STUDIES ON THE NATURE AND ACTIVATION OF O_2^--FORMING NADPH OXIDASE OF LEUKOCYTES. II. RELATIONSHIPS BETWEEN PHOSPHORYLATION OF A COMPONENT OF THE ENZYME AND OXIDASE ACTIVITY

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The activation of O_2^--formation by neutrophil NADPH oxidase is associated with phosphorylation of several membrane and cytosolic proteins. In the membranes a phosphoprotein of 32 kDa belonging to the NADPH oxidase-cytochrome b_{-245} system (P. Bellavite *et al., Free Rad. Res. Commun.,* 1, **11** (1985)) showed the highest relative increase of ^{32}Pi incorporation. Concomitant with the phosphorylation, a shift of the apparent molecular mass of the protein from 31 to 32 kDa occurred. The time-course, the sensitivity to trifluoperazine and the dose-dependence of phosphorylation were similar to those of O_2^- forming activity, except that the latter showed a longer lag-time than the former. The increase of the 32 kDa phosphoprotein was also comparable to the kinetics of cytochrome b_{-245} reduction by anaerobically activated neutrophils. The phosphorylation and the NADPH oxidase were triggered by various stimulants including phorbol myristate acetate, opsonized zymosan, arachidonic acid and sodium fluoride. With arachidonic acid the O_2^- formation was highly active but the phosphorylation was low. With fluoride the enzyme activity was reversible upon removal of the stimulant but the phosphorylation of the 32 kDa peptide was not reversible. Neutrophils treated with PMA at 17°C showed phosphorylation but not activation. The results indicate that phosphorylation of a component of NADPH oxidase is a fundamental but probably not sufficient event in the activation mechanism of the enzyme.

KEY WORDS: Superoxide formation, NADPH oxidase, phosphorylation, phagocyte metabolism, cytochrome b_{-245}.

ABBREVIATIONS: PMA, phorbol-12-myristate-13-acetate; PMSF, phenylmethansulphonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylendiaminotetraacetic acid; EGTA, ethylene glicol-bis(-aminoethyl ether),N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; TFP, trifluoperazine.

INTRODUCTION

The activation of superoxide (O_2^-) production by phagocytes plays a fundamental role in non-specific host defences against infections. The O_2^- generation is catalyzed by NADPH oxidase, a membrane bound enzyme which is dormant in resting cells and is turned on by the interaction of the phagocytes with phagocytosable and soluble stimuli.[1-4] This enzyme has not been fully characterized, but is thought to be composed of various constituents including flavoprotein[5,6] cytochrome b_{-245},[7,8] phos-

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pholipids,[9,10] quinones[11,12] and unknown cytosolic factors.[13,14] The mode of association of these components and the mechanism of electron transport are still unknown.

One of the most relevant and fascinating problems regarding the NADPH oxidase system is the mechanism of its activation upon cell stimulation. Several papers citing evidence indicating a role for protein phosphorylation in the triggering of O_2^- production by the oxidase have been recently published.[15-19] Moreover, we have described in neutrophil membranes a novel polypeptide with molecular mass of 32 kDa, which is phosphorylated in phorbol myristate acetate (PMA)-treated cells and in cell-free system by protein kinase C[20]. Partial purification of this protein from both guinea pig[10] and pig[21] neutrophils indicated that it is a component of NADPH oxidase and probably belongs to cytochrome b_{-245}. Enhanced cytochrome b_{-245} phosphorylation in PMA-activated neutrophils has been also reported by others,[22] although discrepancies on the molecular weight of the cytochrome are still present in the literature (for a review see reference 4). The identification of cytochrome b_{-245} as the target of the phosphorylation process is relevant in order to clarify the activation mechanism of the oxidase. However the relationship between the phosphorylation of a component and the enzymatic function remains to be established. Theoretically, the observed phosphorylation could be: 1) A first step which modifies one of the components in order to facilitate a subsequent interaction with other factors, or 2) the final conformational change of the enzyme directly associated with the catalytic function, or even 3) the effect of inactivation or de-activation, considering that the oxidase activity is short-lasting.[23]

We have approached these problems by investigating the correlation between the phosphorylation of the 32 kDa membrane protein and NADPH oxidase activity in different conditions of cell stimulation. Pig neutrophils were activated by variable PMA concentrations for different periods of time and by other phagocytosable and soluble stimulants. The protein phosphorylation pattern of the isolated membranes and the corresponding NADPH dependent O_2^- forming activity were then evaluated. We have also examined the reduction of cytochrome b_{-245} in cells activated in anaerobiosis, taken as a gross estimate of the number of cytochrome molecules that enter into an active (functioning) state.[24,25] The findings indicate that the various investigated phenomena are strictly correlated but also suggest that the phosphorylation of the 32 kDa protein is only one of the events which occur in the NADPH oxidase activation process.

MATERIALS AND METHODS

Materials

PMA, PMSF, cytochrome c (type VI), NADPH (type III), arachidonic acid, zymosan A were purchased from Sigma, St. Louis, Mo, USA. Zymosan was opsonized with pig serum as described.[26] SDS, acrylamide, N,N'-methylenebisacrylamide, tetramethylenediamine and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad, Richmond, USA; Ficoll 400 and molecular weight standards from Pharmacia, Uppsala, Sweden; carrier-free ^{32}Pi from Amersham, England. Superoxide dismutase was a gift from Dr. J.V. Bannister (Cranfield Biotechnology Centre, Bedford, England), TFP was a gift from Smith, Kline and French Laboratories, Herts, England. All other reagents were the highest grade available.

Methods

Isolation of Neutrophils and Labeling. Neutrophils were isolated from pig blood by dextran sedimentation and purification over Ficoll as previously described.[27] The cells were suspended in 30 mM Hepes buffer, pH 7.4, containing 110 mM NaCl, 5 mM KCl, 0.6 mM $CaCl_2$, 10 mM glucose, 1 U/ml of heparin ("incubation buffer") at a concentration of 2×10^8/ml and incubated with 0.15 mCi/ml of ^{32}Pi for 90 min at 37°C under continuous shaking. The cells were then diluted with six volumes of incubation buffer, centrifuged and resuspended in the incubation buffer without ^{32}Pi at the concentration of 4×10^7/ml.

Cell Activation and Fractionation. For each incubation condition, 10 ml of neutrophil suspension were supplemented with the indicated activating agent and were incubated with continuous shaking at 37°C unless otherwise stated. After the time indicated, the incubation was stopped by the addition of a ten-fold excess of ice-cold incubation buffer followed by centrifugation for 10 min at $400 \times g$. All the subsequent preparative steps were performed at 0–4°C. The cell pellet was suspended in 4 ml of 40 mM Tris/HCl buffer, pH 7.4, containing 10 mM EDTA, 1 mM $MgSO_4$, 2 mM NaN_3, 2 mM PMSF, 2 μM leupeptin and 2 μM pepstatin ("Tris buffer") containing 105 sucrose and sonicated with three to five 10 sec bursts at 100 watts with a Labsonic 1510 sonifier until 80–90% of the cells were disrupted. The homogenate was centrifuged for 10 min at $800 \times g$ in order to sediment nuclei and unbroken cells and the supernatant was overlayed over a discontinuous sucrose gradient made of 2.5 ml of Tris buffer containing 50% sucrose and of 2.5 ml of Tris buffer containing 20% sucrose. After centrifugation for 60 min at $100,000 \times g$ the fraction of 10% sucrose containing the cytosolic proteins and the band at the 20/50% interfacie containing the membranes were collected. One volume of the membrane fraction was mixed with two volumes of Tris buffer containing 0.45 M NaCl and centrifuged for 10 min at $10,000 \times g$. The precipitated proteins were discarded and the supernatant containing the NaCl-washed membranes was centrifuged for 45 min at $100,000 \times g$. The final membrane pellet was suspended in 0.2 ml of 20 mM sodium phosphate buffer, pH 8.0, containing 20% (v/v) glycerol, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM NaN_3 and 2 mM PMSF and assayed for protein content, NADPH oxidase activity and subjected to electrophoresis and autoradiography.

Electrophoresis and Autoradiography. SDS-polyacrylamide electrophoresis was performed according to Laemmli[30] using 20×18 cm slab gels. The final concentrations in the separation gel were as follows: 0.37 M Tris/HCl, pH 8.8, 12% polyacrylamide, 6 M urea, 0.1% SDS. The stacking gel contained 0.1 M Tris/HCl, pH 6.8, 4% polyacrylamide, 0.1% SDS. The gels were polymerized by the addition of 0.05% (v/v) tetramethylenediamine and 0.025% ammonium persulphate. 0.1 ml of each sample was supplemented with 10 μl of 0.25 M Tris/HCl, pH 6.8, containing 20% SDS and with 2 μl of 2-mercaptoethanol and incubated for 60 min at 50°C. Electrophoresis was run in a vertical apparatus, with 0.025 M Tris/HCl, pH 8.45, 0.192 M glycine and 0.1% SDS as electrode buffer, for 14–16 h at 100 volts (constant). Gels were fixed and stained with 45% methanol, 7% acetic acid and 0.05% Coomassie Blue R-250 and destained in 10% ethanol and 7% acetic acid. For the autoradiography the gels were equilibrated for 3 h in 45% methanol and 7% acetic acid, essiccated with a gel drier and exposed to X-ray films (Kodak X-Omat R) into a radiographic cassette for 4–8

days. Densitometer profiles of gels and of autoradiograms were performed with a Ultroscan Laser Densitometer (LKB, Bromma, Sweden). Quantitation of the auto-radiograms was accomplished by cutting and weighing the peak of interest from xerox copies of the scans in triplicate for each electrophoresis lane.[18]

Other Assays.   NADPH oxidase activity was measured as O_2^- generation by monitoring the superoxide-dismutase-inhibitable cytochrome c reduction.[27] Cytochrome b_{-245} reduction was measured with a Perkin Elmer 576 spectrophotometer by monitoring the absorbance difference at 426–405 nm using the extinction coefficient of $106 \, mM^{-1} cm^{-1}$.[28] Proteins were measured by the method of Lowry.[29]

RESULTS

Protein Phosphorylation in Membranes and Cytosolic Fraction of PMA activated Neutrophils

Figure 1 shows the protein phosphorylation pattern of membranes and cytosolic fraction from both resting and PMA-activated pig neutrophils. Many proteins showed increased labeling upon cell stimulation. In a series of seven separate experiments, the proteins whose phosphorylation was constantly enhanced by PMA treatment had the following molecular weight (in kDa ± S.D.): in the membranes 20.7 ± 1.0, 32.1 ± 0.7, 42.9 ± 1.1, 49.0 ± 2.3, 54.4 ± 0.8, 76.0 ± 3.6, 81.8 ± 1.8, 94.5 ± 7.8 and an high-molecular weight protein of about 200 kDa; in the cytosol 18.8 ± 0.8, 23.0 ± 1.0, 25.8 ± 0.3, 49.3 ± 2.5, 54.0 ± 2.0, 70.0 ± 0.2, 93.4 ± 0.9. PMA treatment caused the disappearance of a cytosolic phosphoprotein of 20 kDa, a finding in agreement with previous reports.[18] Among the membrane proteins, the band of 32 kDa exhibited the largest proportional increase of phosphorylation in activated cells. Since previous investigations indicated that it is associated with NADPH oxidase and cytochrome b_{-245},[4,10,21] subsequent studies were focused on this band. The dramatic increase of the 32 kDa phosphoprotein is also illustrated by the densitometric scanning of the autoradiographic film of a representative experiment where neutrophils were activated by increasing doses of PMA (Figure 2). It should be pointed out that the method of isolation of the membranes included a washing with 0.3 M NaCl in order to remove the loosely-bound proteins and to improve the purification of NADPH oxidase, cytochrome b_{-245} and 32 kDa-peptide.[21] This implies that cytochrome b_{-245} (or one of its putative subunits) is the main integral membrane protein phosphorylated in PMA-activated neutrophils.

Comparison of the phosphoprotein patterns with the Coomassie Blue-stained protein patterns revealed that the 32 kDa-peptide, which is phosphorylated in membranes from PMA treated cells, runs with a lower apparent molecular weight in membranes from resting cells. The densitometer scanning of the region of the gel where this shift of apparent molecular weight was observed is reported in Figure 3. Membranes from resting cells (trace 1) showed one definite protein peak of 31 kDa, while those from PMA-treated cells showed in that region two peaks, one of 31 kDa and a second of 32 kDa (trace b). Only this latter peak was phosphorylated in activated membranes (traces c and d). The other protein and phosphoprotein peaks had the same apparent molecular weight in resting and activated membranes. Cal-
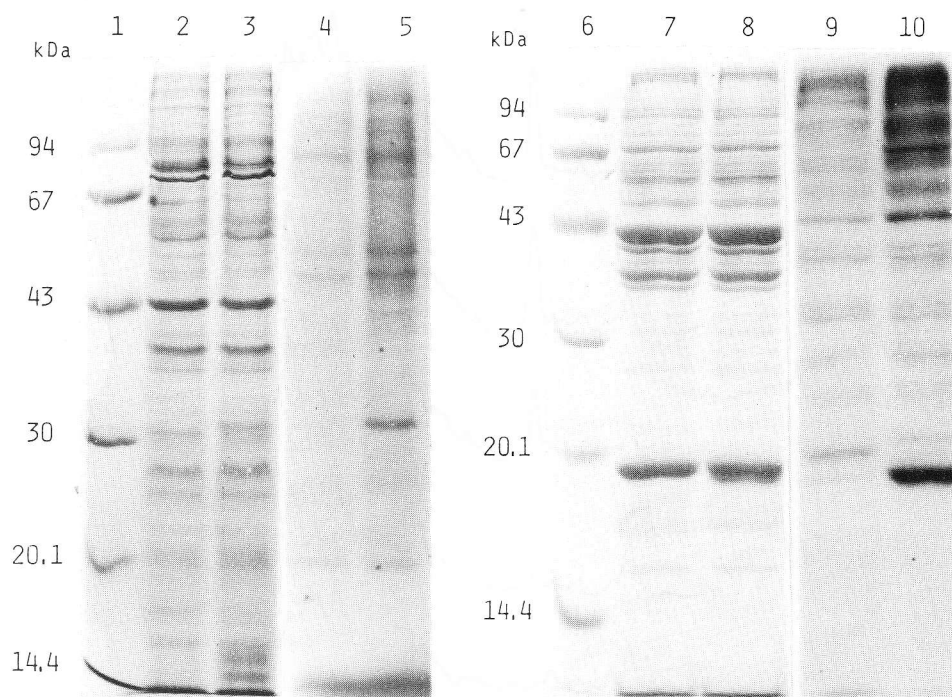
FIGURE 1 *Protein phosphorylation in resting and PMA-activated neutrophils.* Pig neutrophils, prelabeled with ^{32}Pi, were divided in two aliquots, one of which was activated by exposure to 100 ng/ml of PMA for 10 minutes. Control cells were treated with an equal volume of Me_2SO. The membrane and cytosolic fractions were then isolated and subjected to SDS-polyacrylamide electrophoresis and autoradiography as described under "Experimental procedures". Lane 1 and 6, protein standards. Lane 2 and 4, membranes from resting cells; lane 3 and 5, membranes from PMA-activated cells; lane 7 and 9, cytosol from resting cells, lane 8 and 10, cytosol from activated cells. Lane 1–3 and 6–8, proteins stained with Coomassie Brilliant blue; lane 4–5 and 9–10, autoradiogram.

culation of the peak areas with a 3390-A Hewlett Packard integrator indicated that the 31 kDa peak of trace a represents 11.2% of the proteins analysed by scanning, while the 31 kDa and 32 kDa peaks of trace b represent 4.8% and 5.95% of proteins respectively. The fact that the sum of the two protein peaks of activated membranes (10.75% of proteins) corresponds to the peak of resting membranes (11.2%) suggests that the same protein is present in activated membranes in two forms, one of which is phosphorylated and changes its apparent molecular weight. The experiment reported in Figure 3 was done under conditions of maximal stimulation of the cells with PMA. Lower doses of PMA induced lower phosphorylation and proportional minor increase of the protein peak of 32 kDa (not shown). A maximum of 55–60% of the protein exhibited the described shift of the apparent molecular weight in a series of five experiments with maximal doses of PMA.

Time-course and Dose-dependence of Phosphorylation and of NADPH Oxidase Activation

The extent of the phosphorylation of the 32 kDa-peptide was compared with the NADPH-dependent O_2^- forming activity of the same membrane preparation in
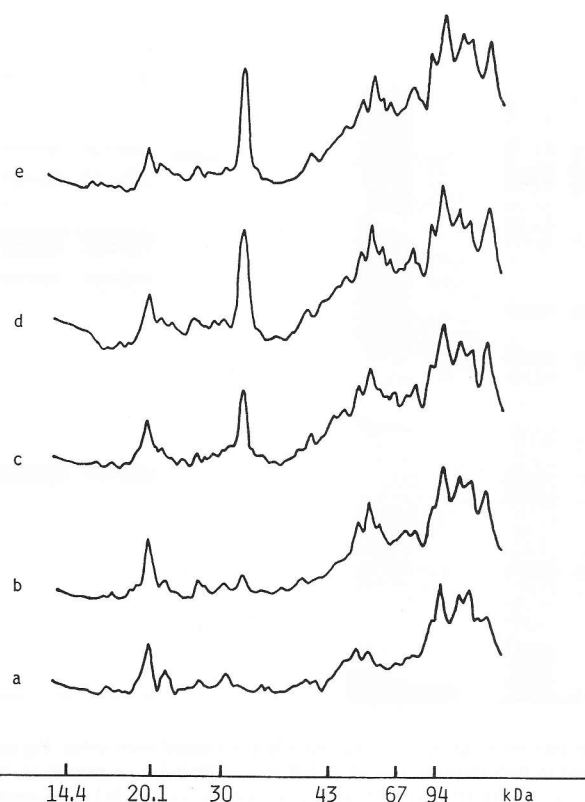
FIGURE 2 *Densitometric profiles of autoradiograms of membranes from neutrophils activated by increasing doses of PMA.* Membranes were isolated from unstimulated cells (trace a) and from cells treated for 10 min with 4 ng/ml PMA (trace b), 20 ng/ml PMA (trace c), 50 ng/ml PMA (trace d), 500 ng/ml PMA (trace e).

variable conditions of cell activation. Figure 4 reports the time-course (panel A) and the dose-dependence (panel B) curves of the two events. The phosphorylation of membranes of resting cells was almost absent and also the O_2^- formation was, as expected, undetectable. The increase of the 32 kDa phosphoprotein started immediately after addition of PMA and progressively increased until 10–15 minutes (panel A). The NADPH oxidase activity was elicited after a lag of 1–3 minutes, then increased and reached a maximum at 10–15 minutes. This suggests that the phosphorylation precedes the activation of the enzyme, a prerequisite for establishing a causal relationship between the two phenomena. In separate tubes, the protein kinase inhibitor, trifluoperazine[31] was added to the cell suspension after ten minutes of activation and the incubation was carried out for a further five minutes. The inhibitor caused a parallel decrease of phosphorylation and of O_2^- forming activity of the membranes.

The dose-response curves (Figure 4B) demonstrated a substantial correlation between the extent of the oxidase activation and the increase of the 32 kDa phosphoprotein. The doses of PMA necessary to induce the phosphorylation were in the same range of those necessary to induce the expression of NADPH oxidase activity.
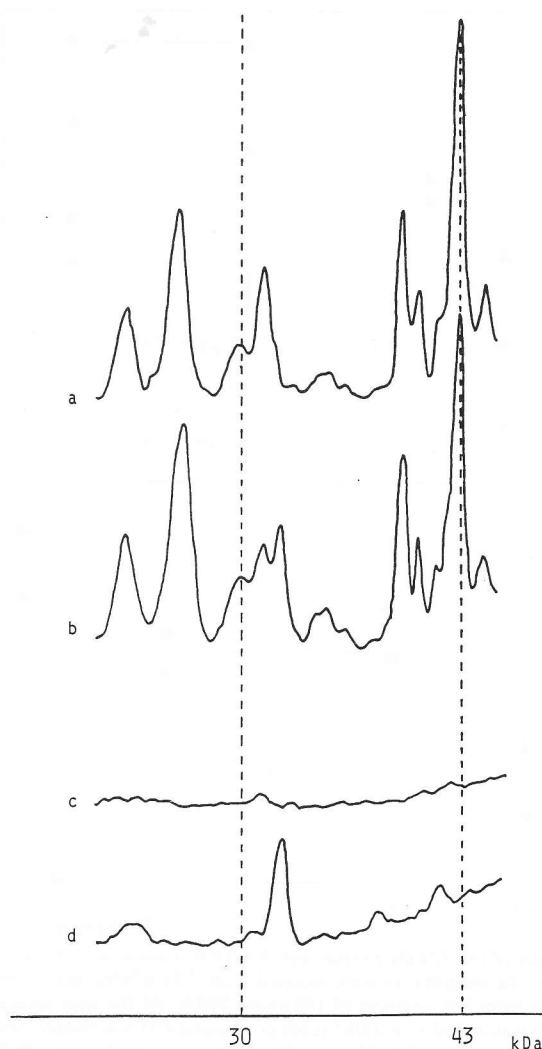
FIGURE 3 *Effect of phosphorylation on the apparent molecular mass of the 32 kDa-peptide.* The traces are densitometric profiles of polyacrylamide electrophoresis gels stained for proteins (a and b) and of the corresponding autoradiograms (c and d). Trace a and c, membranes from resting neutrophils; trace b and d, membranes from neutrophils activated by 100ng/ml PMA.

Cytochrome b_{-245} Reduction in Anaerobiosis

One of the main evidence in favor of the involvement of cytochrome b_{-245} in the electron transport from NADPH to oxygen is the appearance of the reduced spectrum of the heme in neutrophils[24,25] and macrophages[32] activated under anaerobic conditions. This allows quantitation of the number of cytochrome molecules that are "activated". We have therefore considered the possibility that the ability of the cytochrome b_{-245} of being reduced in intact cells is in some way related to its
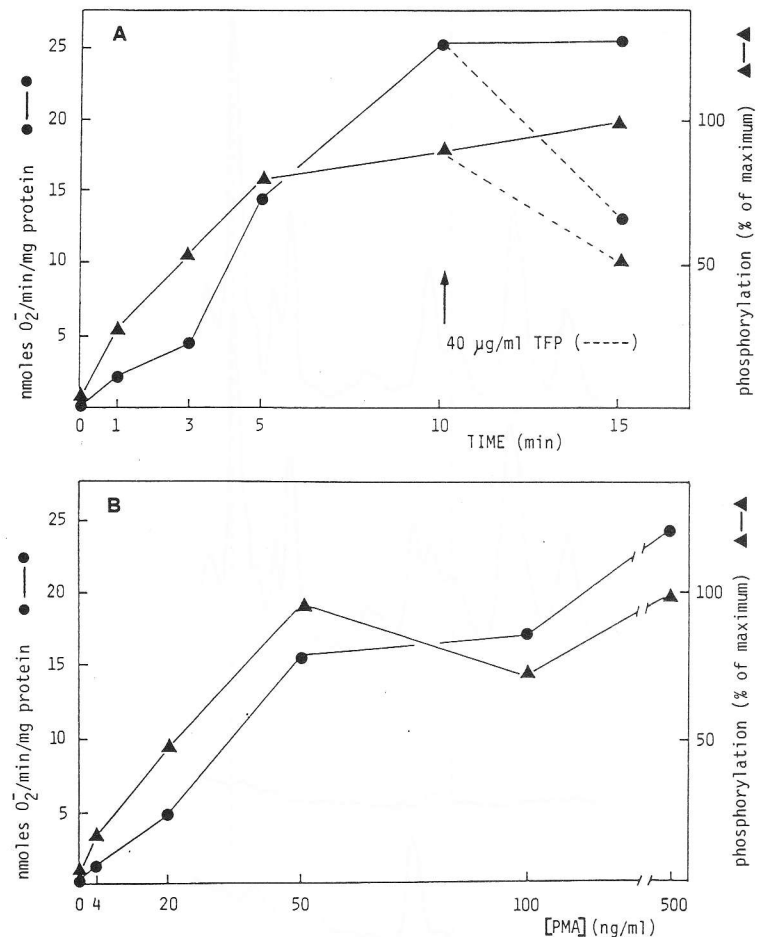
FIGURE 4 *Phosphorylation of the 32 kDa-peptide and NADPH oxidase activity in PMA-activated neu-trophil membranes.* Panel A: the membranes were isolated from ^{32}Pi-labeled neutrophils just before and after different time intervals from the addition of 100 ng/ml PMA. At the time indicated by the arrow, 40 μM trifluoperazine (TFP) was added to parallel tubes containing PMA-activated neutrophils that were then incubated for further five min. Panel B: The membranes were isolated from ^{32}Pi-labeled neutrophils incubated for 10 min with the indicated doses of PMA. The NADPH oxidase activity ($\bullet$) and the intensity of phosphorylation of the 32 kDa band ($\blacktriangle$) were quantitated as described under "Experimental procedures".

phosphorylation. In order to verify this hypothesis the kinetics of cytochrome b_{-245} reduction in neutrophils activated by variable doses of PMA were investigated. As can be seen from Figure 5, cytochrome b_{-245} was not reduced in control cells, i.e. due to anaerobiosis by itself. Increasing doses of PMA caused the progressive appearance of the reduced spectrum of the cytochrome which reached a maximum at 100 ng/ml of the stimulant and after 6–8 minutes. In these conditions the cytochrome b_{-245} reduced by endogenous mechanisms was 60–65% of the total cell cytochrome b reducible by sodium dithionite. By comparing the data of Figure 5 with those of Figures 3 and 4 it can be concluded that the kinetics of the response to different doses of PMA and
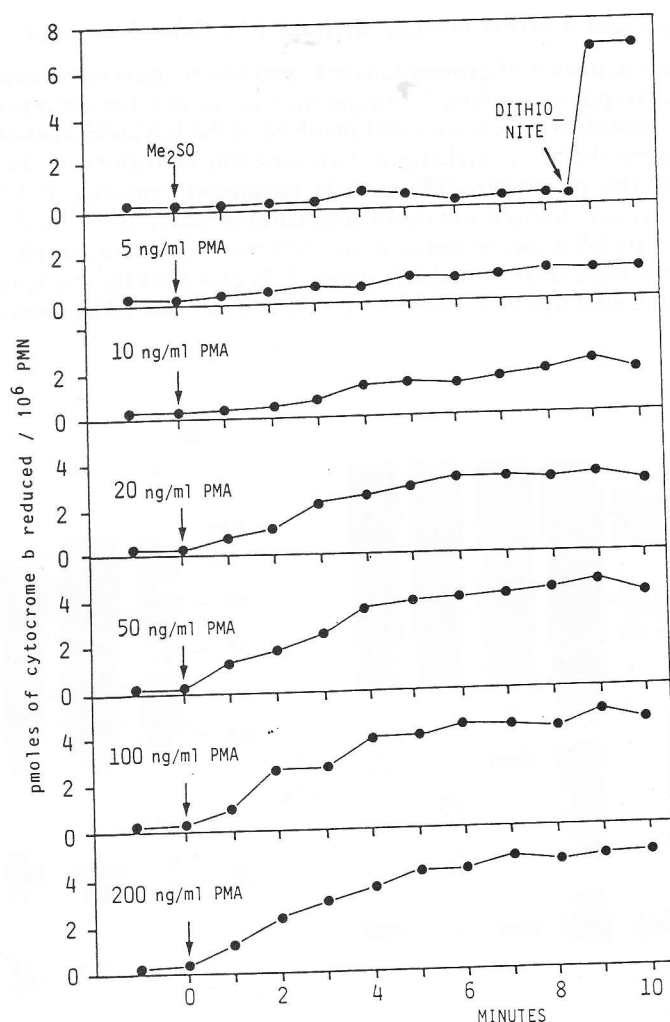
FIGURE 5 *Kinetics of endogenous reduction of cytochrome b_{-245} by PMA-activated neutrophils.* Two cuvettes were filled with 2 ml of neutrophil suspension (4×10^7 cells/ml) in incubation buffer supplemented with 200 U/ml catalase and placed in the compartment for turbid samples of a double beam spectrophotometer thermostated at 37°C and equipped with magnetic stirring. In this experiment the cells were not pre-labeled with ^{32}Pi. To the sample cuvette 0.4 mg of glucose oxidase were added and the cuvette was immediately closed with a plug bearing two needles, one for a continuous inlet of oxygen-free nitrogen and the other for the outlet of the gas and the addition of the stimulant. The reference cuvette was not closed. After 10 min of equilibration, the indicated doses of PMA were added to the sample cuvette and spectra of reduced-oxidized cytochrome b were recorded at time intervals. Full reduction was achieved by addition of 20 μl of a fresh saturated solution of sodium ditionite.

the maximum percentage of molecules involved in the anaerobic reduction of cytochrome b_{-245} are very similar to the behavior of the phosphorylation of the 32 kDa protein.

Phosphorylation and NADPH Oxidase Activation by Other Stimulatory agents

PMA is a direct activator of protein kinase C and can by-pass some important steps of the stimulus-response coupling.[33] The question arises whether the phosphorylation of the 32 kDa-peptide is a constant event involved in the NADPH oxidase activation. Neutrophils were therefore challenged with different activators of the respiratory burst, such as the phagocytosable particle opsonized zymosan and two soluble compounds that act through unknown mechanisms, arachidonic acid and sodium fluoride. In Figure 6A it can be seen that the membranes from neutrophils treated for 10 minutes with these three stimulants (lanes 3–5) exhibited the phosphorylation of the 32 kDa band, although with less intensity than those from PMA-treated cells (lane 2).
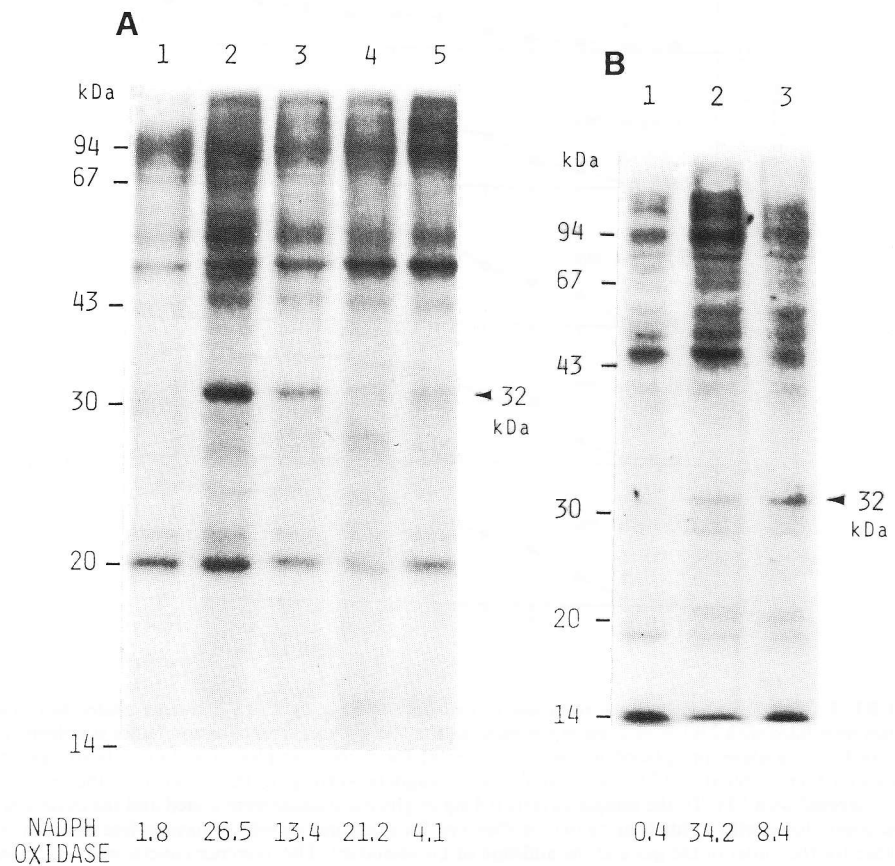

FIGURE 6 *Phosphoprotein patterns and NADPH oxidase activity of membranes from neutrophils activated by various agents.* ^{32}Pi-labeled neutrophils were incubated for 10 min without stimulant (A-1), with 100 ng/ml of PMA (A-2), with 4 mg/ml of opsonized zymosan (A-3), with 100 μM arachidonic acid (A-4), with 20 mM sodium fluoride (A-5) and for 20 min without stimulant (B-1), with 20 mM sodium fluoride (B-2) and with 20 mM sodium fluoride followed by washing and incubation for further 10 min without fluoride (B-3). The incubation was stopped by dilution with an excess of ice-cold buffer and the cells were centrifuged, homogenized and fractionated. The membranes were then subjected to SDS-polyacrylamide electrophoresis and autoradiography and the NADPH oxidase activity was assayed as described under "Experimental procedures".

The NADPH oxidase activity of the membrane preparations whose phosphorylation is shown in Figure 6 is reported at the bottom of each lane. Opsonized zymosan elicited an oxidase activity of about half that elicited by PMA and also the corresponding phosphorylation of the 32 kDa band was roughly a half. Sodium fluoride at 10 minutes caused low activation and corresponding low phosphorylation. With this agent a full expression of the phosphorylation required at least 20 minutes (lane 2 of panel B) and this is in accord with the slow kinetics of neutrophil activation by fluoride.[34] A discrepancy between the two considered parameters was found with arachidonic acid as stimulant (lane 4 of panel A). This compound was able to induce high NADPH oxidase activity, whereas the phosphorylation was very low (but not absent).

Since it was reported that the activation of the oxidase by fluoride is reversible on removal of the stimulant,[34] the possibility that the phosphorylation of the 32 kDa-peptide was also reversible was considered. The experiment reported in Figure 6B showed that this is not the case. Cells were incubated with sodium fluoride for 20 minutes (lane 2) and an aliquot washed and incubated for further ten minutes in the absence of fluoride (lane 3). This procedure did not cause a decrease of the 32 kDa phosphoprotein in the washed cells, while it caused a marked reversal of NADPH oxidase activity. This experiment was repeated twice with the same result.
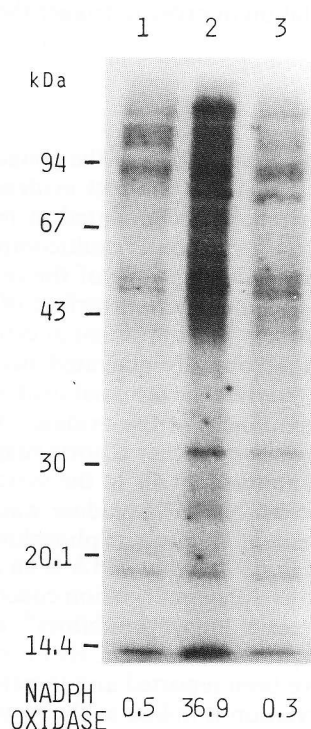

FIGURE 7 *Effect of temperature on phosphorylation of membrane proteins and on NADPH oxidase activity*. The membranes isolated from ^{32}Pi-labeled neutrophils incubated for 10 min at 37°C in the absence of stimulant (lane 1), at 37°C with 100 ng/ml of PMA (lane 2) and at 17°C with 100 ng/ml of PMA (lane 3) were subjected to SDS-polyacrylamide electrophoresis and autoradiography and assayed for NADPH oxidase activity as described under "Experimental procedures".

Effect of Temperature on the Phosphorylation and the Activation of the Oxidase

Some findings reported in the preceding section indicated that the phosphorylation of the 32 kDa-peptide is not always directly proportional to the actual oxidase activity contained in the membranes. These apparent discrepancies suggest that besides the phosphorylation other factors control the activation or the activity of the enzyme. This concept is also in agreement with the observation that phosphorylation seems to precede the expression of the oxidase in the membranes (Figure 3).

It has been reported that the activation of the neutrophil respiratory burst by PMA occurs only at temperatures above 17°C, possibly because below this temperature the membrane fusion is blocked.[35] On the other hand the binding of PMA to its receptor protein kinase C occurs also at low temperatures.[36] It was therefore interesting to verify whether the lack of activation at low temperature was due to lack of phosphorylation. Figure 7 shows that membranes from neutrophils treated with PMA for 10 minutes at 17°C were completely devoid of O_2 forming activity, but the 32 kDa band was clearly phosphorylated. The intensity of the phosphorylation at 17°C was lower than that at 37°C but was comparable with the intensity of the phosphorylation induced at 37°C by other stimulants such as zymosan or fluoride which activated the enzyme (Figure 6). This experiment described a further condition where phosphorylation was dissociated from activation and showed that a temperature-sensitive step is required besides the phosphorylation in order to trigger the NADPH oxidase system.

DISCUSSION

The precise nature and the mode of activation of the phagocyte NADPH oxidase are still unknown, but a series of direct and indirect evidence of the participation of particular factors or systems has been accumulated in recent years. The NADPH oxidase is considered to be a membrane-bound multicomponent system which, in its active form transports electrons from the inside of the cell (NADPH) to molecular oxygen which is released as O_2^- at the external surface of the membrane.[4] A major constituent of the enzyme complex is the low potential cytochrome b_{-245}. The reduction of cytochrome b_{-245} in anaerobically activated neutrophils[24,25] and its rapid reoxidation by oxygen[6] suggest that it is the terminal oxidase of the electron transport chain. Another fundamental component of the oxidase could be a flavoprotein that would act as NADPH dehydrogenase.[37] Other reports suggested that quinones[11,12] or non heme iron[38] could play an important role in the system.

The activation mechanism of the NADPH oxidase was a mystery until few years ago, when the possible involvement of protein phosphorylation was indicated by several lines of evidence, as the discovery that PMA is an activator of protein kinase C[33], the finding of enhanced protein phosphorylation concomitantly with phagocyte's activation,[16,17] the effect of protein kinase inhibitors[39] and the activation of O_2^- formation in cell-free system by protein kinase C[19]. However, a number of phosphorylated proteins in leukocytes have been reported and therefore it is difficult to identify the protein(s) whose phosphorylation is linked to the respiratory burst function. On the basis of kinetic studies and of the effect of inhibitors some authors suggested that a 46 kDa cytosolic protein,[40] or two proteins of 64 and 21 kDa[16] could be involved in the induction of superoxide production. Another candidate is a 44–48 kDa phosphoprotein which is lacking in cells from chronic granulomatous disease patients, where the activation of the oxidase is absent.[41,42] The identity of these proteins remains to

be established, apart from the 21 kDa protein which is probably the myosin light chain.[16]

In our laboratory a protein of 31.5–32 kDa belonging to NADPH oxidase was found to be phosphorylated in the enzyme complex isolated from PMA-treated neutrophils.[4,20,21] This polypeptide was associated with cytochrome b_{-245} after extraction from the membranes and during various purification procedures such as gel filtration, glycerol density gradient centrifugation and dissociation of the enzymatic complex with high detergent and salt concentrations.[10,21] On this basis we have suggested that the 32 kDa-peptide was the cytochrome b_{-245} or one of its putative subunits.[4,21] In vitro experiments showed that the phosphorylation of this protein takes place in a Ca^{2+} and phospholipid dependent fashion.[20] A preliminary report from another laboratory indicated that also in human neutrophils the cytochrome b_{-245} is phosphorylated.[22]

The data reported in this paper confirm and extend the previous observations regarding the role of protein phosphorylation in activated neutrophils. A series of at least nine integral membrane proteins and seven cytosolic proteins exhibited an increased ^{32}Pi incorporation in PMA-activated cells. This series included also most of the phosphoproteins whose existence had been already pointed out by others.[15–18, 40–42] In the membranes the most striking relative increase was shown by the 32 kDa band which was totally dephosphorylated in resting cells and became one of the major labeled bands after PMA treatment. The kinetics and the dose-dependence of phosphorylation and of NADPH oxidase activation were very similar, with the exception that phosphorylation slightly preceded the activation. This indicates that the phosphorylation is not related to some de-activation or inactivation mechanism.

The finding of the shift of apparent molecular mass from 31 to 32 kDa occurring in the phosphorylated form of the protein (Figure 4) is important for three reasons. Firstly, it suggests that the incorporation of phosphate markedly changes either the shape of the protein or its capacity to bind SDS. It is conceivable that such a modification of the physicochemical properties is related to the change of activity or of reactivity into the membrane. It should be pointed out that this is the only membrane protein that exhibits a similar change in the electrophoretic migration upon phosphorylation. Alteration of the electrophoretic behavior of proteins phosphorylated by protein kinase C has been observed in other biological systems.[43] Secondly, by increasing the phosphorylation there is not an increase in the distance of the shift of apparent molecular weight but an increase in the number of molecules that occupy the 32 kDa position (Figures 2 and 4). The lack of intermediate peaks suggests that the phosphorylation is an all-or-nothing event for the protein and selectively affects a single site of the molecule. Finally, the 31–32 kDa shift allowed a quantitative estimation of the proteins that were subjected to phosphorylation. A maximum of 55–60% of the protein present in the membrane was phosphorylated under conditions of maximal activation. The reason for the lack of complete phosphorylation is unknown but it is interesting to note that neither the reduction of cytochrome b_{-245} in anaerobiosis was complete in the same conditions of stimulation. The fact that only a portion of the cytochrome b_{-245} is reduced in PMA-stimulated neutrophils has been previously reported.[44] It has been suggested that there are two distinct pools of cytochrome b_{-245} in neutrophils, only one of which is reducible in anaerobically activated cells.[45]

The phosphorylation of the cytochrome b_{-245}-associated protein of 32 kDa was observed with three other stimulants that presumably act through transduction

pathways that are at least partially different from PMA. Although quantitative differences between the various stimulants were noted (Figure 6), there was no condition of oxidase activation occurring without appearance of the 32 kDa phosphoprotein. The phosphorylation induced by serum treated zymosan, a phagocytosable stimulant that interacts with the membrane through complement and glucose and mannose residues is particularly noteworthy. Since phagocytosis is the most physiological stimulus of the neutrophils, this finding demonstrates that the phosphorylation of the 32 kDa band is not an artifactual effect of some stimulant but is a common step of the stimulus-response coupling system. The lower intensity of phosphorylation by zymosan with respect to PMA can be explained considering that the phagocytosable particle interacts with only a portion of the cell surface and that only the oxidase that is incorporated in the phagocytic vacuole is activated.[46]

The reason for the high enzymatic activity associated with low phosphorylation in membranes from arachidonate-treated neutrophils is unclear. It is possible that arachidonic acid directly interacts with some component of the NADPH oxidase, because this agent is able of triggering the O_2^- production in a cell-free system.[13,14] It has been suggested that dissolution of fatty acids in the plasmalemma lipid phase leads to a modification of the properties of the lipid bilayer such as crucial membrane proteins might interact more easily.[47]

The phosphorylation of the 32 kDa band by fluoride provides the basis for understanding the mechanism of activation of the respiratory burst by this halide. It is possible that fluoride affects the phosphorylation state of the cytochrome b_{-245} either by inhibiting phosphatase activities or by activating a protein kinase. This latter mechanism seems to be the most conceivable because fluoride elicits calcium mobilization possibly through activation of a guanine nucleotide binding protein.[48]

Some data in the present investigation point out that there is not a simple and direct relationship between ^{32}Pi incorporation and enzymatic activity. In fact the association of a substantial phosphorylation of the 32 kDa protein with low or absent O_2^- formation was found in three experimental conditions, i.e. in the first 1–3 minutes after addition of PMA (Figure 3), in cells activated with fluoride and then de-activated by removal of the stimulant (Figure 6B) and after incubation of neutrophils with PMA at 17°C (Figure 7). These observations suggest that the presence of a phosphorylated form of the 32 kDa-peptide is not sufficient to perform the electron transport from NADPH to oxygen. The nature of the other change(s) required for optimal enzymatic function remains to be clarified. The effect of temperature is consistent with the data indicating that the lateral mobility of the proteins in the membrane,[49] or the protein-lipid interaction,[9] or the fusion between the membrane and the granules[35] could play an important role in the activation or in the activity of the oxidase. It is also possible that the phosphorylation triggers the activation but this latter event is labile in the absence of the concomitant occurrence of other membrane modifications.

Most of the data of the present investigation are consistent with the conclusion that the phosphorylation of cytochrome b_{-245} could play a key role in the activation process of the NADPH oxidase system. The mechanism by which the phosphorylation changes the activity of the enzyme is only a matter of speculation because little is known of the interaction between the various components of the electron transport system. Assuming that the 32 kDa-peptide belongs to the cytochrome b_{-245}, its phosphorylation would allow the interaction with the neighbouring oxido-reducing components, thus assembling a functional electron-transport chain (Figure 8). The
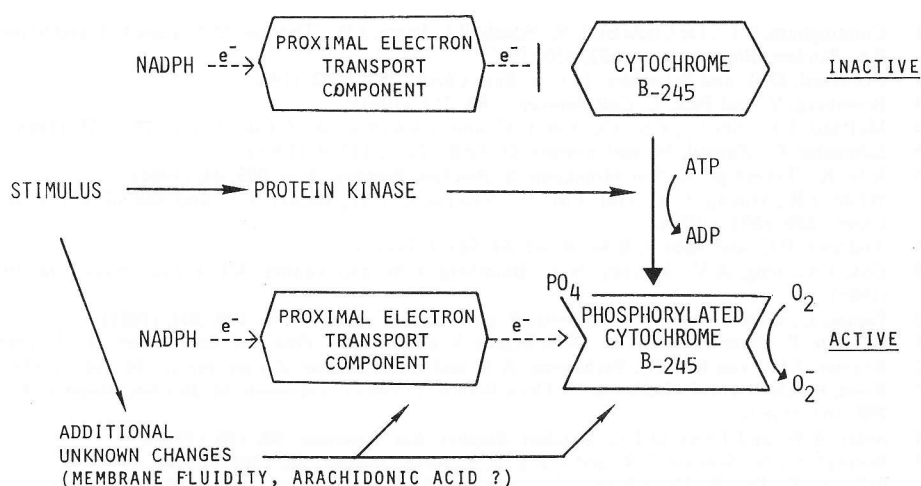
FIGURE 8   *Hypothetical model of the activation mechanism of NADPH oxidase.*

similarity of the kinetics of reduction of cytochrome b_{-245} by intact cells with those of its phosphorylation is compatible with this model. The presented hypothesis is also consistent with the finding that in resting membranes the electrons may flow from NADPH through flavoprotein and quinones but cannot reach the cytochrome b_{-245}, while in activated membranes the electrons reach the cytochrome and then O_2^- is generated.[50] Other unknown modifications of the complex would enhance the velocity of the reaction or stabilize the enzymatic activity. Possibility some activating agents (arachidonic acid) may by-pass the phosphorylation process when exerting their effects.

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