Protein tyrosine phosphorylation in rabbit peritoneal neutrophils

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Protein tyrosine phosphorylation in rabbit peritoneal neutrophils was examined by immunoblotting with antibodies specific for phosphotyrosine. Stimulation of the neutrophils with chemotactic factor fMet-Leu-Phe (10 nM) caused rapid increases of tyrosine phosphorylation of several proteins with apparent molecular masses of (Group A) 54–58 kDa and 100–125 kDa and (Group B) 36–41 kDa. Stimulation of Group A proteins was observed by fMet-Leu-Phe (10 nM, maximum at 20 s) and A23187 (1 μ M, 1 min). Stimulation of Group B proteins was observed by fMet-Leu-Phe (ED₅₀ 0.15 nM, 1 min), leukotriene B₄ (ED₅₀ 0.15 nM, 1 min), phorbol 12-myristate 13-acetate (PMA) (ED₅₀ 25 ng/ml, 10 min) and partially by ionophore A23187 (1 μ M, 1 min). Pretreatment of the cell with the protein kinase inhibitor H-7 (25 μ M, 5 min) and PMA (0.1 μ g/ml, 3 min) partially inhibited the fMet-Leu-Phe effect. However, pretreatment of the cells with quin 2/AM (20 μ M, 10 min) completely inhibited the fMet-Leu-Phe effect. The results indicate that rapid regulation of tyrosine phosphorylation is an early event occurring in stimulated neutrophils. Furthermore the effect of fMet-Leu-Phe on tyrosine phosphorylation may require Ca²⁺ mobilization and may partially require the activity of H-7-sensitive protein kinases.

INTRODUCTION

Neutrophils undergo diverse responses such as chemotaxis, degranulation and superoxide generation upon stimulation with various chemotactic factors such as fMet-Leu-Phe [1,2]. In fMet-Leu-Phe-stimulated neutrophils, increases in the levels of Ca²⁺ and diacylglycerol have been observed and linked to the activation of protein kinase C [3]. The tumour promoter phorbol 12-myristate 13-acetate (PMA), an analogue of diacylglycerol, can also activate the neutrophil, probably through the activation of protein kinase C [4]. However, an inhibitor of protein kinase C and cyclic nucleotide-dependent protein kinase, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7), does not inhibit most of the responses initiated by fMet-Leu-Phe stimulation [5]. This raises the question of whether other protein kinases resistant to H-7 may be involved in signal transduction in fMet-Leu-Phe-stimulated neutrophils.

We previously reported that fMet-Leu-Phe stimulates an H-7-resistant histone H4 kinase in rabbit peritoneal neutrophils [6]. Recently, we observed that protein tyrosine kinase activity in membranes of rabbit neutrophils is also resistant to H-7 inhibition (see below). Tyrosine kinase activity in human and rabbit neutrophils has been observed [7–9]. In the present paper, we studied the regulation of tyrosine phosphorylation in stimulated neutrophils. Preliminary results of this work have been reported [10].

EXPERIMENTAL

Materials

Leukotriene B₄ (LTB₄) was obtained from Biomol Research Lab (Philadelphia, PA, U.S.A.); cytochalasin B (CB) and diisopropyl fluorophosphate (DFP) were from Aldrich; Hepes, PMA, fMet-Leu-Phe, ATP, A23187, NNN'-tetrakis-(2-pyridylmethyl)ethylenediamine and quin 2/AM were obtained from Sigma; ¹²⁵I-Protein A (NEX-146L) was from New England

Nuclear; H-7 was obtained from Seikagaku America Inc. (St. Petersburg, FL, U.S.A.) Antibodies against phosphotyrosine (1 mg/ml) were prepared from rabbits and characterized as described in [11]. It was used at a 1:1000 dilution in immunoblotting experiments. The antibody solutions were used for up to 5 times for immunoblotting experiments.

The isolation of rabbit peritoneal neutrophils and preparation of the membrane fraction from the neutrophils were described in [10]. Cells were suspended in Hanks buffer (137 mM-NaCl, 5 mM-KCl, 0.7 M-KH₂PO₄, 1 mM-CaCl₂, 10 mM-Hepes, 17 mM-NaHCO₃, pH 7.2) containing 1 mM-DFP at 37 °C for 5 min before use.

Stimulation of neutrophils and immunoblotting of neutrophil proteins

Neutrophils (5 × 10⁷ cells/ml) were stimulated with various concentrations of fMet-Leu-Phe, LTB₄, A23187 or PMA in a final volume of 0.1 ml. At indicated time points, samples were mixed with 50 μ l of SDS-stopping solution (9% SDS, 6% β -mercaptoethanol, 10% glycerol and a trace amount of Bromophenol Blue dye in 0.196 M-Tris/HCl, pH 6.7) followed by SDS/PAGE analysis (6%-acrylamide gel). The gels were then subjected to immunoblotting experiments using the antibodies against phosphotyrosine as described previously [10]. Antibodies bound to the nitrocellulose papers were detected by ¹²⁵I-Protein A and autoradiography [10,12]. Protein standards (SDS-Blue) obtained from Sigma were used to estimate the molecular masses of the neutrophil proteins.

Endogenous phosphorylation of membrane fraction

Neutrophil membranes (1 mg of protein/ml) in 10 mM-Hepes/1 mM-EGTA/25 mM-MgCl₂ were phosphorylated at room temperature by addition of 1 mM of unlabelled ATP or [γ -³²P]ATP (10³ c.p.m./pmol) (final volume 100 μ l) for various time points in the presence or absence of H-7 (100 μ M) or quin 2/AM (100 μ M). The reaction was stopped by the addition of 50 μ l of

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; DFP, di-isopropyl fluorophosphate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; LTB₄, leukotriene B₄; CB, cytochalasin B; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid.

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SDS-stopping solution. Controls were treated similarly but without addition of ATP. For membranes which had been phosphorylated with unlabelled ATP, samples were analysed by SDS/PAGE, followed by immunoblotting with antibodies against phosphotyrosine. For membranes which had been labelled with [32 P]ATP, samples were analysed by SDS/PAGE and autoradiography, and phosphoamino acid analysis of labelled protein bands was done as described previously [6,10].

Other methods

Protein concentration was determined as described in ref. [13] with BSA as the standard. Densitometric scans of the autoradiographs were performed with a Hoefer GS 300 densitometer.

RESULTS

Initial attempts to study protein tyrosine phosphorylation in neutrophils by using 32 P-labelled cells were unsuccessful, owing to the low specific radioactivity of 32 P incorporated into neutrophil proteins and the low level of [32 P]phosphotyrosine [14]. Immunoblotting using antibodies prepared against phosphotyrosine has been widely used to study the changes in tyrosine phosphorylation in intact cells [15]. We have also used this method to study the effect of fMet-Leu-Phe on the tyrosine phosphorylation of neutrophils. The specificity of the anti-phosphotyrosine antibody has been studied. The antibodies recognize phosphotyrosine but not phosphoserine or phosphothreonine [II]; C.-K. Huang, unpublished work).

Fig. 1. Autoradiograph showing tyrosine phosphorylation of membrane proteins and the effect of H-7 and quin 2/AM on the tyrosine phosphorylation

Neutrophil membranes (100 μ l of 1 mg/ml) were phosphorylated with MgCl_2 (25 mM) and ATP (1 mM) for 20 s (lanes 2, 5, 8), 40 s (lanes 3, 6, 9) and 60 s (lanes 4, 7) and then stopped by SDS-stopping solution (50 μ l). Lane 1: control, membranes without MgATP treatment. Lanes 2-4: membranes treated with MgATP. Lanes 5-7: membrane treated as lanes 2-4 in the presence of H-7 (100 μ M). Lanes 8 and 9: membrane treated as lanes 2 and 3 in the presence of quin 2/AM (100 μ M). Proteins were analysed by SDS/PAGE, and immunoblotted with anti-phosphotyrosine antibodies and 125 I-Protein A as described in the text.

Preliminary studies using various subcellular fractions indicated that protein tyrosine kinase activity was most enriched in the membrane fraction of neutrophils. The antibodies were used to monitor the tyrosine phosphorylation of membrane proteins. As shown in Fig. 1, the membrane fraction contained very little phosphotyrosine. Addition of unlabelled ATP to the membrane increased the amount of phosphotyrosine in several proteins, including a major protein of 56 kDa (pp56). pp56 was previously identified as pp62 [10]. It is recognized by anti-*src*-peptide antibodies [10,12], and is most likely a *src*-like tyrosine kinase. A similar protein was also found to be tyrosine-

Fig. 2. Effect of various concentrations of fMet-Leu-Phe on the tyrosine phosphorylation of neutrophil proteins

Neutrophils were treated with either Hanks buffer or fMet-Leu-Phe (0.1–10 nM). At various times, samples of the cells (100 μ l each) were withdrawn from the solution and mixed with SDS-stopping solution (50 μ l). Proteins were analysed by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies and 125 I-Protein A, followed by autoradiography as described in the text. Densitometric scans of the autoradiographs shown in (a): A, control cells; B, fMet-Leu-Phe (10 nM, 20 s)-treated cells; C, fMet-Leu-Phe (10 nM, 60 s)-treated cells. Densitometric scans of the autoradiographs shown in (b): A, control cells; B, fMet-Leu-Phe (0.2 nM, 60 s)-treated cells; C, fMet-Leu-Phe (1 nM, 60 s)-treated cells. Numbers above peaks are in kDa.

Fig. 3. Effect of various stimuli on the tyrosine phosphorylation of neutrophil proteins

Cells were stimulated with fMet-Leu-Phe, A23187, LTB₄, CB or PMA. Samples (100 μ l) of the cells were withdrawn from the reaction and stopped by SDS-stopping solution (50 μ l). Proteins were analysed by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies and ¹²⁵I-Protein A, followed by autoradiography as described in the text. Densitometric scans of the autoradiographs shown in (a): A, control cells; B, fMet-Leu-Phe (10 nM, 60 s)-treated cells; C, A23187 (1 μ M, 60 s)-treated cells; D, LTB₄ (30 nM, 60 s)-treated cells. Densitometric scans of the autoradiographs shown in (b): A, control cells; B, CB (5 μ g/ml, 1 min)-treated cells; C, PMA (0.1 μ g/ml, 5 min)-treated cells. Numbers above the peaks are in kDa.

Fig. 4. Dose/response curves of the tyrosine phosphorylation of pp41 induced by fMet-Leu-Phe (10 pM–10 nM, 60 s), LTB₄ (3 pM–30 nM, 60 s) or PMA (12.5–100 ng/ml, 10 min)

The reaction was stopped by SDS-stop solution and the proteins were analysed by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies and ¹²⁵I-Protein A. The amount of tyrosine phosphorylation on p41 was analysed by densitometric scanning of the p41 bands on the autoradiographs. Average values of three different experiments are shown, taking the maximum level of phosphorylation of p41 induced by each stimulus as 100 %.

phosphorylated in intact neutrophils (see below). Addition of H-7 (100 μ M) or quin 2/AM (100 μ M) to the membrane did not inhibit the rate of phosphorylation of membrane proteins. The result indicated that neutrophil membranes contained tyrosine kinase activity, and that the phosphorylation reaction carried out by the kinase(s) was not sensitive to the protein kinase inhibitor H-7 or the intracellular Ca²⁺ chelator quin 2/AM (100 μ M). In addition, membrane tyrosine kinase activity was detectable with poly(Glu,Tyr) as the substrate [9]. This also was not inhibited by H-7 (result not shown).

The phosphoamino acid residues in pp56 were analysed by phosphorylating the proteins with [γ -³²P]ATP followed by phosphoamino acid analysis as described previously [6,10]. It was found that [³²P]phosphotyrosine was the only phosphoamino acid detected within 20 s of phosphorylation. With longer time (> 40 s) of phosphorylation, both [³²P]phosphotyrosine and [³²P]phosphoserine were detected (results not shown).

The effect of fMet-Leu-Phe on the protein tyrosine phosphorylation of intact neutrophils was studied (Fig. 2). At a high concentration of fMet-Leu-Phe (10 nM), stimulation of tyrosine phosphorylation of proteins with molecular masses of 56 kDa, 58 kDa and 125 kDa (Group A) was observed within 20 s of stimulation (Fig. 2a). The result confirmed what we reported previously [10] on the effect of fMet-Leu-Phe (10 nM, 20 s) on two proteins with molecular masses of 125 kDa and

Fig. 5. Autoradiograph showing the effect of various pretreatments of neutrophils on fMet-Leu-Phe-stimulated protein tyrosine phosphorylation

Cells were pretreated with Hanks buffer (lanes 1, 2), H-7 (25 μ M, 5 min; lanes 3, 4), quin 2/AM (10 μ M, 10 min; lanes 5, 6), PMA (0.1 μ g/ml, 3 min; lanes 7, 8) and CB (5 μ g/ml, 5 min; lanes 9, 10). At the end of preincubation, the samples were treated with Hanks buffer (lanes 1, 3, 5, 7, 9) or fMet-Leu-Phe (FMLP; 10 nM, 40 s; lanes 2, 4, 6, 8, 10). The reaction was stopped by adding SDS-stop solution (50 μ l). Proteins were analysed by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies and 125 I-Protein A. The two lanes within each labelled lane are duplicate samples.

62 kDa. In the present study longer gels were used for electrophoresis, and the proteins in the 62 kDa region have now been resolved into three bands, with apparent molecular masses of 58 kDa, 56 kDa and 54 kDa. The protein of 56 kDa (pp56) was the dominant phosphoprotein. At low concentrations of fMet-Leu-Phe (1 nM) the effect of fMet-Leu-Phe on pp56 required a longer incubation time (40–60 s) to observe a stimulatory effect (Fig. 2b). In all cases the maximum effect of fMet-Leu-Phe on pp56 was about double the phosphorylation of control cells as determined by densitometric scanning. A group of bands with molecular masses of 36 kDa, 38 kDa and 41 kDa (pp36, pp38, pp41; Group B) was also detectable, which had a latent period of 20 s for phosphorylation and took about 60–80 s to reach maximum phosphorylation. The effect is strong; almost a 10-fold increase of tyrosine phosphorylation was observed in fMet-Leu-Phe-treated cells compared with control. The relative intensity of pp36–pp41 to pp56 varied from preparation to preparation, and the pp36 band was not always detectable in all the experiments (cf. Figs. 2b and 3a). No other low-molecular-mass phosphoproteins were observed when gels with higher acrylamide concentrations were used (result not shown). Tyrosine phosphorylation of a protein of 100 kDa was also stimulated by fMet-Leu-Phe in some experiments (Fig. 3a).

The effects of other stimuli (LTB₄, A23187, PMA and CB) on the tyrosine phosphorylation of neutrophil proteins were studied (Fig. 3). Densitometric scans were prepared in order to compare the effects of these stimuli with those induced by fMet-Leu-Phe (10 nM, 1 min) (Figs. 3a and 3b). LTB₄ (30 nM, 1 min) stimulated tyrosine phosphorylation with a pattern similar to that induced by fMet-Leu-Phe. However, the maximal effect of LTB₄ on pp41 and pp38 was only 51 % and 73 % of that produced by fMet-Leu-Phe respectively. A23187 (1 μ M, 1 min), stimulated the tyrosine phosphorylation of Group A proteins and pp38 (45 % of that induced by fMet-Leu-Phe), but stimulated only weakly pp41 phosphorylation (7 % of that induced by fMet-Leu-Phe). PMA (0.1 μ g/ml, 5 min or longer) stimulated tyrosine phosphorylation of Group B proteins (pp41, 36 % of that produced by fMet-Leu-Phe; pp38, 46 % of that produced by fMet-Leu-Phe), but not Group A proteins. CB (5 μ g/ml, 1 min) stimulated the phosphorylation of both Group A and Group B proteins (pp41, 47 % of that produced by fMet-Leu-Phe; pp38, 68 % of that produced by fMet-Leu-Phe) (Fig. 3b). The effect of CB was transient, being maximum within 1 min and then decaying slowly thereafter. Similar results were observed with dihydrocytochalasin B (not shown). Although CB stimulated tyrosine phosphorylation,

Table 1. Effects of various pretreatments on fMet-Leu-Phe-stimulated tyrosine phosphorylation of pp41

Cells (250 μ l; 5×10^7 cells/ml) in Hanks buffer were pretreated with various agents (PMA, H-7, quin 2/AM or CB) as indicated, and then 100 μ l portions of the cells were treated with either Hanks buffer (control) or fMet-Leu-Phe (10 nM, 40 s). The reactions were stopped with SDS-stopping solution (50 μ l). Proteins were analysed by SDS/PAGE, and immunoblotted with anti-phosphotyrosine antibodies and 125 I-Protein A. The relative levels of tyrosine phosphorylation of pp41 were estimated by densitometric scanning of the autoradiographs. Results shown are the average of two experiments with duplicate samples. The level of tyrosine phosphorylation of pp41 in fMet-Leu-Phe-stimulated cells without any pretreatments was defined as 100.

Pretreatment	Relative phosphorylation	
	Control	+fMet-Leu-Phe
A	10 $\pm$ 1	100
B PMA (0.1 μ g/ml, 3 min)	15 $\pm$ 5	42 $\pm$ 3
C H-7 (25 μ M, 5 min)	8 $\pm$ 1	59 $\pm$ 7
D Quin-2/AM (10 μ M, 10 min)	2 $\pm$ 2	15 $\pm$ 3
E CB (5 μ g/ml, 5 min)	26 $\pm$ 2	43 $\pm$ 4

prolonged pretreatment of the cells with CB (5 μ g/ml, 5 min) resulted in the inhibition of fMet-Leu-Phe-stimulated tyrosine phosphorylation of pp41 (Fig. 5 and Table 1).

The large increase in tyrosine phosphorylation of pp41 induced by various stimuli allowed us to study the dose/response curve of pp41 phosphorylation. The ED₅₀ values for fMet-Leu-Phe, LTB₄ and PMA were 0.15 nM, 0.15 nM and 25 ng/ml respectively (Fig. 4). The ED₅₀ for PMA is close to that for protein kinase C activation [16]. The ED₅₀ values for fMet-Leu-Phe and LTB₄ were close to those for Ca²⁺ mobilization induced by fMet-Leu-Phe and LTB₄ [17].

To test whether Ca²⁺ mobilization is required for fMet-Leu-Phe-stimulated tyrosine phosphorylation of pp41, cells were pretreated with PMA (0.1 μ g/ml, 3 min) (Fig. 5). Such pretreatment has been shown to inhibit Ca²⁺ mobilization induced by fMet-Leu-Phe [18]. PMA pretreatment inhibited 50 % of the effect of fMet-Leu-Phe on pp41 phosphorylation. Ca²⁺ mobilization can also be inhibited by pre-loading the cells with quin 2/AM [19]. Quin 2/AM pretreatment inhibited almost completely the effect of fMet-Leu-Phe on pp41 phosphorylation

(Fig. 5 and Table 1). Pretreatment of the cells with a heavy-metal-ion chelator, *NNN'*-tetrakis-(2-pyridylmethyl)ethylene-diamine (10 μ M, 10 min), did not inhibit the effect of fMet-Leu-Phe (result not shown). Quin 2/AM had very little effect on the tyrosine phosphorylation of isolated membranes (Fig. 1). Thus the effect of quin 2/AM in intact cells is most likely due to its chelation of intracellular Ca^{2+} . More recently, we have observed results similar to those of quin 2/AM treatment in neutrophils treated with bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid acetoxymethyl ester (BAPTA/AM) so as to inhibit the Ca^{2+} mobilization induced by fMet-Leu-Phe (results not shown). Prolonged incubation of cells in buffers containing EGTA (10 mM, 10 min) also inhibited the fMet-Leu-Phe stimulation of tyrosine phosphorylation (results not shown). The effect of H-7 on fMet-Leu-Phe stimulation was also tested (Fig. 5 and Table 1). H-7 inhibited partially (40%) the effect of fMet-Leu-Phe on phosphorylation of p41, but had very little effect on the tyrosine phosphorylation of isolated membranes (Fig. 1). The effect of H-7 on tyrosine phosphorylation in intact cells may be due to its inhibitory effect on protein phosphorylation mediated by protein kinase C [5] or other H-7-sensitive kinases [20,21].

DISCUSSION

Phosphorylation of proteins on tyrosine residues generally has been associated with the regulation of cell growth and transformation [24]. However, the presence of high tyrosine kinase activity in non-proliferative tissues and cells [25] suggests that protein tyrosine phosphorylation also may be involved in other regulatory processes such as signal transduction. The results in the present paper clearly demonstrate that rapid regulation of tyrosine phosphorylation is an early event occurring in stimulated neutrophils.

In this work, we observed that tyrosine phosphorylation of two groups of proteins (A and B) were regulated differently in fMet-Leu-Phe-stimulated neutrophils. fMet-Leu-Phe (10 nM, 20 s) transiently stimulated the tyrosine phosphorylation of Group A proteins (56–58 kDa and 100–125 kDa), whereas both high and low doses of fMet-Leu-Phe (0.1 nM and higher) stimulated the phosphorylation of Group B proteins (36–41 kDa). The phosphorylation of Group B proteins occurred with a 20 s delay, but was sustained for up to 2 min. LTB_4 , a weaker stimulus than fMet-Leu-Phe in activating various neutrophil responses [26], produced a weaker effect on tyrosine phosphorylation. The Ca^{2+} ionophore A23187 stimulates the tyrosine phosphorylation of Group A proteins and p38, but only weakly that of p41 of Group B proteins. In contrast, the protein kinase C activator PMA was able only to stimulate the Group B protein. A23187 or PMA alone was not as potent as fMet-Leu-Phe. That a combination of both Ca^{2+} increase and protein kinase C activation may be required for full stimulation of tyrosine phosphorylation is supported by the following results. (a) The increase in intracellular Ca^{2+} and tyrosine phosphorylation induced by fMet-Leu-Phe can be inhibited by the pretreatments of cells with quin 2/AM and PMA (Table 1; [18]). (b) Pretreatment of the cells with H-7, an inhibitor of protein kinase C, cyclic-AMP-dependent protein kinase [27] and Ca^{2+} /calmodulin-dependent protein kinase [28], inhibited the tyrosine phosphorylation induced by fMet-Leu-Phe. (c) The ED_{50} values for p41 phosphorylation induced by PMA, fMet-Leu-Phe and LTB_4 have been determined (Fig. 4): that for PMA is close to the ED_{50} for protein kinase C activation [16], and those for fMet-Leu-Phe and LTB_4 were close to the ED_{50} values for Ca^{2+} mobilization induced by fMet-Leu-Phe and LTB_4 [17]. (d) Under certain conditions, A23187 and PMA can stimulate tyrosine phosphorylation synergistically (see below). (e) Other data in-

dicate that a pertussis-toxin-sensitive GTP-binding protein may be involved in fMet-Leu-Phe-stimulated tyrosine phosphorylation [10,29]. This would fit with a requirement for both Ca^{2+} mobilization and protein kinase C activation.

The levels of tyrosine phosphorylation of neutrophil proteins may be regulated by the activities of protein kinases, phosphoprotein phosphatases or changes in the conformation of the substrates. In neutrophils, various types of protein tyrosine kinase such as *c-fgr* [30], *c-fes/fps* [31], *c-hck* [32] and *c-src* [33] have been found. How these kinases are regulated in neutrophil remains to be studied. Recent work suggested that *c-fgr* in human neutrophils may be translocated from granules to the plasma membrane upon degranulation [30]. The C-terminal portion of *c-hck* may regulate its kinase activity [34]. With respect to phosphatases, a membrane-associated phosphotyrosine phosphatase activity has been observed in human neutrophils [5]. CD45, a phosphotyrosine phosphatase, is mobilized from specific and/or tertiary granules to the plasma-membrane fraction when neutrophils are stimulated by fMet-Leu-Phe or CB [35]. *In vitro*, the specific activities of phosphotyrosine phosphatases are much higher than those of tyrosine kinases [36]. It has been suggested that the level of tyrosine phosphorylation *in vivo* may be determined mostly by the regulation of phosphatase activity [36–38]. If this is true, mobilization of phosphatase during degranulation may serve as a mechanism for the dephosphorylation of Group A proteins, which appear to localize in the plasma membrane fraction (Fig. 1). Recently, by using the anion-channel blocker DIDS [39] to inhibit the degranulation of neutrophils, we were able to demonstrate a synergistic effect of PMA and A23187 in stimulating tyrosine phosphorylation of Group A proteins (results not shown). Without the DIDS treatment, the synergistic effect of A23187 and PMA was difficult to detect. This could be due to the rapid degranulation induced by A23187 and PMA.

Phosphotyrosine-containing proteins (p130, p70, p60) had been found in the detergent-insoluble cytoskeleton fraction of Rous-sarcoma-virus-transformed fibroblasts [40]. Similar proteins have been observed at the focal contact at the termini of actin-containing filament bundles in normal fibroblasts and at the intercellular junctions in epithelial cells [41]. We have also observed most of the tyrosine-phosphorylated protein localized in the Triton-X-100-insoluble (cytoskeleton) fraction of neutrophils (results not shown). The stimulatory effect of CB on tyrosine phosphorylation may be due to its effect on disrupting the actin filaments, which could result in increasing interaction between tyrosine kinases and their substrates. Prolonged pretreatment of neutrophils with CB resulted in the inhibition of fMet-Leu-Phe-stimulated tyrosine phosphorylation of p41 (Fig. 5 and Table 1). This may be due to that CB pretreatment inducing mobilization of CD45 from granules to the membrane fraction [35] and thus increasing the phosphatase activity in the membrane. This hypothesis should be tested.

Recently, by using inhibitors for protein tyrosine kinases or phosphatases, several groups suggested that tyrosine phosphorylation may be important for superoxide production [38,42,43]. However, the possibility that these inhibitors may not be specific and may interfere directly with some components of the superoxide-generation system cannot be ruled out. Nevertheless, it is noteworthy that recent gene cloning of two cytosolic factors (p46 and p65) involved in superoxide production revealed that both factors contained amino acid sequences similar to those of *src*-like protein tyrosine kinases [44]. The possible role of protein tyrosine kinases in superoxide production merits further study.

In the present work, rabbit peritoneal neutrophils were used. Compared with human blood neutrophils, rabbit peritoneal neutrophils are more sensitive to fMet-Leu-Phe and A23187 and

secrete more of the intracellular granular contents [45,46]. The peritoneal neutrophils may be considered as pre-activated cells. However, the results of many experiments using rabbit peritoneal neutrophils were found to be valid when human peripheral-blood neutrophils were used instead [1]. Preliminary studies indicate that patterns of protein tyrosine phosphorylation stimulated either by fMet-Leu-Phe or PMA in human peripheral-blood neutrophils [42,47] or by guanosine 5'-[γ -thio]triphosphate in permeabilized human neutrophils [29] are similar to what is described in this paper. Further work is required to demonstrate whether the regulation of tyrosine phosphorylation is identical in both peritoneal and blood neutrophils.

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