Tyrosine phosphorylation and activation of NADPH oxidase in human neutrophils: a possible role for MAP kinases and for a 75 kDa protein

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Challenge of neutrophils with concanavalin A (ConA), formyl-methionyl-leucyl-phenylalanine (FMLP), and phorbol 12-myristate 13-acetate (PMA) induced the tyrosine phosphorylation of several proteins. Among these proteins we have identified two mitogen-activated protein kinase (MAPK) isoforms of 43 kDa (p43 MAPK) and 45 kDa (p45 MAPK) molecular mass. Moreover here we show that: (1) FMLP induced the tyrosine phosphorylation of the p43 MAPK, and ConA that of p45 MAPK, while PMA induced the tyrosine phosphorylation of both p43 and p45 MAPK; all these agonists induced the tyrosine phosphorylation of a 75 kDa protein (p75). (2) With FMLP or ConA as agonists, tyrosine phosphorylations of MAPK and p75 can be involved in the process of NADPH oxidase activation. On the contrary, PMA can activate the respiratory burst independently of these phosphorylations. (3) In Ca^{2+}-depleted neutrophils, where phospholipid hydrolysis did not take place, ConA or FMLP did not activate the respiratory burst, but while ConA induced the tyrosine phosphorylation of p45 MAPK and p75, FMLP was not able to phosphorylate p43 MAPK and p75. (4) As previously observed in our laboratory, a double stimulation of Ca^{2+}-depleted neutrophils with ConA plus FMLP induced a respiratory burst in the absence of activation of second messengers derived from phospholipase C, D and A2 activity. This respiratory burst was accompanied by tyrosine phosphorylation of both p43 and p45 MAPKs. These results indicate that when FMLP is the agonist, both the tyrosine phosphorylation of p43 MAPK and p75, and the activation of NADPH oxidase, are coupled to Ca^{2+}-dependent mechanisms. On the contrary, ConA can induce the tyrosine phosphorylation of p45 MAPK and p75 independently of calcium, but an unknown Ca^{2+}-dependent mechanism is necessary for the activation of NADPH oxidase by this agonist. This mechanism could be substituted by the induction of tyrosine phosphorylation of both p43 MAPK and p45 MAPK when Ca^{2+}-depleted neutrophils are stimulated with ConA plus FMLP.

INTRODUCTION

It is well established that the serine/threonine phosphorylation by protein kinases of several components such as cytosolic factors p47phox and p67phox, and the gp91phox and p22phox subunits of cytochrome b_{558}, is involved in the activation of NADPH oxidase, the superoxide-generating enzymic system of phagocytes [1–7]. However, the signalling events that lead to the activation of the protein kinases involved, as well as the role of phosphorylation of oxidase components in the assembly and activity of the enzymic system, have not been fully elucidated. In this respect, it has been proposed that the activation of tyrosine kinases may also be involved in the activation of NADPH oxidase. In fact, several investigations have shown that the neutrophil respiratory burst that occurs in response to several agonists such as phorbol 12-myristate 13-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (FMLP), granulocyte–macrophage colony-stimulating factor (GM-CSF), C5a, leukotriene B4 (LTB4), platelet-activating factor (PAF), heat-aggregated IgG, and guanosine 5′-[γ-thio]triphosphate in permeabilized cells, was associated with the tyrosine phosphorylation of many proteins [8–22]. Moreover, it has been demonstrated that tyrosine kinase activation is responsible for the activation of phospholipases D [23–27] and Cγ2 [28], two processes involved in the transmembrane signallings for the activation of the neutrophil respiratory burst [1,3,4]. In particular, it has been reported that the onset of the respiratory burst in response to FMLP is associated with the tyrosine phosphorylation of proteins of 40–44 kDa [10,11,18]. These proteins have been identified as members of the mitogen-activated serine/threonine protein kinases (MAP kinases) family [29,30], also named extracellular signal-regulated kinases (ERK kinases), that are involved in the transmembrane signalling of several growth factors [31–33].

The potential role of MAP kinases in the activation of the NADPH oxidase remains to be clarified. It has been reported recently that p41 MAP kinase/ERK 2 was able to phosphorylate a synthetic peptide of p47phox [34], and to phosphorylate and activate a purified cytosolic phospholipase A2 [35]. In this paper, we present evidence that tyrosine phosphorylation of p43 and p45 MAP kinases and of a 75 kDa protein is involved in the signalling pathways for the activation of the neutrophil NADPH oxidase induced by FMLP and ConA. The possible identity of the 75 kDa protein is also discussed.

MATERIALS AND METHODS

Materials

PMA and FMLP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; SDS, acrylamide, *N*,*N*′-methylene-bisacrylamide, tetramethylenediamine and blotting nitrocellulose membranes were purchased from Bio-Rad, Richmond, U.S.A.; Ficoll 400 and molecular-mass standards were from Amersham,

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; ConA, concanavalin A; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; GM-CSF, granulocyte–macrophage colony-stimulating factor; LTB4, leukotriene B4; PAF, platelet-activating factor; PMSF, phenylmethanesulphonic acid; mAb, monoclonal antibody; p47phox and p67phox, cytosolic phagocyte oxidase factors of 47 and 67 kDa respectively; PAO, phenylarsine oxide; NP-40, Nonidet P-40; MBP, myelin basic protein.

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Bucks., U.K.; IgG2bk anti-phosphotyrosine mouse monoclonal antibody (mAb) from hybridoma 4G10 was obtained from UBI, Lake Placid, NY, U.S.A.; ERK 1 (691), an affinity-purified rabbit polyclonal antibody raised against a peptide which corresponds to amino acids 305–327 within the XI subdomain of the ERK 1 protein and reacting with both the 43-kDa ERK 1, the 41-kDa ERK 2, and a third ERK protein of 45 kDa, and Raf-1(C-12), an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to the 12 C-terminal amino acid residues of Raf-1, were from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.; erk1-CT, an affinity-purified rabbit polyclonal antibody that recognizes the 43 kDa, 42 kDa and 44 kDa MAP kinases encoded by the *erk1* gene, *mapk* gene and *mpk* gene respectively was from UBI, Lake Placid, NY, U.S.A.

Neutrophil preparation

Human neutrophils were prepared from venous blood of healthy donors as previously described [36]. Cells were resuspended in phosphate-buffered saline, then treated at 4 °C for 10 min with 2 mM di-isopropyl fluorophosphate (DFP; Sigma Chemical Co.), washed and resuspended in Hanks' balanced salt solution buffered with 20 mM Hepes (pH 7.4), and containing 0.5 mM $CaCl_2$ and 5.6 mM glucose.

Cellular Ca^{2+} depletion

When required, a portion of the cells was suspended at a final concentration of 6×10^7 cells/ml in Hanks' balanced salt solution without calcium. These cells were depleted of calcium as previously described [37]. Briefly, neutrophils were incubated with 1 mM EGTA and 30 μM Quin 2/AM for 60 min at 37 °C under agitation. At the end of incubation the cells were washed twice and resuspended in Hanks' balanced salt solution containing 1 mM EGTA. This treatment results in a decrease of the free cytosolic calcium concentration to less than 10 nM, monitored according to [38].

Metabolic studies

Oxygen consumption was measured at 37 °C with a Clark oxygen electrode using $1.5–2.0 \times 10^7$ neutrophils/ml in Hanks' balanced salt solution containing 2 mM NaN_3, 5 μg/ml cytochalasin B and, in the case of Ca^{2+}-depleted neutrophils, 1 mM EGTA. When PMA was used as the stimulus, cytochalasin B was omitted. When required, 120 μM genistein was added to the cells 20 min before the stimuli.

Neutrophil activation

Human neutrophils (2×10^7/ml) were treated with different stimuli for various lengths of time at 37 °C, in a water bath under continuous shaking. Reactions were stopped by diluting the cells with a 10-fold excess of ice-cold Hanks' balanced salt solution containing 1 mM PMSF, 2 mM Na_3VO_4, 10 μM phenylarsine oxide (PAO) and, in the case of Ca^{2+}-depleted neutrophils, 1 mM EGTA. Neutrophils were then centrifuged (500 ***g*** for 7 min at 4 °C), and resuspended in 0.5 ml of prewarmed electrophoresis sample buffer [60 mM Tris/HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol]. The protein content was measured by the method of Bradford [39].

Electrophoresis and immunoblotting

Aliquots of samples from resting and activated neutrophils that contained the same amount of proteins (30 μg) were subjected to SDS/PAGE according to Laemmli [40]. Proteins were electroblotted as previously described [6]. The blots were incubated for 120 min in TBS buffer (50 mM Tris, pH 7.5, 170 mM NaCl) containing 5% (w/v) BSA and 0.2% (v/v) Nonidet-P-40 (NP-40) (blocking buffer), before incubation overnight at 4 °C with 2 μg/ml anti-phosphotyrosine mAbs 4G10, anti-(MAP kinases), or anti-(Raf-1) antibodies in blocking buffer. After various washings with TBS containing 0.2% (v/v) NP-40, the blots were incubated for 60 min in horseradish peroxidase-labelled anti-(rabbit IgG) or anti-(mouse IgG) (Amersham, U.K.), diluted 1:15000 and 1:2000 respectively in blocking buffer. After further washings, bound antibodies were revealed by enhanced chemiluminescence (ECL) detection reagents (Amersham, U.K.). When required, blots were stripped as previously described [6].

Immunoprecipitation

After the desired incubation time with neutrophil stimuli, reactions were stopped and cells pelleted as described above. Pellets were resuspended in 400 μl of solubilization buffer [25 mM Tris, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS], containing 2 mM Na_3VO_4, 10 μM PAO, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1 mM EDTA, 1 mM PMSF, extracted for 30 min on ice and centrifuged at 15000 ***g*** for 5 min to remove insoluble material. The supernatants were incubated for 120–180 min at 4 °C under rotation with 7 μg of anti-phosphotyrosine mAb 4G10 conjugated to trisacryl–Protein A (Pierce), and washed three times with 700 μl of ice-cold solubilization buffer. Immunoprecipitated proteins were eluted by boiling the beads for 3 min at 95 °C in 50 μl of electrophoresis sample buffer [60 mM Tris/HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol]. The beads were pelleted by centrifugation and supernatants were subjected to SDS/PAGE and immunoblotting as described above.

Assay for MAP kinase activity

Aliquots of proteins (700 μg) were incubated for 120 min at 4 °C with an anti-(MAP kinase) polyclonal antiserum (erk1-CT, UBI) that specifically recognizes the 43 kDa, 42 kDa and 44 kDa MAP kinases. Immunocomplexes were collected with trisacryl-Protein A, and washed twice with solubilization buffer and once with a buffer containing 25 mM Tris, pH 7.5, 137 mM NaCl, 40 mM $MgCl_2$ and 10% (v/v) glycerol. The immunocomplexes were incubated for 30 min at 30 °C in a reaction mixture containing 40 mM Hepes, pH 7.4, 40 mM $MgCl_2$, 1 mM DL-dithiothreitol, 100 μM ATP and 5 μCi of [γ-^{32}P]ATP with 10 μg of myelin basic protein (MBP) as a substrate. The reactions were analysed by electrophoresis on SDS/14% PAGE and exposure of the dried gel to X-ray film.

RESULTS AND DISCUSSION

Protein tyrosine phosphorylation

Figure 1(a) shows the tyrosine phosphorylation patterns observed after challenge of neutrophils with FMLP (5 μM) for 1 min or with Con A (300 μg/ml) or PMA (100 ng/ml) for 4 min. These time points were chosen because they correspond to those at which a maximal rate of NADPH oxidase activity was observed in response to these agonists. All the stimulants induced the

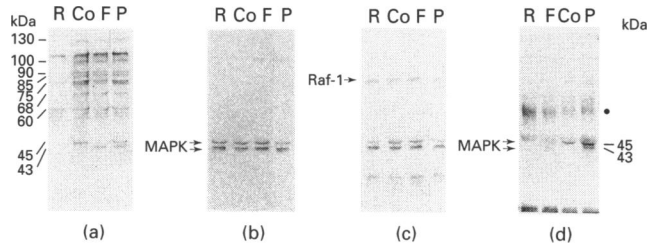

Figure 1 Protein tyrosine phosphorylation patterns and identification of raf-1 and MAP kinase isoforms in human neutrophils

Neutrophils (2×10^7/ml) were incubated at 37 °C under agitation with FMLP (5 μM, 1 min) (F), ConA (300 μg/ml, 4 min) (Co) or PMA (100 ng/ml, 4 min) (P); the reactions were stopped and the sample boiled in electrophoresis sample buffer. Lysates were subjected to SDS/10% PAGE, and immunoblotted with anti-phosphotyrosine mAb 4G10 (**a**). The blot was then stripped and reprobed with anti-(MAP kinase) polyclonal antibody (**b**), and again with anti-(raf-1) polyclonal antibody (**c**). In (**c**), besides the raf-1, the 43 and 45 kDa bands of MAP kinases are still present. This is due to the fact that anti-(MAP kinase) antibody was not removed before incubation with the anti-(raf-1) antibody. Part of the neutrophils was extracted in solubilization buffer (see the Materials and methods section) and immunoprecipitated with anti-phosphotyrosine mAb 4G10. The immunoprecipitated material was subjected to SDS/8% PAGE and immunoblotted with anti-(MAP kinases) antibody (**d**). Immunoglobulin molecules are indicated by a dot. R = resting neutrophils. The data are from one experiment representative of three.

tyrosine phosphorylation of proteins with apparent molecular masses of 130, 100, 90, 85, 75 and 68 kDa. While the 75 kDa protein was more phosphorylated by ConA and PMA than by FMLP, the other proteins were phosphorylated to a similar extent by all three agonists. Moreover, ConA (but not FMLP) induced the tyrosine phosphorylation of a 45 kDa protein, and FMLP (but not ConA) elicited the phosphorylation of a 43 kDa protein, while PMA induced the phosphorylation of both 43 and 45 kDa proteins. The specificity of the anti-phosphotyrosine antibody was confirmed by inhibition of the binding with an excess of phosphotyrosine (results not shown). Collectively, the FMLP- and PMA-induced tyrosine phosphorylation patterns reported herein are similar to those reported by other groups [13,20,21]. With respect to ConA, this is the first demonstration that it can induce protein tyrosine phosphorylation in human neutrophils.

Identification of the 43, 45 and 75 kDa tyrosine phosphorylated proteins

Because certain isoforms of MAPK/ERK, a family of serine-threonine kinases that are regulated by phosphorylation on both tyrosine and serine/threonine residues [31,33], have apparent molecular masses similar to those observed herein for the 43 and 45 kDa phosphoproteins, we investigated whether these two phosphoproteins may be identified as MAP kinases. For this purpose we used an affinity-purified rabbit polyclonal antibody that recognizes the 43 kDa MAP kinase/ERK 1, the 41 kDa MAP kinase/ERK 2, and as yet uncharacterized 45 kDa MAP kinase [ERK1(691), Santa Cruz Biotechnology]. Figure 1(b) shows that this antibody recognized protein bands at 43 kDa and 45 kDa, which co-migrated precisely with the 43 kDa and 45 kDa proteins that were tyrosine phosphorylated in response to FMLP, ConA and PMA stimulation. A 41 kDa protein that migrated immediately under the 43 kDa band was also weakly recognized by the anti-MAPK antibody. However, this protein was not detected by the anti-phosphotyrosine antibody, indicating that it was very weakly tyrosine phosphorylated. It is likely that this protein corresponds to a 40 kDa MAP kinase species reported to be tyrosine phosphorylated in FMLP-stimulated neutrophils [30].

To confirm that the 43 kDa and 45 kDa phosphoproteins were indeed members of MAP kinases family, extracts from resting and stimulated neutrophils were immunoprecipitated with anti-phosphotyrosine antibodies, prior to immunoblotting with the anti-(MAP kinase) antibody (Figure 1d). It can be seen that anti-phosphotyrosine antibody immunoprecipitated a 43 kDa MAP kinase from FMLP-stimulated neutrophils, a 45 kDa MAP kinase from ConA-stimulated neutrophils and both 43 kDa and 45 kDa MAP kinases from PMA-stimulated cells. The same results have been obtained probing the proteins immunoprecipitated with anti-phosphotyrosine antibody with a different anti-(MAP kinase) antibody that recognizes the 43 kDa, 42 kDa and 44 kDa MAP kinases encoded by *erk-1* gene, *mapk* gene and *mpk* gene respectively (erk1-CT, UBI) (results not shown).

The above data demonstrate that the tyrosine-phosphorylated 43 kDa and 45 kDa proteins can be identified as MAP kinase isoforms, which will be referred to as p43 MAPK and p45 MAPK hereafter. The formal proof that these kinases correspond to the previously described p43 ERK-1 and p45 ERK-4 [41,42], however, will require purification and sequence analysis. Our finding that p43 MAPK and p45 MAPK are selectively phosphorylated depending upon the agonist used, suggests that their respective activities are regulated by different mechanisms. That tyrosine phosphorylation of MAP kinases correlates with their activation [30,43–45] further supports this view.

It has been shown that both serine/threonine and tyrosine phosphorylation of MAP kinases can be catalysed by a kinase, termed MAP kinase kinase or MAPKK, which in turn is activated by the serine/threonine kinase, raf-1 [46–48]. Since raf-1 becomes tyrosine phosphorylated in myeloid cells challanged with several agonists, we examined whether the tyrosine phosphorylated 75 kDa protein observed in our experiments could be identified as raf-1, since its molecular mass corresponds to that of raf-1 [48]. When we tried to immunoprecipitate this protein with a specific anti-raf-1 antibody, this antibody failed to immunoprecipitate raf-1. We therefore reprobed the blot depicted in Figure 1(b) with the anti-raf-1 antibody (Figure 1c). The superimposition of the two blots revealed that the bands detected by the anti-raf-1 antibody (panel c of Figure 1) exactly coincided with the 75 kDa tyrosine-phosphorylated protein observed after neutrophil activation by ConA, PMA and FMLP (panel a of Figure 1). These results indicate that the 75 kDa protein might be the serine-threonine kinase raf-1. Figure 1(c) also shows that neutrophil stimulation with FMLP, ConA and PMA increased the apparent molecular mass of the 75 kDa protein. This shift is considered to be a characteristic of raf-1 activation, due to its phosphorylation [48–50]. The formal proof that this 75 kDa phosphoprotein corresponds to raf-1 will require further investigation. Therefore it will be henceforth referred to as p75.

Taken together, the results so far presented demonstrate that neutrophil activation in response to different agonists is associated with an increase of tyrosine phosphorylation of p75 (raf-1 ?) and of p43 MAPK and p45 MAPK, that, on the basis of previous reports [30,43–45,51,52], presumably corresponds to their activation.

Relationship between the tyrosine phosphorylation of p43 MAPK, p45 MAPK and p75, and the activation of NADPH oxidase in human neutrophils

In many cell types, raf-1 and MAP kinases have been implicated in gene regulation and cell-cycle control [31–33,48]. However,

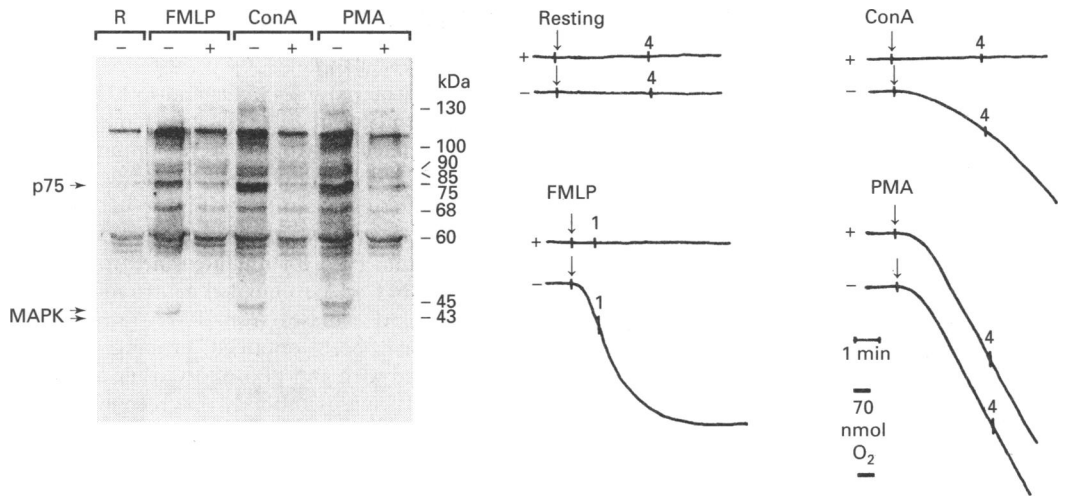

Figure 2 Effect of genistein on protein tyrosine phosphorylation and respiratory burst induced by PMA, ConA and FMLP in normal neutrophils

Neutrophils (1×10^7/ml) were incubated for 20 min at 37 °C in the absence (−) or presence (+) of 120 μM genistein. Cells were then stimulated with FMLP, ConA or PMA at the same doses and for the same times as in Figure 1. Reactions were stopped and samples were boiled in electrophoresis sample buffer, subjected to SDS/10% PAGE, and immunoblotted with anti-phosphotyrosine mAb 4G10. Oxygen consumption was also determined in parallel assays (numbers indicate the time points in minutes at which protein tyrosine phosphorylation was investigated). Abbreviation: R, resting neutrophils. The data are from one experiment representative of three.

their role in non-dividing cells such as neutrophils remains poorly understood. It is known that two kinds of responses are induced in neutrophils following challenge with PMA, ConA or FMLP: some are rapid, such as the activation of NADPH oxidase responsible for the respiratory burst [1,3–5], some others are slow, such as cytokine production [53]. Here, we tried to elucidate whether tyrosine phosphorylation of MAP kinases and of p75 (raf-1 ?) induced by PMA, ConA or FMLP may be involved in the activation of NADPH oxidase. To this end, we used three approaches: (a) we examined the effect of genistein, an inhibitor of tyrosine kinases, on both tyrosine phosphorylation and activation of NADPH oxidase; (b) we compared the kinetics of tyrosine phosphorylation with those of NADPH oxidase activation as determined by the measurement of oxygen consumption in whole cells; (c) we investigated the tyrosine phosphorylation pattern induced by FMLP, ConA and PMA in Ca^{2+}-depleted neutrophils in parallel with the activation of NADPH oxidase under these conditions.

(a) Effect of genistein on protein tyrosine phosphorylation and on the induction of the respiratory burst in normal neutrophils

Figure 2 shows the effect of genistein (120 μM) on tyrosine phosphorylation and on the respiratory burst induced by FMLP, ConA and PMA, under the same conditions reported in Figure 1. At this genistein concentration, the cell viability was not affected as determined by Trypan Blue exclusion (results not shown). When FMLP or ConA were used as stimuli, genistein depressed the tyrosine phosphorylation of all protein bands including that of p75, while it completely suppressed that of p43 MAPK and p45 MAPK. Under these conditions, the respiratory burst induced by FMLP or ConA was also completely abrogated by genistein. When PMA was used as stimulus, genistein pretreatment decreased the tyrosine phosphorylation of all the proteins, including that of p75, and suppressed that of both p43 MAPK and p45 MAPK, but it did not affect the PMA-elicited NADPH oxidase activity. Similar results were obtained pretreating the cells for 60 min at 37 °C with 10 μg/ml erbstatin, another tyrosine kinase inhibitor (results not shown). These data indicate that the activation of NADPH oxidase could involve protein tyrosine phosphorylation in neutrophils stimulated with ConA and FMLP, but not with PMA. Consistent with this conclusion is that similar observations have been reported in the case of PMA and FMLP [10,11,18,54,55]. That genistein was found to be most effective in inhibiting the tyrosine phosphorylation of p43 MAPK and p45 MAPK suggests that these kinases could play a prominent role in NADPH oxidase activation induced by ConA and FMLP. However, the involvement of other proteins, whose tyrosine phosphorylation is also depressed by genistein, cannot be ruled out.

(b) Time course of tyrosine phosphorylation and oxygen consumption in normal neutrophils in response to FMLP and ConA

The possibility that p43 MAPK, p45 MAPK and p75 could be involved in the FMLP- or ConA-induced activation of NADPH oxidase was further supported by comparing the time courses of their tyrosine phosphorylation with those of the respiratory burst. Indeed, it was found that the kinetics of the respiratory burst only showed a good correlation with those of p75, p43 MAPK and p45 MAPK tyrosine phosphorylation. Figure 3 shows that (1) oxygen consumption in response to FMLP started at 30 s, reached a maximal velocity within 1 min, rapidly decreased at 3 min and completely ceased at 5 min; (2) a significant tyrosine phosphorylation of both p43 MAPK and p75 was detectable as early as 30 s after FMLP stimulation, was maximal at 1 min, decreased at 3 min and was markedly depressed at 5 min; (3) tyrosine phosphorylation of the other proteins (i.e. 68, 85, 90, 100, and 130 kDa) became evident at 30 s and remained constant up to 5 min.

When ConA was used as stimulus: (1) the tyrosine phosphorylation of both p45 MAPK and p75, as well as oxygen consumption, started at 30 s and continuously increased up to 5 min; (2) tyrosine phosphorylation of 68, 85, 90, 100, and 130 kDa proteins was clearly detectable at 30 s and then remained constant up to 5 min. These results were confirmed by

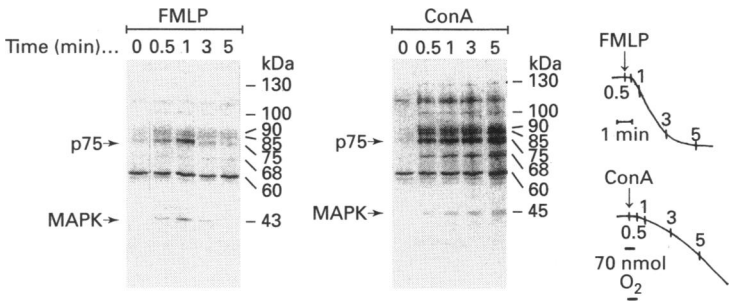

Figure 3 Time course of protein tyrosine phosphorylation and oxygen consumption induced by FMLP and ConA in normal neutrophils

Neutrophils (2×10^7/ml) were stimulated at 37 °C under agitation with (**a**) FMLP (5 μM) or (**b**) ConA (300 μg/ml) for the indicated times; reactions were stopped and samples were boiled in sample buffer, subjected to SDS/9% PAGE and immunoblotted with anti-phosphotyrosine mAb 4G10. Values for oxygen consumption (**c**), which were determined in parallel assays, are also reported (numbers indicate the time points in minutes at which protein tyrosine phosphorylation was investigated). The data are from one experiment representative of four.

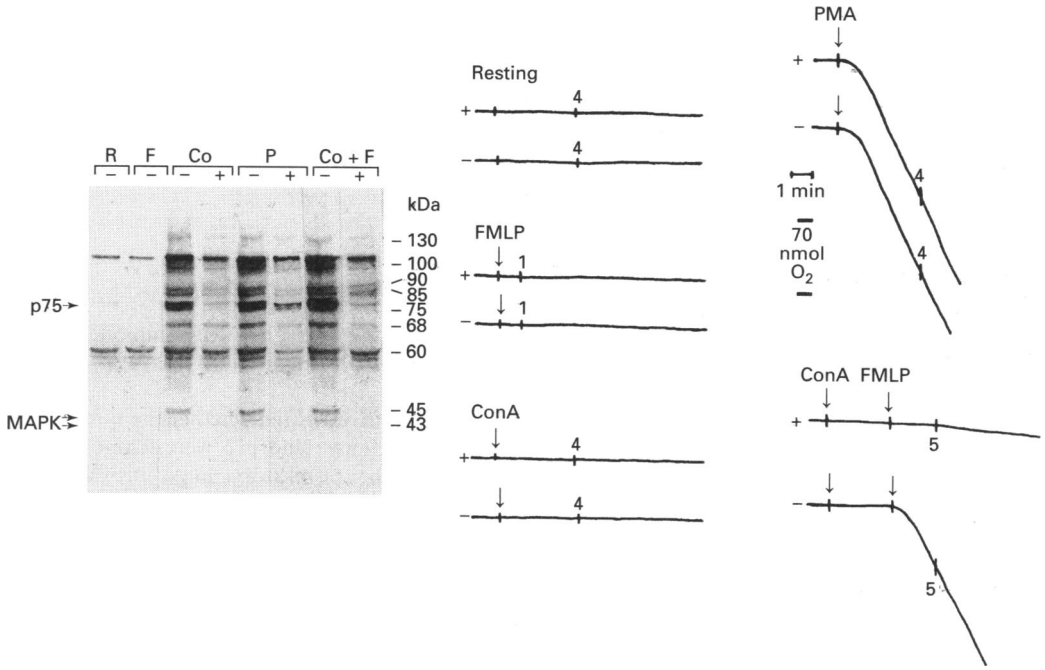

Figure 4 Effect of genistein on protein tyrosine phosphorylation and on the induction of the oxygen consumption in Ca^{2+}-depleted neutrophils

Neutrophils were depleted of Ca^{2+}, then incubated for 20 min at 37 °C in the absence (−) or presence (+) of 120 μM genistein, and stimulated with FMLP (F), ConA (Co), and PMA (P) at the same doses and for the same times as in Figure 1, or with FMLP (5 μM) added after 3 min of pretreatment with ConA (300 μg/ml) (Co + F). In this latter case, the reaction was stopped 5 min after stimulation with ConA (i.e. 2 min after addition of FMLP). Cells were then lysed and subjected to SDS/10% PAGE. Blots were probed with anti-phosphotyrosine mAb 4G10. Oxygen consumption was determined in parallel assays (numbers indicate the time points in minutes at which protein tyrosine phosphorylation was investigated). Abbreviation: R, resting neutrophils. Data are from one experiment representative of five.

densitometric analysis of tyrosine phosphorylated bands (results not shown).

These findings suggest that the respiratory burst induced by FMLP correlated with the increase in tyrosine phosphorylation of p43 MAPK and p75, while the respiratory burst induced by ConA correlated with the increase of p45 MAPK and p75 tyrosine phosphorylation. It is worth pointing out that in response to both ConA and FMLP, the kinetics of p45 MAPK and p43 MAPK tyrosine phosphorylation respectively, were paralleled by those of p75, suggesting a strict correlation between the tyrosine phosphorylation of p75 and that of these MAP kinases. This further suggests that p75 may represent raf-1, which may indirectly account for the tyrosine phosphorylation of both MAP kinases, especially since such an occurrence has been reported in different cell types [46,47].

(c) Protein tyrosine phosphorylation and respiratory-burst induction in Ca^{2+}-depleted neutrophils

On the basis of previous work from our laboratory [6,56,57], it is known that Ca^{2+}-depleted neutrophils (1) do not respond to FMLP or ConA (as sole stimuli) with a respiratory burst and with an increased production of second-messenger inositol phosphates, phosphatidic acid, arachidonic acid, and diacylglycerol from the stimulation of phospholipase C, D and A2; (2) are capable of generating a respiratory burst, in spite of the

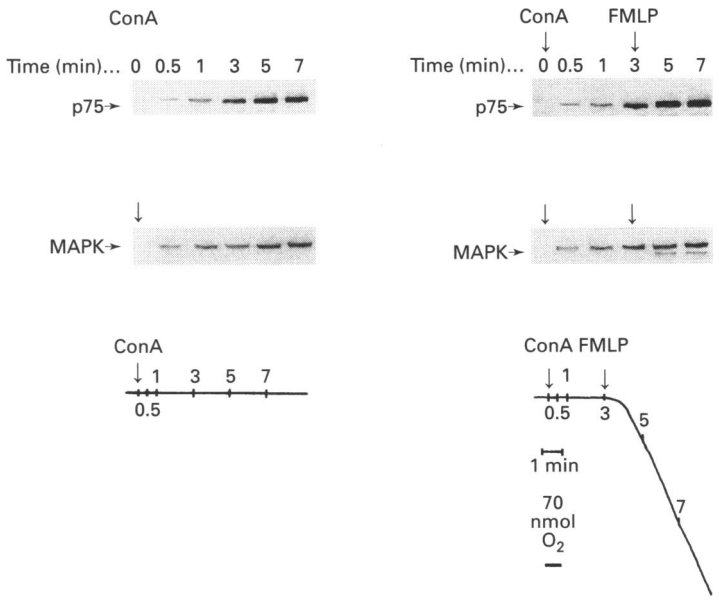

Figure 5 Time courses of tyrosine phosphorylation of p75 and MAP kinases, and of the respiratory burst, in Ca^{2+}-depleted neutrophils

Ca^{2+}-depleted neutrophils were stimulated at 37 °C under agitation with ConA alone (**a**) (300 μg/ml) or ConA (300 μg/ml) followed by FMLP (5 μM) (**b**) as described in Figure 4, for the indicated times. Samples were subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine mAb 4G10. Only the tyrosine phosphorylation of p75 and of the MAP kinases are shown. Oxygen consumption was also determined in parallel assays (numbers indicate the time points in minutes at which the sampling was made). The data are from one experiment representative of five.

absence of Ca^{2+} changes and production of second messengers from phospholipid hydrolysis, in response to ConA and FMLP if these agents are simultaneously or sequentially added; and (3) remain able to respond with a respiratory burst to PMA.

We therefore characterized the tyrosine phosphorylation pattern and the stimulation of the respiratory burst in Ca^{2+}-depleted neutrophils challenged with FMLP, ConA and PMA alone, or with ConA and FMLP added together, in the presence or absence of 120 μM genistein. Figure 4 shows that the PMA-induced respiratory burst and protein tyrosine phosphorylation were similar to those induced in normal (i.e. not Ca^{2+}-depleted) neutrophils, with regard to the pattern and the extent of protein phosphorylation, as well as to the inhibition by genistein. This therefore indicates that the PKC-mediated mechanisms of activation of tyrosine kinases are Ca^{2+}-independent. Figure 4 also shows that FMLP or ConA alone failed to induce a respiratory burst in Ca^{2+}-depleted cells. Similarly, FMLP did not induce protein tyrosine phosphorylation in these cells. However, the ConA-induced protein tyrosine phosphorylation pattern was essentially the same in Ca^{2+}-depleted neutrophils, relative to normal cells (see Figures 1 and 2). Accordingly, genistein profoundly inhibited the ConA-induced tyrosine phosphorylation of p45 MAPK and p75, as observed in normal cells (see Figure 2).

As expected on the basis of our previous results [56], the sequential stimulation of Ca^{2+}-depleted neutrophils with ConA and FMLP resulted in the generation of a respiratory burst, which was paralleled by a protein tyrosine phosphorylation. Interestingly, this was accompanied by tyrosine phosphorylation of both p43 MAPK and p45 MAPK, while cell stimulation by these agents used as single stimuli had led to the phosphorylation of only one MAPK species, i.e. p43 MAPK with FMLP alone in normal neutrophils, and p45 MAPK with ConA alone in normal or Ca^{2+}-depleted neutrophils (Figures 2 and 4). Moreover, the sequential stimulation of Ca^{2+}-depleted cells with ConA and FMLP induced a hyperphosphorylation of p75. Under these conditions, genistein markedly inhibited p75, p45 MAPK and p43 MAPK phosphorylation, as well as the respiratory burst (Figure 4).

We next investigated whether the tyrosine phosphorylation of the MAP kinases and p75 were temporally correlated with the onset of the respiratory burst in Ca^{2+}-depleted neutrophils stimulated with ConA and/or FMLP. Figure 5 shows that when ConA was used as a single stimulus, tyrosine phosphorylation of p45 MAPK and p75 was detectable at 0.5 min, and increased with time (up to 7 min) in the absence of respiratory burst. In comparison, when the cells were stimulated with FMLP following ConA exposure, the tyrosine phosphorylation of p75 was further increased, and that of p43 MAPK became detectable. Under these conditions, the respiratory burst was also restored. In contrast, the tyrosine phosphorylation of the 130, 100, 90, 85, and 68 kDa proteins was detectable after 0.5 min of ConA stimulation, but did not increase with time, regardless of whether FMLP was added as second agonist (results not shown).

In a final series of experiments, we investigated whether the tyrosine phosphorylation of the MAP kinases is correlated with their activation. For this purpose we have immunoprecipitated the MAP kinases from lysates of both normal and Ca^{2+}-depleted neutrophils with a specific antibody that recognizes the 43 kDa, 42 kDa, and 44 kDa MAP kinases encoded by the *erk-1* gene, *mapk* gene, and *mpk* gene respectively (erk1-CT, UBI). The enzymic activity of MAP kinases was determined by an immunocomplex kinase assay using MBP as substrate. Figure 6 shows that MBP was phosphorylated by MAP kinases in normal neutrophils stimulated with FMLP or ConA, that induced the tyrosine phosphorylation of p43 MAPK and p45 MAPK respectively (Figures 1, 2 and 3), and in Ca^{2+}-depleted neutrophils stimulated with ConA alone or ConA followed by FMLP, that caused the tyrosine phosphorylation of p45 MAPK and both p43 MAPK and p45 MAPK respectively (Figures 4 and 5). On the contrary FMLP alone, which in Ca^{2+}-depleted neutrophils did

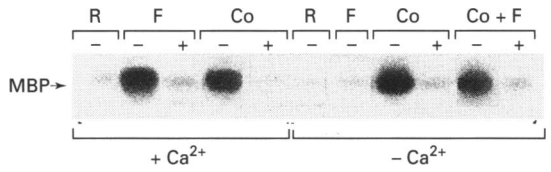

Figure 6 Activation of MAP kinases in normal and Ca^{2+}-depleted neutrophils

MAP kinases were immunoprecipitated with specific antibody from 700 μg of lysates of normal (+ Ca^{2+}) and Ca^{2+}-depleted (– Ca^{2+}) neutrophils stimulated with FMLP (F), ConA (Co) or ConA followed by FMLP (Co + F) at the same doses and for the same times as in Figures 1 and 4, in the presence (+) or absence (–) of 120 μM genistein. Immunocomplexes were collected and incubated with [γ-^{32}P]ATP and myelin basic protein (MBP) as substrate. The MAP kinase assays were analysed by electrophoresis on a SDS/14% polyacrylamide gel and exposure of the dried gel to X-ray film. MBP is indicated by an arrow. Abbreviations: R, resting neutrophils. The data are from one experiment representative of three.

not induce the tyrosine phosphorylation of p43 MAPK (Figure 4), was not able to activate MAP kinases in these cells (Figure 6). Figure 6 also shows that pretreatment of neutrophils with 120 μM genistein, which suppressed the tyrosine phosphorylation of MAP kinases induced by all the stimulants (Figures 2 and 4), also inhibited the MAP kinase activity induced by FMLP or ConA in normal neutrophils and by ConA alone or ConA followed by FMLP in Ca^{2+}-depleted neutrophils. These results indicate that the tyrosine phosphorylation of p43 MAPK and p45 MAPK correlates with their activation, and further proves that both these proteins are members of the MAP kinases family.

Concluding remarks

The results reported in this paper indicate that tyrosine kinases are involved in the mechanisms of activation of the neutrophil NADPH oxidase by FMLP and ConA. Among the proteins that are tyrosine phosphorylated following stimulation with these agonists, we have identified two MAP kinase isoforms of apparent molecular masses 43 and 45 kDa, as well as a 75 kDa protein that might represent raf-1. The data presented indicate that these three proteins could play a prominent role in the onset of the respiratory burst. In fact, (1) the time course and the extent of tyrosine phosphorylation of p43 MAPK and p75 induced by FMLP, and of p45 MAPK and p75 induced by ConA, closely correlated with those of the respiratory burst induced by the same agonists; and (2) the tyrosine kinase inhibitor genistein inhibited both the respiratory burst and tyrosine phosphorylation of p43 MAPK, p45 MAPK and p75 induced by ConA and FMLP.

It can be postulated, on the basis of the findings presented by others, that tyrosine kinases are involved in activation of phospholipases D [23–27] and A2 [35,58], that the phosphorylation of p75 (raf-1 ?), p43 MAPK and/or p45 MAPK by ConA and FMLP is involved in the activation of NADPH oxidase through the activation of phospholipase A2 and/or D.

The results of the experiments in Ca^{2+}-depleted neutrophils allow us to better understand some properties of the above sequence of reactions. The fact that in Ca^{2+}-depleted neutrophils ConA, when added alone, is unable to activate the NADPH oxidase (Figures 4 and 5) demonstrates that the signalling sequence, tyrosine phosphorylations, stimulation of phospholipase A2 and/or phospholipase D, and activation of NADPH oxidase, are Ca^{2+}-dependent. The finding that in conditions of Ca^{2+}-depletion, where we have previously shown that phospholipases A2 and D are not stimulated [56], ConA was able to induce the phosphorylation of p45 MAPK and p75 as in normal neutrophils, suggests that with this agonist, the Ca^{2+}-dependent step of the above sequence of activation of NADPH oxidase is the stimulation of phospholipase A2 and/or D, while the tyrosine phosphorylation of p45 MAPK and p75 is Ca^{2+}-independent. Interestingly, in spite of the lack of stimulation of these phospholipases, the activation of NADPH oxidase can be achieved in Ca^{2+}-depleted neutrophils also by the addition to ConA-treated cells of a second agonist, FMLP, which, as with ConA, was ineffective when added alone. The activation of NADPH oxidase resulting from the second stimulus coincided with the appearance of the phosphorylation of p43 MAPK in addition to that of p45 MAPK. Thus, one can speculate that the activation of the respiratory burst in the absence of Ca^{2+} and of the activation of phospholipases A2 and/or D is possible when both p43 MAPK and p45 MAPK are tyrosine phosphorylated. If this is the case, the tyrosine phosphorylations of p45 MAPK and p75 are unable to activate the NADPH oxidase in the absence of the stimulation of phospholipases A2 and D, but the phosphorylation of p43 MAPK could substitute for the functions of phospholipase A2 and/or of phospholipase D. It remains to be elucidated the mechanism(s) by which p43 MAPK can substitute for phospholipase A2 or phospholipase D so that the contemporaneous phosphorylation of the two MAP kinases can activate the NADPH oxidase in the absence of calcium and stimulation of phospholipid hydrolysis.

In contrast with what happens with ConA, when FMLP is the agonist the stimulation of the tyrosine kinase responsible for the phosphorylation of p75 and p43 MAPK is coupled to an upstream Ca^{2+}-dependent mechanism. This Ca^{2+}-dependent mechanism for the phosphorylation of p75 and p43 MAPK can be substituted by the previous phosphorylation of both p75 and p45 MAPK, as occurs when FMLP is added to Ca^{2+}-depleted neutrophils pretreated with ConA. The mechanism by which a previous phosphorylation of p75 and/or p45 MAPK allows p43 MAPK to be phosphorylated independently of calcium remains to be investigated.

We wish to thank Dr. P. McDonald for the critical reading of this manuscript. This work was supported by grants from Consiglio Nazionale delle Ricerche (contributo no. 93.04629.CT04), from M.U.R.S.T. (Fondi 40%), and Associazione Italiana Ricerca sul Cancro (A.I.R.C.).

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Received 9 March 1994/20 June 1994; accepted 24 June 1994