phosphorylation in vitro of the 31.5 kDa protein was increased by phorbol myristate acetate and was inhibited by trifluoperazine. The data are compatible with an involvement of protein kinase C in the activation of NADPH oxidase.

NADPH oxidase *Cytochrome* b_{245} *Phosphorylation* *Protein kinase* *Neutrophil activation* *Respiratory burst*

1. INTRODUCTION

The enzyme, or enzyme system, NADPH oxidase of phagocytes is dormant in resting cells but is activated when they engulf bacteria or when they are stimulated by soluble compounds [1–3]. As a consequence of this activation, large amounts of oxygen-derived free radicals are produced and discharged in the phagocytic vacuole and in the surrounding medium.

On the basis of much indirect evidence, it has been suggested that a phosphorylation process, possibly catalyzed by Ca^{2+} and phospholipid-dependent protein kinase C, may be involved in the activation of NADPH oxidase [4–7]. However, the following remain to be established: (i) the molecule which is the target of the phosphorylation process, (ii) the type of protein kinase which is involved and (iii) the relationship between phosphorylation and enzyme activation.

Recent work from this laboratory dealt with extraction and partial purification of NADPH oxidase from the plasma membrane of neutrophils. The active enzyme was obtained in the form of a high-M_r proteolipid complex containing various protein species including cytochrome b_{-245} [8–11].

Electrophoretic analysis of such enzyme preparations from guinea pig [9] and pig [11] neutrophils demonstrated that a protein of 31.5 kDa is a part of the NADPH oxidase complex and probably corresponds to cytochrome b_{-245}. This protein is phosphorylated in vivo when the cells are stimulated with phorbol myristate acetate [11].

The present report provides further evidence of the phosphorylation of the 31.5 kDa protein both in vivo and in vitro and shows that this polypeptide is phosphorylated by Ca^{2+} and phospholipid-dependent protein kinase C.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Phorbol 12-myristate 13-acetate (PMA), phosphatidylserine (PS) and phenylmethanesulphonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). PMA was dissolved in dimethyl sulfoxide (DMSO). Trifluoperazine (TFP) was a gift from Smith, Kline and French Laboratories (Herts, England). Carrier-free [^{32}P]orthophos-

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phoric acid and [γ-^{32}P]ATP were purchased from Amersham (England). All other reagents were of the highest grade available.

2.2. *Phosphorylation in vivo and partial purification of NADPH oxidase*

Neutrophils, isolated from pig blood [10], were labeled with ^{32}P by incubation of the cells (350×10^6/ml) with 0.11 mCi/ml of ^{32}P for 90 min at 37°C as described [11]. After centrifugation and washing, the cells were divided into 2 aliquots, one of which was treated with PMA (1 μg/ml) for 5 min at 37°C. Resting cells were treated only with an equivalent volume of DMSO. Then the cells were homogenized by sonication, the membranes isolated by sucrose density gradient centrifugation, and NADPH oxidase extracted by solubilization of the membranes and partially purified by gel filtration chromatography through Ultrogel AcA34 essentially according to [11]. By using this method, NADPH oxidase was eluted from the chromatographic column as a high-M_r proteolipid complex which was sedimentable upon 3-fold dilution with water and centrifugation for 2 h at 100000 $\times$ g. When necessary, ultracentrifugation of NADPH oxidase complexes was used to concentrate the enzyme before electrophoresis or phosphorylation in vitro.

2.3. *Phosphorylation in vitro of partially purified NADPH oxidase*

A partially purified NADPH oxidase preparation was obtained from resting cells as described in section 2.2 but omitting the treatment of the cells with ^{32}P. A cytosolic fraction from resting neutrophils was prepared as follows. 5×10^8 pig neutrophils were suspended in 3 ml ice-cold 20 mM Tris-Cl buffer, pH 7.5, containing 10 mM β-mercaptoethanol, 5 mM EGTA, 2 mM EDTA and 1 mM PMSF. The cells were sonicated at 0°C with 3 pulses of 10 s at 150 W with a Labsonic 1510 (B. Brown) sonifier and then centrifuged for 60 min at 100000 $\times$ g. The supernatant cytosolic fraction was used as the source of protein kinase.

The standard reaction mixture for the phosphorylation in vitro contained, in a final volume of 0.4 ml, 50 mM Tris-Cl buffer, pH 7.5, 10 mM $MgCl_2$, 1 mM NaF, 0.01% Triton X-100, 20 μM [^{32}P]ATP (10^6 cpm/nmol), 120 μg partially purified NADPH oxidase, 10 μl cytosolic fraction and, where indicated, 0.5 mM Ca^{2+}, 50 μg PS, 40 ng PMA, 0.4 mM TFP. Where Ca^{2+} was omitted, 1 mM EGTA was added to the reaction mixture. The reaction was started by the addition of [^{32}P]ATP and the incubation was carried out for 10 min at 30°C. The reaction was terminated by addition of 2 ml ice-cold 50 mM Tris-Cl buffer, pH 7.5, containing 1 mM EGTA and 0.3 M NaCl and the samples were centrifuged for 2 h at 100000 $\times$ g. The pellets containing NADPH oxidase complexes were suspended in 150 μl of 50 mM Na-phosphate buffer, pH 7.5, containing 10% glycerol, 1 mM EGTA and 1 mM PMSF and were subjected to electrophoresis. When phosphorylation of the cytosolic proteins in the absence of NADPH oxidase was examined, the final centrifugation was omitted and samples were directly subjected to electrophoresis.

2.4. *Electrophoresis and autoradiography*

Samples were treated with 2% SDS and 3% (v/v) β-mercaptoethanol and incubated for 1 h at 50°C in the presence of 1 mM PMSF and 10% glycerol. Electrophoresis was run for 7–8 h at 150 V (constant) on slab gels ($13 \times 20 \times 0.1$ cm) consisting of a linear polyacrylamide gradient (4–20%), with 40 mM Tris-Cl buffer, pH 8.0, containing 1% SDS, 1 mM EDTA and 20 mM Na-acetate as electrode buffer. The gels were stained for protein with Coomassie brilliant blue R250, destained, dried and exposed to Kodak X-OMAT R films in a radiographic cassette for 4–6 days at −70°C.

3. RESULTS AND DISCUSSION

3.1. *Protein phosphorylation in vivo*

^{32}P-labeled neutrophils were divided into 2 aliquots, one of which was stimulated with PMA. The activated (A) and resting (R) cells were processed according to [11] until a partially purified preparation of NADPH oxidase was obtained by gel filtration chromatography. Fig.1 shows the phosphorylated proteins in the postnuclear supernatant (lanes 1A, 1R), in plasma membranes isolated by sucrose density gradient (lanes 2A, 2R) and in partially purified NADPH oxidase obtained by gel filtration chromatography (lanes 3A, 3R). The band of 31.5 kDa which is a component of the

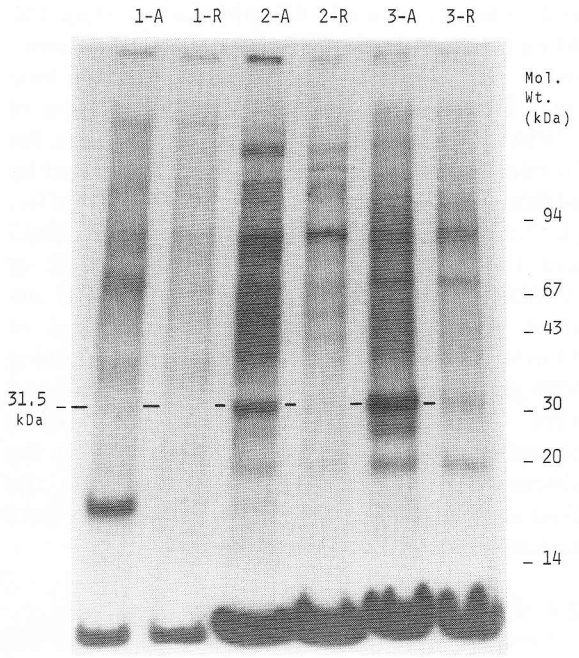

Fig.1. Autoradiograph of neutrophil [^{32}P]phosphoproteins at different stages of purification of NADPH oxidase. ^{32}P-labeled neutrophils were incubated with PMA (A) or with DMSO (R), homogenized and centrifuged at low speed to obtain the postnuclear supernatant fraction (lanes 1-A and 1-R). Membranes (lanes 2-A and 2-R) were isolated by sucrose density gradient centrifugation of the postnuclear supernatant and partially purified NADPH oxidase (lanes 3-A and 3-R) was obtained by gel filtration chromatography of extracts of the membranes. SDS-polyacrylamide gel electrophoresis and autoradiography were done as described in section 2. 50 μg protein were subjected to electrophoresis in each lane.

NADPH oxidase [9,11] was markedly phosphorylated in the preparations from activated cells and progressively increased in the 3 purification steps. It has been shown [11] that using this purification procedure NADPH oxidase specific activity and cytochrome b_{-245} specific content increased by about 6-fold in the plasma membrane compared to the postnuclear supernatant and by further 3-fold in the peak eluted from gel filtration chromatography compared to the plasma membrane. Therefore, the 31.5 kDa phosphorylated protein increased in parallel with the purification of the oxidase and this indicates that this polypeptide is the component of the enzyme which is the target of the phosphorylation.

3.2. *Protein phosphorylation in vitro*

In the experiment shown in fig.1 the phosphorylation occurred in vivo, i.e. in ^{32}P-labeled neutrophils stimulated by PMA. Since PMA is an activator of protein kinase C [12], it is conceivable that the phosphorylation is catalyzed by this type of protein kinase. However, the situation in vivo is complicated and activation of other kinases, such as those dependent on calmodulin or on cyclic nucleotides, cannot be excluded. The involvement of protein kinase C could be reinforced if phosphorylation of the 31.5 kDa protein in vitro was (i) dependent on Ca^{2+} and phospholipids, (ii) stimulated by PMA and (iii) inhibited by protein kinase C inhibitors. The following data show that these are the cases.

Partially purified NADPH oxidase prepared from resting neutrophils was incubated with the cytosol of resting neutrophils in the presence of [γ-^{32}P]ATP and in the presence or absence of Ca^{2+}, PS, PMA and TFP. After incubation, NADPH oxidase was pelleted by ultracentrifugation to remove excess ATP and cytosolic proteins that interfere with electrophoretic identification of the phosphorylated proteins. The pellet was then subjected to SDS electrophoresis followed by autoradiography (fig.2). The 31.5 kDa protein was phosphorylated in the presence of Ca^{2+} and PS (lane 2) and the phosphorylation was increased by PMA (lane 3). In the absence of Ca^{2+} and PS (lane 1) phosphorylation of this protein was practically undetectable and the same pattern of lane 1 was obtained by omitting Ca^{2+} alone or PS alone in both the presence and absence of PMA (not shown). TFP, an inhibitor of protein kinase C [13], completely abolished the phosphorylation of the 31.5 kDa band (lane 4). No significant phosphorylation of the proteins of the NADPH oxidase complex occurred in the absence of cytosol (lane 5). Lane 6 shows phosphoproteins of the cytosol. The proteins of 18, 21, 45, 55, 70 and 89 kDa correspond to those reported by others in studies on intact cells [14–16], cytoplasts [7] and cell lysates [13]. The identity of these proteins remains to be established, with the exception of that of the 21 kDa protein, which is the light chain of myosin [16].

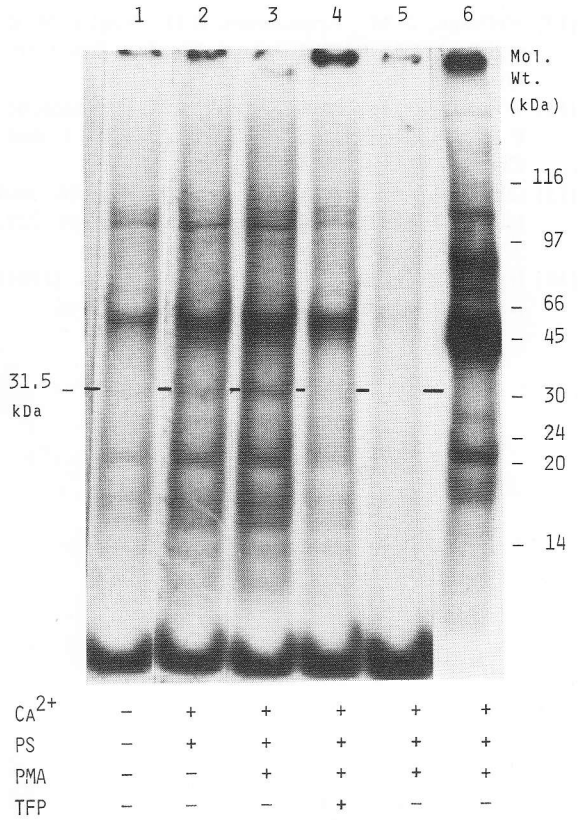

Fig.2. Autoradiograph showing the phosphorylation in vitro of partially purified NADPH oxidase. Partially purified NADPH oxidase obtained by gel filtration chromatography of membrane extracts from resting neutrophils was incubated with cytosol from resting neutrophils in the presence of [γ-^{32}P]ATP and of the compounds indicated beneath the figure. Incubation conditions, electrophoresis and autoradiography were done as described in section 2. Lanes: 1–4, NADPH oxidase plus cytosol; 5, NADPH oxidase without cytosol; 6, cytosol without NADPH oxidase. After incubation samples containing NADPH oxidase (lanes 1–5) were centrifuged as described in section 2 to remove excess cytosolic proteins and the pellets were subjected to electrophoresis. 40 μg protein in lanes 1–5 and 20 μg protein in lane 6 were subjected to electrophoresis.

The cytosolic phosphoproteins of 18, 21, 55 and 70 kDa are also visible in lanes 1–4, but are contaminant proteins which remain stuck to the NADPH oxidase complex during the sedimentation procedure performed after the phosphorylation reaction. The Ca^{2+} and phospholipid dependency and the sensitivity to PMA and to trifluoperazine of the phosphorylation of these proteins were not well established under our experimental conditions and remain to be investigated.

White et al. [14] have observed that in neutrophils most of the proteins phosphorylated following PMA stimulation are cytosolic, some are cytoskeletal and only that of 31 kDa belongs to the plasma membrane. Likely, this is similar to the protein which belongs to NADPH oxidase [9,11] and is phosphorylated in vitro by protein kinase C mechanisms, as shown by the experiments reported here.

We are now investigating the relationship between the phosphorylation and the activation of the enzymatic activity. The possibilities that phosphorylation may cause either the removal of an inhibitor, or the interaction between the enzyme and the substrate (NADPH), or the functional coupling between different components of the enzymatic complex are being considered.

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