Relationship between phosphorylation and translocation to the plasma membrane of p47phox and p67phox and activation of the NADPH oxidase in normal and Ca^{2+}-depleted human neutrophils

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Stimulation of neutrophils with different agonists activates a latent multicomponent NADPH oxidase that reduces molecular oxygen to superoxide anion. Evidence has accumulated that phosphorylation of p47phox (the 47 kDa cytosolic phagocyte oxidase factor) and translocation of the two cytosolic components p47phox and p67phox are essential steps in the activation of NADPH oxidase in response to phorbol esters. We analysed the relationships between activation of the NADPH oxidase and phosphorylation and translocation of p47phox and p67phox in normal and Ca^{2+}-depleted neutrophils stimulated by the receptor-mediated agonists formyl-methionyl-leucyl-phenylalanine and concanavalin A. The results produced the following conclusions: (1) Translocation of p47phox and p67phox is an essential mechanism for activation of the NADPH oxidase. (2) A continuous translocation of p47phox and p67phox is necessary to maintain the NADPH oxidase in an activated state. (3) Only a fraction of p47phox and p67phox translocated to the plasma membrane is functional for the activation of the oxidase. (4) Translocation is independent of protein kinase C, and is linked to transmembrane signalling involving Ca^{2+} transients and production of lipidic second messengers. However, under some conditions, such as in Ca^{2+}-depleted neutrophils, translocation can also occur independently of signalling pathways involving production of second messengers from hydrolysis of phospholipids and Ca^{2+} transients. (5) Phosphorylation of p47phox and p67phox can be quantitatively dissociated from translocation, as staurosporine markedly inhibits phosphorylation but not translocation. (6) The activity of NADPH oxidase is not correlated with the amounts of the phosphorylated proteins present in the plasma membrane.

INTRODUCTION

The nature and the mechanisms of activation of the electron transport system, termed NADPH oxidase, which is responsible for the production of bactericidal toxic oxygen molecules in phagocytes are not completely understood [1–3]. It is known that the activation of the oxidase requires interaction at the plasma membrane level between membrane and cytosolic components; these include the low-potential cytochrome b_{558} [4,5], a flavoprotein [6], the cytosolic proteins p47phox and p67phox (phagocyte oxidase factors of 47 and 67 kDa respectively) [7–10] and the small GTP-binding proteins rap1A, rac-1 and rac-2, [11–14].

Studies performed with neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA) have shown that: (1) translocation of the cytosolic p47phox and p67phox to the plasma membrane is an essential process for the activation of NADPH oxidase, and translocation of p47phox is preceded by phosphorylation ([15–27]; for reviews see [3,28]); and (2) continuous phosphorylation of p47phox is necessary to maintain NADPH oxidase in an activated state [29, 30]. It is widely accepted that these PMA-stimulated events are due to activation of protein kinase C and phosphorylation of appropriate substrates [24,30–33].

The issue of the mechanism(s) responsible for the activation of NADPH oxidase by receptor-mediated agonists is much more controversial. In this case, more than a single signalling pathway can be activated, involving (or not) second messengers derived from hydrolysis of phospholipids, generation of cytosolic Ca^{2+} transients, protein phosphorylation or G-proteins [1–3,34].

In this paper we have used formyl-methionyl-leucyl-phenylalanine (FMLP) and Concanavalin A (Con A) to investigate: (1) the relationship between the activation of the NADPH oxidase and the translocation and phosphorylation of p47phox and p67phox, and (2) the signalling pathways involved in these processes. For this purpose we have used both normal and Ca^{2+}-depleted neutrophils. The rationale behind using Ca^{2+}-depleted neutrophils is that they can respond to receptor-mediated agonists with an activation of NADPH oxidase in spite of the lack of activation of signalling pathways involving Ca^{2+} transients and production of second messengers from hydrolysis of phospholipids by phospholipase C, D and A2 [35–38].

MATERIALS AND METHODS

Materials

Quin-2/AM and staurosporine were purchased from Calbiochem–Boehringer (La Jolla, CA, U.S.A.); PMA, FMLP, Con A and cytochalasin B were from Sigma Chemical Co., St. Louis, MO, U.S.A. SDS, acrylamide, *NN'*-methylenebisacrylamide, tetramethylenediamine, Coomassie Brilliant Blue R-250 and blotting nitrocellulose membranes were purchased from Bio-Rad, Richmond, CA, U.S.A. Ficoll 400 and molecular mass standards were from Pharmacia, Uppsala, Sweden.

Neutrophil preparation

Human neutrophils were prepared from the venous blood of healthy donors as previously described [39]. Cells suspended in

Abbreviations used: p47phox and p67phox, cytosolic phagocyte oxidase factors of 47 and 67 kDa respectively; PMA, phorbol 12-myristate 13-acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; Con A, concanavalin A; ECL, enhanced chemiluminescence.

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PBS were treated at 4 °C for 10 min with 2 mM di-isopropyl-fluorophosphate (Sigma Chemical Co.), washed and suspended to a final concentration of 60×10^6/ml in Hanks' balanced salt solution containing 20 mM Hepes, 0.5 mM $CaCl_2$ and 5.6 mM glucose (pH 7.4).

Cellular Ca^{2+} depletion

When required, a portion of the cells was suspended to a final concentration of 60×10^6/ml in Hanks' balanced salt solution without calcium. These cells were depleted of calcium as previously described [40]. This treatment results in a decrease of the free cytosolic Ca^{2+} concentration to less than 10 nM, monitored according to [41].

Metabolic studies

Oxygen consumption was measured at 37 °C with a Clark oxygen electrode using 30×10^6 neutrophils/ml in Hanks' balanced salt solution containing 2 mM NaN_3, 5 μg/ml cytochalasin B and 0.5 mM $CaCl_2$ or, in the case of Ca^{2+}-depleted neutrophils, 1 mM EGTA. In the experiments in which PMA was used as stimulus, cytochalasin B was omitted. When required, 200 nM staurosporine was added to the cells 5 min before the stimulants.

Neutrophil activation and fractionation

Neutrophils (30×10^6) were treated with different stimuli in the conditions described above. After different time periods, cells were diluted with a 10-fold excess of ice-cold Hanks' balanced salt solution containing 0.5 mM $CaCl_2$ (normal) or 1 mM EGTA (Ca^{2+}-depleted), centrifuged (4 °C) at 500 *g* for 7 min and finally suspended in 0.6 ml of 'relaxation buffer' (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 3 mM phenylmethanesulphonyl fluoride, 10 mM Pipes, pH 7.3, containing 20 μg/ml leupeptin and 20 μg/ml pepstatin). All the subsequent steps were performed according to Clark and co-workers [20]. The final pellet was resuspended in 70 μl of electrophoresis sample buffer [60 mM Tris/HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, pH 6.8]. The protein content was measured by the method of Bradford [42].

Electrophoresis and Immunoblotting

Aliquots of membranes from resting and activated neutrophils that contained the same amount of protein (40–50 μg) were subjected to SDS/PAGE on 9% gels, according to Laemmli [43]. Proteins were transferred from the gels to nitrocellulose membranes (Bio-Rad) using a Bio-Rad Trans Blot Cell apparatus; blotting was performed at 500 mA for 180 min in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3. To ensure that comparable amounts of proteins had been transferred to the nitrocellulose membranes, proteins were revealed on the nitrocellulose membranes by staining with 0.02% (v/v) Ponceau S (Sigma) for 1 min. The blots were rinsed in water and incubated for 120 min in blocking buffer [3% (w/v) BSA, 0.025% (v/v) Tween 20 in PBS, pH 7.4], before incubation with purified rabbit anti-p47phox antibodies, diluted 1:200 in blocking buffer (anti-p47phox antibodies were kindly provided by Dr. A. W. Segal, Department of Medicine, University College London) [44]. The blots were rinsed with several changes of PBS containing 0.1% (v/v) Tween 20 and then incubated for 60 min in horseradish peroxidase-labelled donkey anti-rabbit IgG (Amersham UK) at a dilution of 1:15000 in blocking buffer. After further washing, bound antibodies were revealed by enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham). The blots were then rinsed with PBS and stripped for 60 min at 20 °C in a solution containing 7 M guanidinium hydrochloride, 50 mM Tris/HCl, 2 mM EDTA and 50 mM dithiothreitol, pH 7.5. This treatment removed both primary and secondary bound antibodies, and enabled us to reuse the blot with a different primary antibody. After washing with an excess of PBS containing 0.1% (v/v) Tween 20, the blots were incubated with purified rabbit anti-p67phox antibodies, diluted 1:300 in blocking buffer. Anti-p67phox antibodies were also provided by Dr. A. W. Segal. All the subsequent steps were performed as described for the anti-p47phox antibodies.

Phosphorylation studies

Human neutrophils (60×10^6/ml), suspended in phosphate-free Hanks' balanced salt solution containing 0.025% (w/v) BSA, were incubated for 2 h at 37 °C with 400 μCi/ml [^{32}P]P_i (Amersham). After 1 h, 1 mM EGTA and 20 μM quin-2 were added to a portion of the cells in order to deplete them of Ca^{2+}, according to [40]. At the end of incubation the cells were washed twice and resuspended in Hanks' balanced salt solution containing 0.5 mM $CaCl_2$ (normal neutrophils) or 1 mM EGTA (Ca^{2+}-depleted neutrophils). Oxygen consumption was measured as described under Metabolic studies, above. For measurement of the phosphorylation of p47phox and p67phox on the plasma membrane, the ^{32}P-labelled neutrophils were activated and fractionated as described above, except that the relaxation buffer was supplemented with 5 mM EDTA, 10 mM NaF and 0.5 mM okadaic acid. In preliminary experiments we established that the samples were taken after the maximal extent of phosphorylation of p47phox and p67phox had been reached with all of the stimulants. The samples were run on a 0.75 mm-thick slab gel according to the method of Laemmli [43]. The gel consisted of a 4% (w/v) acrylamide stacking gel and a 9% (w/v) separating gel. The samples were run at a constant voltage of 110 V overnight. The gels were stained with Coomassie Blue, destained, dried under vacuum in a Bio-Rad 583 gel dryer and autoradiographed using Kodak X-Omat AR film. Autoradiographs were scanned with an LKB 2202 Ultroscan laser densitometer. The phosphorylation of p47phox and p67phox was estimated by measuring the height of the densitometric peaks [30].

RESULTS AND DISCUSSION

Kinetics of translocation of p47phox and p67phox and activation of NADPH oxidase in human neutrophils stimulated with different agonists

Figure 1 shows the translocation of p47phox and p67phox to the plasma membrane at different time points after stimulation with Con A (300 μg/ml), FMLP (100 nM) and PMA (100 ng/ml), and the polarographic traces of the oxygen consumption in parallel assays. PMA induced translocation of p47phox and p67phox, and this increased linearly with time up to 7 min. Oxygen consumption stimulated by PMA under analogous conditions reached a maximum within 1 min and then remained linear. FMLP induced a rapid translocation of p47phox and p67phox, which reached a maximal value within 1 min. After this time the amounts of protein translocated to the plasma membrane remained nearly constant. In this case the oxygen consumption reached a maximal velocity within 1 min, but rapidly decreased

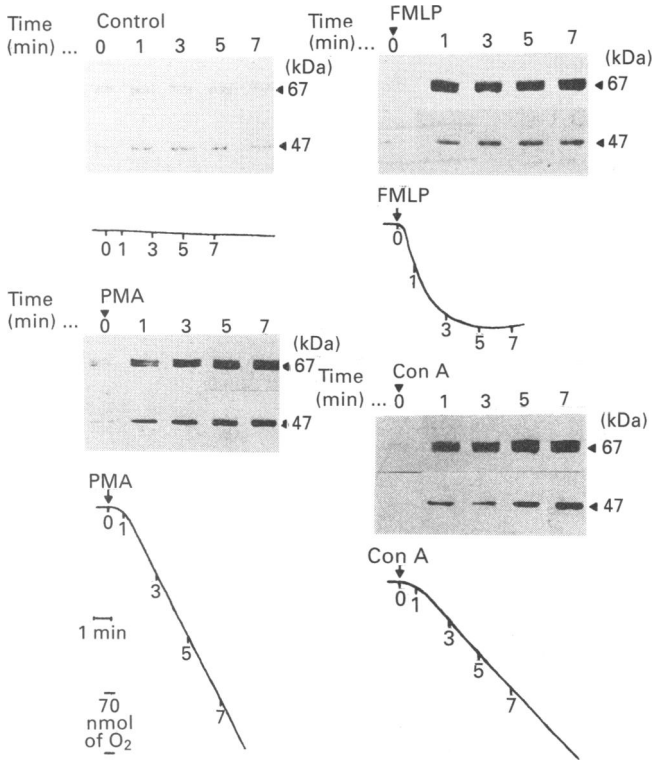

Figure 1 Kinetics of p47phox and p67phox translocation to the plasma membranes of normal neutrophils

Only results with the membrane fractions are shown. The cells (30×10^6/ml) were incubated at 37 °C with PMA (100 ng/ml), FMLP (100 nM) and Con A (300 μg/ml), then blocked at the indicated times, sonicated and fractionated (see the Materials and methods section). The membrane fraction was subjected to electrophoresis by SDS/PAGE on a 9% gel. Proteins were electroblotted to nitrocellulose paper, probed with anti-p47phox antiserum, incubated with horseradish peroxidase-labelled donkey anti-rabbit IgG, exposed to ECL detection reagents and visualized on X-ray film. The blots were then washed, stripped and exposed to anti-p67phox antiserum. The locations of the 47 kDa and 67 kDa bands are indicated. The polarographic traces of O_2 consumptions in parallel assays with the same agonists and at the doses reported above are shown under each blot (numbers indicate the time in minutes). The figure shows the results of an experiment representative of four.

at approx. 2 min and had completely ceased within 5 min. When Con A was the stimulus, translocation of p47phox and p67phox was detectable at 1 min and increased continuously over the period of observation. In response to Con A, oxygen consumption reached a maximum within 90 s and then remained linear.

These results demonstrate the following. (1) With all of the stimuli, i.e. PMA and the receptor-mediated agonists Con A and FMLP, the respiratory burst was accompanied by translocation of p47phox and p67phox. (2) With PMA and Con A, whereas oxygen consumption reached a maximal rate within 1–2 min and then continued linearly, the amounts of p47phox and p67phox translocated to the plasma membrane continued to increase over the period of observation. This means that continuous translocation is necessary to maintain the linearity of the respiratory burst. (3) This conclusion is reinforced by the results obtained with FMLP, which caused translocation of p47phox and p67phox and activation of the respiratory burst with a time course different from those observed with PMA and Con A. In fact, the respiratory burst activated by FMLP ceased very rapidly, while protein translocation to the plasma membrane remained constant. This means that activation of NADPH oxidase is terminated when the process of translocation of p47phox and p67phox ceases. Phosphorylation of p47phox and p67phox in response to FMLP decreased in parallel with the decrease in the respiratory burst [31,45–48], and phosphorylation of p47phox and p67phox in response to PMA remained constant in parallel with the linearity of oxygen consumption [21,29,31,46]. One can therefore postulate that, at the plasma membrane, only the phosphorylated proteins are involved in the activation of the NADPH oxidase.

It is widely accepted that phosphorylation of p47phox is a prerequisite for its translocation to the plasma membrane and activation of NADPH oxidase [24,26,27]. Thus it appears that some protein kinases are involved directly (such as in activation by PMA), or indirectly (such as in activation by FMLP or Con A) through production of second messengers. We addressed this problem in two ways, by investigating (1) the effect of staurosporine on the phosphorylation and translocation of p47phox and p67phox induced by PMA, FMLP and Con A, and (2) the phosphorylation and translocation of these proteins in Ca^{2+}-depleted neutrophils, which lack the transmembrane signals that generate second messengers for the activation of protein kinases, diacylglycerol, phosphatidic acid, arachidonic acid and an increase in cytosolic Ca^{2+} [35–38].

Effect of staurosporine on translocation of p47phox and p67phox and on activation of NADPH oxidase in normal neutrophils

When PMA was the stimulant, both translocation of p47phox and p67phox and the respiratory burst were nearly completely inhibited by staurosporine (Figure 2). However, when the agonist was Con A or FMLP, translocation of p47phox and p67phox was completely unaffected by staurosporine. Thus translocation of p47phox and p67phox may involve either activation of protein kinase C, as in the case of PMA, or other protein kinase C-independent mechanisms, as in the case of FMLP and Con A. Stimulation of the respiratory burst by Con A or FMLP was only slightly depressed by staurosporine; in eight independent experiments with different batches of neutrophils, inhibition of the responses to Con A and FMLP was 30 ± 10% and 14 ± 7% respectively.

Kinetics of the translocation of p47phox and p67phox and the activation of NADPH oxidase in Ca^{2+}-depleted neutrophils stimulated with different agonists

Figure 3 shows the kinetics of translocation to the plasma membrane of p47phox and p67phox, and of the activation of the respiratory burst, in response to different agonists in Ca^{2+}-depleted neutrophils. In these cells, the lipid-based transmembrane signalling does not occur [35–38]. PMA-induced translocation of p47phox and p67phox and stimulation of oxygen consumption were similar to the responses observed in normal neutrophils (see Figures 1 and 2), with regard to kinetics, magnitude and inhibition of staurosporine. However, FMLP or Con A alone was unable to stimulate the respiratory burst or the translocation of p47phox and p67phox. This finding suggests that, with these agonists, protein translocation and activation of NADPH oxidase are dependent on the generation of second messengers from hydrolysis of phospholipids and/or on Ca^{2+} fluxes. In agreement with previous results from our laboratory [35], stimulation with Con A followed by FMLP activated the respiratory burst in Ca^{2+}-depleted neutrophils. As shown in Figure 3 this respiratory burst was accompanied by the translocation of the p47phox and p67phox from the cytosol to the

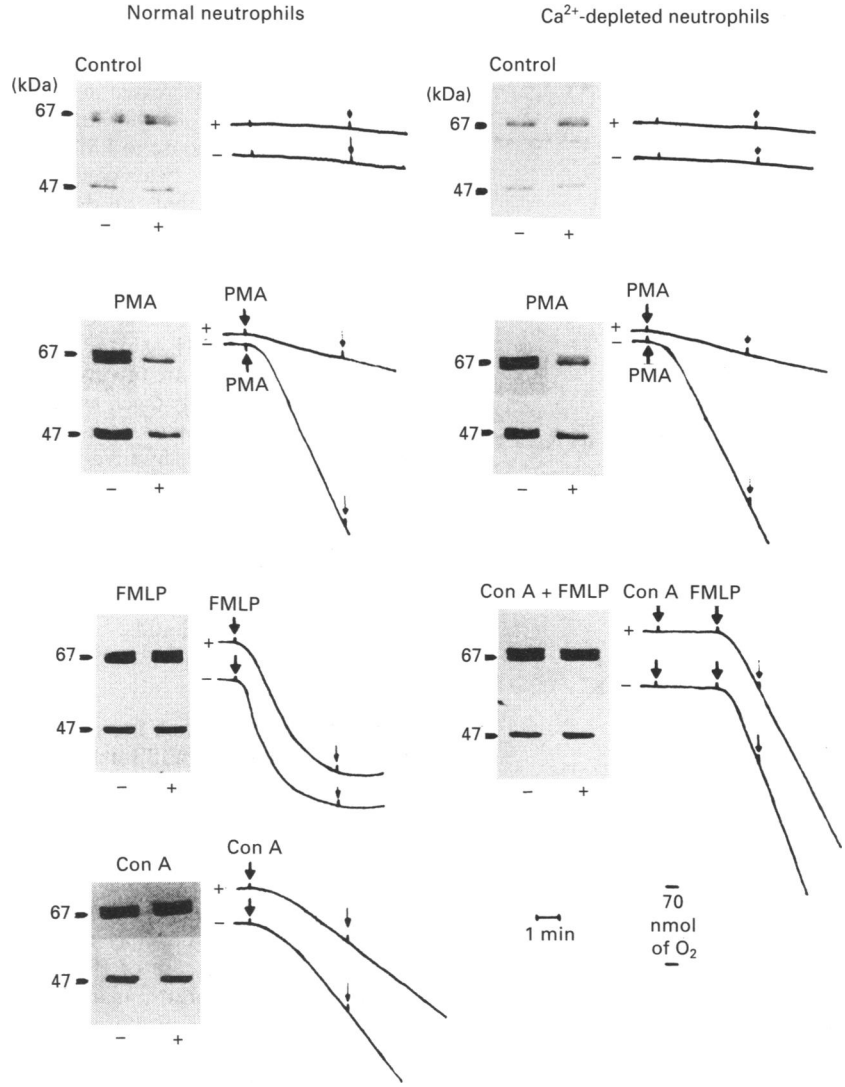

Figure 2 Effects of staurosporine on the translocation of p47phox and p67phox in normal and Ca^{2+}-depleted neutrophils

Only the membrane fractions are shown. The protocol was the same as in Figure 1, except that the experiments were performed in the absence (−) or in the presence (+) of 200 nM staurosporine added 5 min before the stimulants. The period of exposure of neutrophils to the different agonists was 5 min. To the right of each blot, the oxygen consumptions registered in parallel assays are reported. The arrows indicate the time points (5 min) at which translocation of p47phox and p67phox was investigated.

plasma membrane. Both the respiratory burst and protein translocation were insensitive to staurosporine (Figure 2). Densitometric analysis of the translocated p47phox and p67phox (results not shown) demonstrated that in this case also the translocation increased slightly with time, whereas the respiratory burst remained linear.

Effect of staurosporine on the phosphorylation state of p47phox and p67phox translocated to the plasma membrane

In order to understand the relationships between the phosphorylation and translocation of p47phox and p67phox, we investigated the effects of staurosporine on the phosphorylation state of these proteins translocated to the plasma membrane. Activation of neutrophils with PMA caused a marked increase at the plasma membrane of two phosphorylated proteins, of 47 kDa and 67 kDa (Figure 4). While the phosphorylation and translocation of p47phox has been demonstrated in many laboratories [24,26,27], no direct evidence has been presented to date that p67phox is phosphorylated upon activation of NADPH oxidase. Since it has been reported that a protein in the 59–69 kDa range is markedly phosphorylated when neutrophils are stimulated with known activators of NADPH oxidase [15,16,18], and that this phosphorylation shows kinetics consistent with those of the respiratory burst [31,45,47], one can presume that the phosphorylated 67 kDa protein represented p67phox. For this reason, even in the absence of a true identification, we have designated this 67 kDa protein as p67phox throughout the paper. When the stimulant was PMA, staurosporine completely inhibited the appearance of phosphorylated p47phox and p67phox on the plasma membrane in both normal and Ca^{2+}-depleted neutrophils. This depression was paralleled by a marked inhibition of p47phox and p67phox translocation, and by a complete suppression of the activation of NADPH oxidase (see Figure 2). The data on p47phox obtained with PMA in normal neutrophils are in agreement with those of Clark and co-workers [24]. In Ca^{2+}-depleted neutrophils, FMLP or Con A alone did not induce phosphorylation of p47phox and p67phox as well as

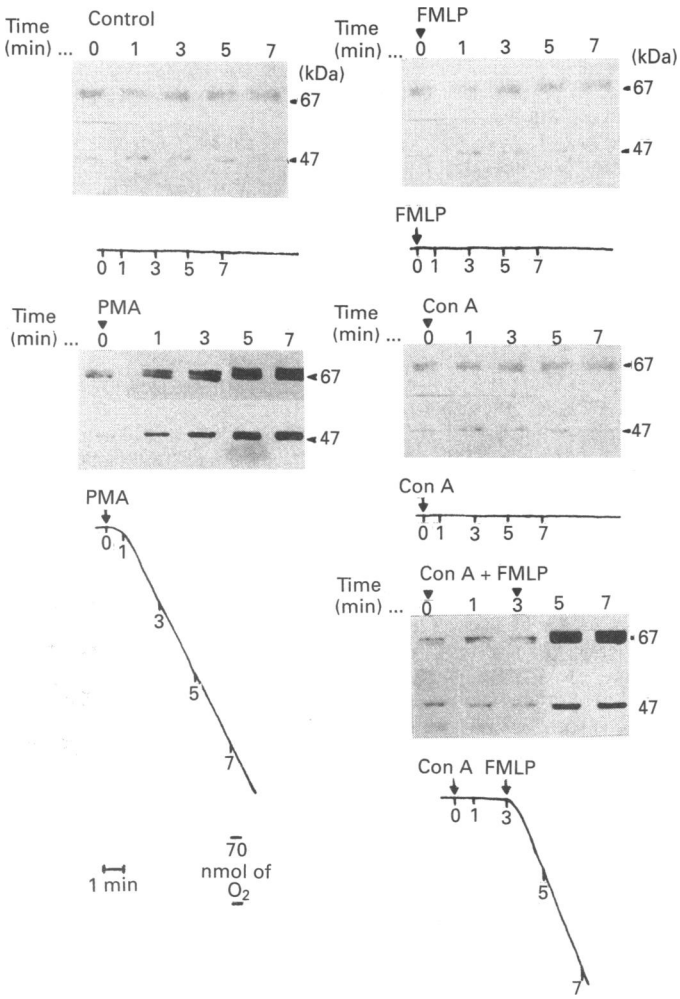

Figure 3 Kinetics of p47phox and p67phox translocation to the plasma membranes in Ca^{2+}-depleted neutrophils

Only the membrane fractions are shown. The protocol was the same as in Figure 1, except that the cells were depleted of Ca^{2+} as described in the Materials and methods section. For each stimulus, cells derived from the same donor were used to perform both the experiments with Ca^{2+}-depleted neutrophils and the experiments with normal neutrophils reported in Figure 1. Under each blot, the trace of oxygen consumption registered in a parallel assay is reported.

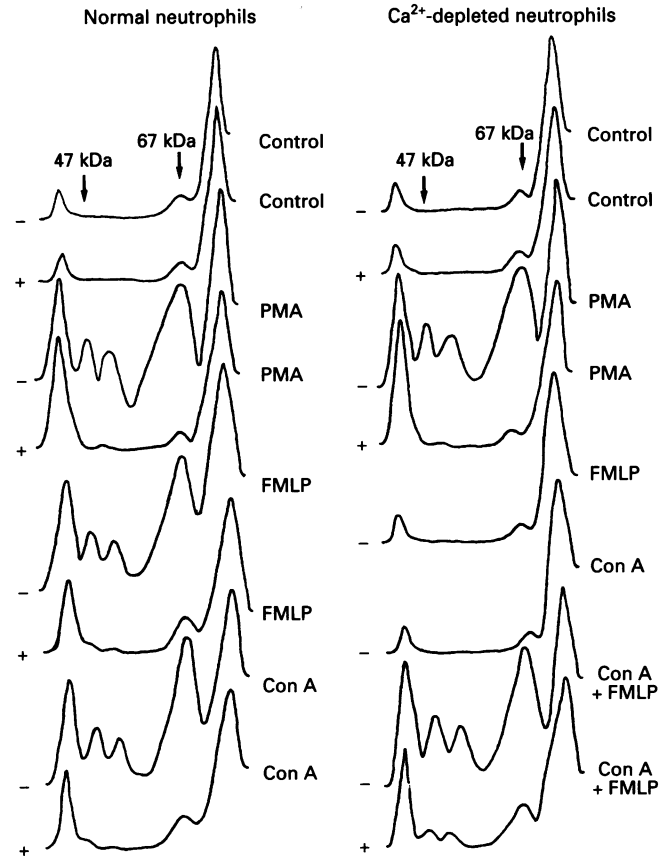

Figure 4 Effect of staurosporine on phosphorylation of p47phox and p67phox present in the membranes of normal and Ca^{2+}-depleted neutrophils

Neutrophils (30×10^6/ml) labelled with $[^{32}P]P_i$ were activated at 37 °C by the same agonists used for the translocation experiments at the doses indicated in Figure 1, in the presence (+) or absence (−) of staurosporine (200 nM). Samples were blocked at times where the maximal velocity of O_2 consumption was reached, i.e. between 1 and 2 min, depending on the stimulus used, and then sonicated and fractionated as described in the Materials and methods section. p47phox and the p67phox are indicated by arrows. The figure shows an experiment representative of five.

their translocation and activation of the respiratory burst (see Figure 3). When the stimulus was FMLP on Con A in normal neutrophils or, in Ca^{2+}-depleted neutrophils, Con A plus FMLP, staurosporine did not inhibit the translocation of p47phox and p67phox (see Figure 2), but did depress the extent of phosphorylation of these proteins on the plasma membrane (Figure 4). On the basis of a densitometric analysis of five experiments, the inhibition of phosphorylation of p47phox was $95 \pm 7\%$ with FMLP, $72 \pm 15\%$ with Con A and $73 \pm 15\%$ with Con A plus FMLP in Ca^{2+}-depleted neutrophils. The depression of phosphorylation of p67phox was $72 \pm 6\%$ with FMLP, $70 \pm 16\%$ with Con A and $72 \pm 17\%$ with Con A plus FMLP. In spite of this inhibition, the respiratory bursts induced by FMLP, Con A or FMLP plus Con A were only slightly affected by staurosporine. Badway and co-workers [49] reported that inhibition of FMLP-induced p47phox phosphorylation by staurosporine occurred in the absence of any effect on the respiratory burst [49].

With all of the stimuli, a phosphorylated protein of 49–51 kDa was always present in the plasma membrane of activated neutrophils (see Figure 4). The behaviour and the sensitivity to inhibitors of this protein were similar to those of p47phox. This protein probably corresponds to that described by Badway and co-workers [29,30,48,49], and its function has not been clarified.

Experiments on protein phosphorylation performed with whole neutrophils have given similar results, including staurosporine sensitivity (results not shown).

Conclusions

The conclusions derived from the results presented in this paper can be summarized in five points. (1) With all of the stimuli used, translocation of p47phox and p67phox is an essential process for activation of NADPH oxidase, and a continuous translocation is necessary to maintain the oxidase in an active state. Thus the linearity of the stimulated O_2 consumption may be assured by the continuous recruitment of new 'units' of NADPH oxidase, which remains only transiently in an activated state, due to translocation of portions of p47phox and p67phox. (2) With PMA, activation of NADPH oxidase is directly correlated with the phosphorylation and translocation of p47phox and p67phox, and with the amount of phosphorylated protein present at the plasma membrane. The primary reaction is an increase in the

phosphorylation of cytosolic proteins by protein kinase C directly activated by PMA, followed by translocation and activation of the oxidase. (3) With receptor-mediated stimuli, translocation of p47phox and p67phox seems to be linked to transmembrane signalling mechanisms involving Ca^{2+} transients and production of second messengers from the hydrolysis of phospholipids. This translocation is staurosporine-insensitive and can be quantitatively dissociated from the process of phosphorylation, which is markedly sensitive to staurosporine. (4) With appropriate receptor-mediated stimuli (double stimulation of Ca^{2+}-depleted neutrophils with Con A plus FMLP), protein translocation can occur with mechanisms that are independent of changes in Ca^{2+} and activation of phospholipid hydrolysis. This translocation is staurosporine-insensitive and can be dissociated from the process of phosphorylation, which is markedly inhibited by staurosporine. (5) The finding that the phosphorylation of p47phox and p67phox does not correlate with the translocation and activation of NADPH oxidase might be explained by assuming either that phosphorylation is not the only modification of the cytosolic proteins that promotes their translocation, as proposed by Miyahara [50] and Heyworth and Badway [28], or that protein translocation and NADPH oxidase activation are regulated by the phosphorylation of a threshold number of sites in each molecule of p47phox and p67phox.

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