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Possible Involvement of Optimally Phosphorylated L-Plastin in Activation of Superoxide-Generating NADPH Oxidase

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The involvement of protein phosphatases in the activation of superoxide (O_2^-)- generating enzyme in human neutrophils was examined using calyculin A, an inhibitor of protein phosphatase type 1 and 2A. Calyculin A inhibited the phorbol myristate acetate (PMA)- and opsonized zymosan (OZ)-activated O_2^- generation by human neutrophils. This inhibitory effect of calyculin A on PMA-activated O_2^- generation was reversed by the addition of KT5926, a specific inhibitor of myosin light chain kinase and Ca^{2+}/calmodulin-dependent protein kinase II. These results suggest that the addition of calyculin A may cause hyperphosphorylation of some protein(s) that plays a crucial role in the PMA-dependent activation of O_2^- generating enzyme, and that this protein hyperphosphorylation may be evoked by a KT5926-sensitive kinase or its downstream kinase. Whereas two-dimensional analysis involving ^{32}P revealed that calyculin A caused the hyperphosphorylation of many proteins, KT5926 mainly reduced the calyculin A-induced hyperphosphorylation of a 67 kDa protein in activated neutrophils, suggesting that the hyperphosphorylation of the 67 kDa protein might inhibit the PMA-dependent activation of NADPH oxidase. The 67 kDa cytosolic protein was moderately phosphorylated on the addition of PMA. On the other hand, in the absence of calyculin A, KT5926 inhibited both PMA-induced O_2^- generation and phosphorylation of the 67 kDa protein. Amino acid sequence analysis of peptides derived from the 67 kDa protein revealed that the 67 kDa protein was identical to L-plastin, an actin-bundling protein. We conclude that optimally phosphorylated L-plastin may play some crucial role in the activation of NADPH oxidase.

Key words: human neutrophils; L-plastin; NADPH oxidase; phosphatase; phosphorylation.

Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; OZ, opsonized zymosan.

Neutrophils act as a first defense against invading bacteria or viruses, and are important mediators of the acute inflammatory response. Neutrophils recognize, ingest, and kill microorganisms in phagocytic vacuoles, releasing oxygen intermediates such as O_2^-, H_2O_2 and OH^-. Generation of O_2^- is catalyzed by NADPH oxidase, which is dormant in resting cells, and consists of membrane-bound and cytosolic components (*1*, *2*). The cytosolic components comprise a 47 kDa protein ($p47^{phox}$), a 67 kDa protein ($p67^{phox}$), a 40 kDa protein ($p40^{phox}$), and rac protein. $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are phosphorylated during the activation of NADPH oxidase (*1*, *3*, *4*). $p47^{phox}$ is phosphorylated by several protein kinases (*5–10*). Phosphorylation of $p47^{phox}$ is crucial for translocation of cytosolic components and assembly of the active NADPH oxidase (*11–13*).

On the other hand, many studies have indicated an important regulatory role for protein serine/threonine phosphatase in a wide variety of cellular functions (*14*, *15*). The involvement of serine/threonine phosphatase in the activation of NADPH oxidase has been investigated using phosphatase inhibitors. *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP)–activated O_2^- generation by human neutrophils was enhanced by okadaic acid and calyculin A, inhibitors of phosphatase type 1 and 2A (*16–19*), while phorbol myristate acetate (PMA)-induced O_2^- generation was inhibited by these phosphatase inhibitors (*18–20*). Inversely, these phosphatase inhibitors enhanced the O_2^- generation activated by suboptimal concentrations of PMA (*21*). These results suggest that the signal transduction pathway in the PMA-induced activation of NADPH oxidase cannot be explained simply by phosphorylation of $p47^{phox}$. One possible explanation is that the activation of NADPH oxidase requires a balance of phosphorylation and dephosphorylation of some proteins (*22–25*).

To further explore the roles of protein phosphorylation and dephosphorylation in the activation of NADPH oxidase, we examined the effects of calyculin A and various kinase inhibitors on the O_2^- generation by fMLP-, PMA-

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and OZ-activated neutrophils. In this study, we obtained evidence that optimal phosphorylation of L-plastin may be required for the PMA- and OZ-dependent activation of NADPH oxidase.

EXPERIMENTAL PROCEDURES

Reagents—Calyculin A, wortmannin and lysyl endopeptidase were purchased from Wako Pure Chemical Industries (Osaka). $^{32}P_i$ was obtained from DuPont NEN. Cytochrome *c* (Type VI), zymosan A and phorbol myristate acetate were obtained from Sigma, St. Louis. Opsonized zymosan was prepared by incubation with freshly prepared human serum at 37°C for 30 min. KT5926, K-252a, staurosporine and herbimycin A were purchased from Kyowa Medex (Tokyo). All other chemicals used were commercial preparations of the highest purity.

Preparation of Neutrophils—Human neutrophils were isolated from peripheral blood obtained from healthy volunteers by the dextran sedimentation method, as previously described (*26*). After lysis of the red cells, the cell suspension was sedimented and resuspended in HEPES-buffered saline (HBS), and then stood at 4°C for 1 h. After the upper layer had been aspirated off, the pellet was suspended in HBS. The isolated leukocyte fraction contained more than 95% of viable neutrophils, as judged on differential staining as previously reported (*26*).

Measurement of O_2^- Generation—The rate of O_2^- generation was measured at 37°C by recording the reduction of cytochrome c as the change in difference absorption between 550 and 540 nm (*27*) using a dual-beam spectrophotometer (Hitachi, Model 557, Tokyo). The assay mixture comprised 0.1 mM cytochrome *c*, 5 mM glucose and 1 $\times 10^6$ neutrophils in HBS. After the neutrophils had been preincubated at 37°C for 10 min with or without inhibitors, O_2^- generation was initiated by the addition of various stimulants, as indicated.

Protein Phosphorylation of Neutrophils—Analysis of the protein phosphorylation of neutrophils was performed according to a previous report (*28*) with slight modifications. Neutrophils were preloaded with $^{32}P_i$ at 30°C for 1 h. The ^{32}P-loaded cells were preincubated with or without inhibitors as above. After preincubation with inhibitors, the cells were stimulated with PMA at 37°C for 2 min. The reaction was terminated with chilled HBS containing 2 mM diisopropyl fluorophosphate, 20 μM leupeptin, 50 μM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride, 0.1 M NaF, 10 mM EDTA, 2 mM *N*-ethylmaleimide, 1 mM ammonium molybdate, 1 mM iodoacetate, and 1 mM benzamidine. After precipitation, the cells were suspended in an aliquot of a 0.25 M sucrose solution containing 10 mM Tris-HCl buffer (pH 7.4) and a mixture of protease inhibitors and phosphatase inhibitors. The cells were then disrupted by sonication at 0°C for 20 s. The sonicates were centrifuged at 500 ×*g* for 15 min at 4°C to remove whole cells and nuclei. The supernatants were then centrifuged at 100,000 ×*g* for 30 min at 4°C to obtain cytosolic and membrane fractions. After separation of the cytosol and membrane fractions, two-dimensional gel electrophoresis was performed. For the first dimension, nonequilibrium isoelectric focusing was performed in 4 × 100-mm cylindrical 4% polyacrylamide gels containing 8.5 M urea, 2% Nonidet P-40, 1.6% pH 3.5–9.5 Ampholines, and 0.4% pH 2.5–4.0 Ampholines. Electrophoresis in the second dimension was carried out by the Laemmli technique using 12.5% polyacrylamide gels. The gels were stained and autoradiographed with Kodak X-OMAT film. Densitometric analysis was performed with an ATTO densitometer (AE6900-2D, ATTO, Tokyo).

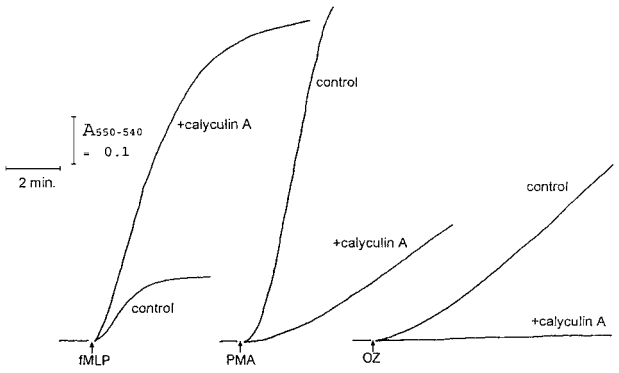

Fig. 1. **Effect of calyculin A on O_2^- generation by human neutrophils.** Human neutrophils were preincubated with or without 100 nM calyculin A at 37°C for 10 min. After preincubation, the cells were activated with 1 μM fMLP, 0.1 μg/ml PMA or 1.25 mg/ml of OZ. The rate of O_2^- release was determined by measuring the reduction of cytochrome *c*. These traces are representative of at least four experiments.

Protein Sequencing—The 67 kDa protein, derived from approximately 5×10^7 cells was excised from two-dimensional gels (25 gels). Then, *in gel*–proteinase digestion with lysyl endopeptidase was performed according to Kawasaki *et al*. (*29*). The resulting peptides were purified by reverse phase high performance liquid chromatography on a BioRad RP-318 column (250 × 4.6 mm). The purified peptides were applied to a gas phase peptide sequencer (PPSQ-10, Shimadzu, Kyoto). BLAST (GenomeNet) was used to search the SWISS-PROT protein database.

RESULTS

Effect of Calyculin A on O_2^- Generation by Human Neutrophils—At first, the effect of calyculin A on O_2^- generation by human neutrophils was examined. One hundred nM of calyculin A resulted in marked enhancement of fMLP-activated O_2^- generation, while PMA- or opsonized zymosan(OZ)-activated O_2^- generation was strongly inhibited by calyculin A (Fig. 1). We confirmed that the reduction of cytochrome c was inhibited by the addition of SOD (data not shown). These distinct effects of calyculin A were previously reported by several investigators (*16–19*). The enhancement of calyculin A on fMLP-induced O_2^- generation may be explained as follows; calyculin A inhibits the dephosphorylation of $p47^{phox}$, resulting in the maintenance of the phosphorylation state of $p47^{phox}$, which delays the termination of NADPH oxidase, while in the absence of calyculin A the phosphorylated $p47^{phox}$ induced by fMLP- was rapidly dephosphorylated within 3 min, as previously suggested by Badwey *et al*. (*30*). Calyculin A inhibited the O_2^- generation by PMA- and OZ-

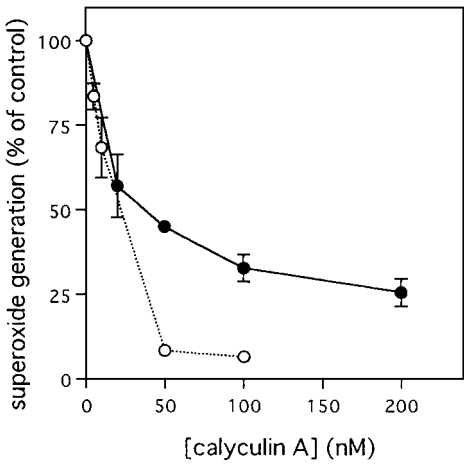

Fig. 2. **Effect of calyculin A on O_2^- generation by PMA- and OZ-activated human neutrophils.** Neutrophils were preincubated at 37°C for 10 min with various concentrations of calyculin A, and then activated with 0.1 μg/ml PMA (solid circles) or 1.25 mg/ml OZ (open circles). Each value was obtained by determination of the maximum velocity from cytochrome c reduction rate. The results are expressed as percentages of control values in the absence of an inhibitor. Data values represent the means ± SE for four experiments.

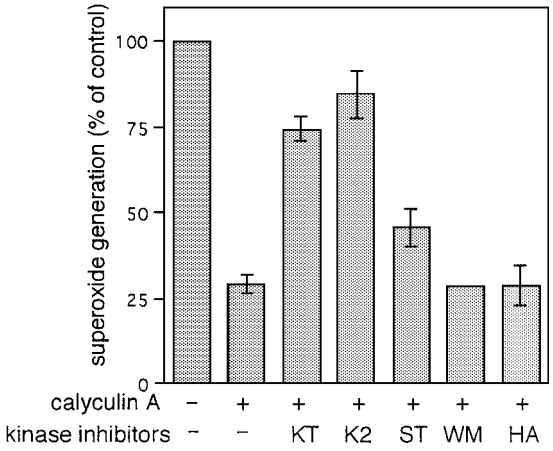

Fig. 3. **Antagonism of calyculin A-induced inhibition of O_2^- generation by kinase inhibitors.** After incubation with 100 nM calyculin A plus DMSO (–), 1 μM KT5926 (KT), 1 μM K-252a (K2), 50 nM staurosporine (ST), 10 μM wortmannin (WM), or 1.74 μM herbimycin A (HA) for 10 min, neutrophils were stimulated with 0.1 μg/ml PMA. Each value was obtained by determination of the maximum velocity from cytochrome c reduction rate. The results are expressed as percentages of control values in the absence of an inhibitor. Data columns represent the means ± SE for three to six experiments.

activated neutrophils in a dose-dependent manner (Fig. 2). In the absence of calyculin A, neutrophils generated 60.9 ± 2 .9 and 41.2 ± 7.9 (mean ± SE for 15 and 4 experiments, respectively) nmol O_2^-/min/10^7 cells in response to PMA and OZ, respectively. These results suggest that inhibition of the dephosphorylation of some protein(s) prevents the PMA- and OZ-induced activation of NADPH oxidase. Suzuki *et al*. reported that dephosphorylation of cofilin, an actin-binding protein, may be involved in the OZ-induced activation of NADPH oxidase (*22*). However, cofilin dephosphorylation did not occur during the PMA-induced activation of NADPH oxidase by PMA (*22*). Therefore, we examined the mechanism of the inhibitory effect of calyculin A on PMA-activated O_2^- generation.

Antagonistic Effects of Kinase Inhibitors on Calyculin A–Dependent Inhibition of O_2^- Generation by Neutrophils— Next, we attempted to antagonize the inhibitory effect of calyculin A with kinase inhibitors such as KT5926, K-252a, staurosporine, wortmannin and herbimycin A. KT5926 and K-252a effectively restored the calyculin A-induced inhibition of O_2^- generation by PMA-activated neutrophils (Fig. 3). Staurosporin partially restored the calyculin A-dependent inhibition. Wortmannin and tyrosine kinase inhibitor herbimycin A were not effective. Among these kinase inhibitors, which were effective as to antagonization of the inhibition by calyculin A, KT5926 is a specific myosin light chain kinase and Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, whereas K-252a exhibits a broad inhibitory spectrum (*31–33*). Therefore, we focused on the KT5926-dependent antagonization of the inhibitory effect of calyculin A. KT5926 also restored the calyculin A-induced inhibition of O_2^- generation by OZ-activated neutrophils (data not shown).

Whereas the inhibitory effect of calyculin A was reversed by KT5926 as an antagonist, in the absence of calyculin A, KT5926 inhibited the PMA-activated O_2^- generation in a dose-dependent manner. The inhibitory effect of 100 nM calyculin A on O_2^- generation was optimally reversed by the addition of 1 μM KT5926 (Fig. 4). On the other hand, when the effects of various concentrations of calyculin A on the PMA-activated O_2^- generation were examined, for which the concentration of KT5926 was fixed at 1 μM, O_2^- generating activity was maximum with 100 nM calyculin A (data not shown). Therefore, we hypothesized that there is some protein whose phosphorylation has optimal state for the activation of NADPH

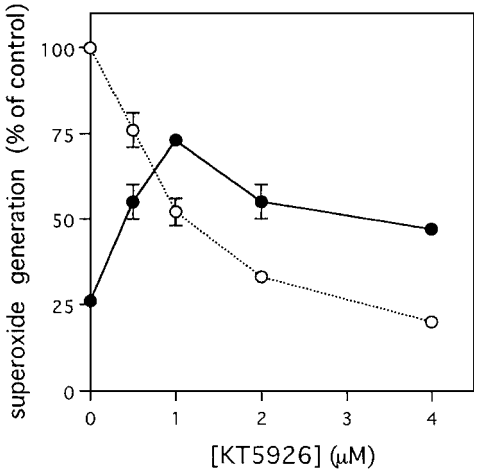

Fig. 4. **Effects of KT5926 and calyculin A on O_2^- generation by human neutrophils.** Neutrophils were treated with various concentrations of KT5926 in the presence (solid circles) or absence (open circles) of 100 nM calyculin A for 10 min, and then activated with 0.1 μg/ml PMA. Each value was obtained by determination of the maximum velocity from cytochrome c reduction rate. The results are expressed as percentages of control values in the absence of an inhibitor. Data values represent the means ± SE for three experiments.

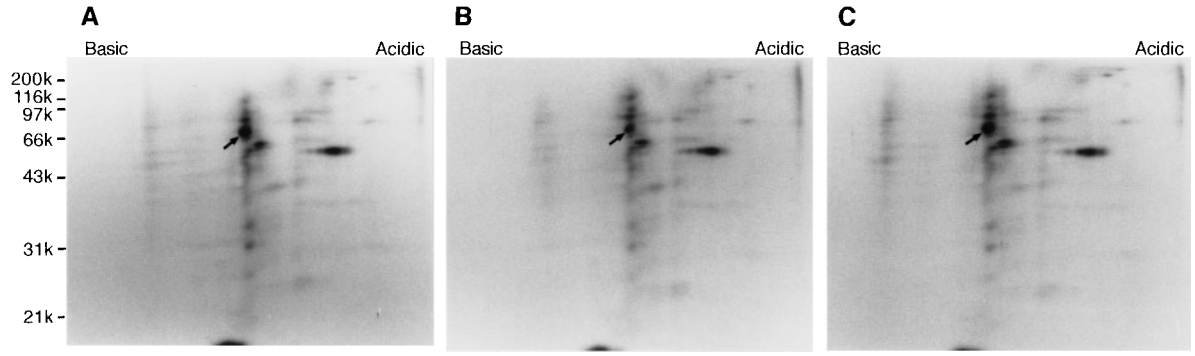

Fig. 5. **Reduction of hyperphosphorylation of the 67 kDa protein by KT5926.** ^{32}Pi-loaded neutrophils were incubated with 100 nM calyculin A (A), 100 nM calyculin A plus 1 μM KT5926 (B), or 100 nM calyculin A plus 10 μM wortmannin (C) for 10 min, and then activated with 0.1 μg/ml PMA at 37°C for 2 min as described under "EXPERIMENTAL PROCEDURES." A portion of the cytosol fraction was subjected to two-dimensional electrophoresis and autoradiography. The arrow in each photograph indicates the 67 kDa protein.

oxidase. In other words, such a protein(s) in an inactive form is phosphorylated to give rise to an active form and further hyperphosphorylated to provide another inactive form.

Effect of Calyculin A on Protein Phosphorylation—Next, we identified the protein(s) that was hyperphosphorylated in the presence of calyculin A and whose hyperphosphorylation was inhibited on the addition of KT5926. In the presence of calyculin A, many proteins in the cytosol and membrane fractions prepared from resting neutrophils were heavily phosphorylated (data not shown). It is well-known that wortmannin is an inhibitor of not only PI3 kinase but also myosin light chain kinase (*34*, *35*). Therefore, it is expected that the target kinases of calyculin A and wortmannin may overlap. Since KT5926 but not wortmannin restored the calyculin A-dependent inhibition of O_2^- generating activity, it may be useful to analyze the phosphorylation state in the presence of KT5926 as compared to that in the presence of wortmannin.

It should be noted that the calyculin A–induced hyperphosphorylation of cytosolic 67 kDa protein (Fig. 5, arrows) in PMA-activated neutrophils was markedly reduced by KT5926. Several proteins (31 kDa and 34 kDa at similar positions corresponding to the first dimension position of the 67 kDa protein) were slightly dephosphorylated on the addition of KT5926 in addition to the 67 kDa protein. Whereas dephosphorylation of these proteins was also observed on the addition of wortmannin, phosphorylation of the 67 kDa protein was only observed in the presence of KT5926. In the absence of calyculin A, phosphorylation of the 67 kDa protein was enhanced on the addition of PMA, and this PMA-dependent enhancement of phosphorylation of the 67 kDa protein was reduced by KT5926. The degree of PMA-induced phosphorylation of the 67 kDa protein in the absence of calyculin A was very similar to that in the presence of both calyculin A and KT5926 (Fig. 6). On the basis of the results obtained on dye-staining, there was no difference in the protein content of the 67 kDa protein in the cytosol fractions prepared from neutrophils treated with these inhibitors (data not shown). These results suggest that whereas optimal phosphorylation of the 67 kDa protein is required for activation of PMA-induced O_2^- generation, hyperphosphorylation of the 67 kDa protein by calyculin A may cause inhibition of O_2^- generation by PMA-activated neutrophils. Calyculin A also induced the hyperphosphorylation of many membrane proteins, and there was almost no difference in protein phosphorylation between membrane fractions prepared from PMA-acti-

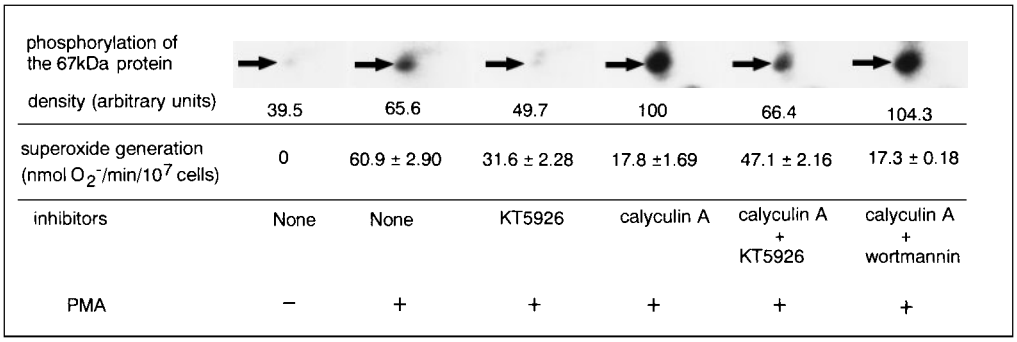

Fig 6. **Effect of calyculin A, KT5926 and/or wortmannin on ^{32}P-incorporation into the 67 kDa protein.** ^{32}P-Loaded neutrophils were preincubated with or without 100 nM calyculin A, 1 μM KT5926 and/or 10 μM wortmannin, and then activated with 0.1 μg/ml PMA as described under "EXPERIMENTAL PROCEDURES." As a control, cells were treated with 0.1% DMSO alone. A portion of the cytosolic fraction was subjected to two-dimensional electrophoresis and autoradiography. The arrow in each photograph indicates the 67 kDa protein. The relative level of ^{32}P-incorporation into the 67 kDa protein was determined by densitometric analysis.

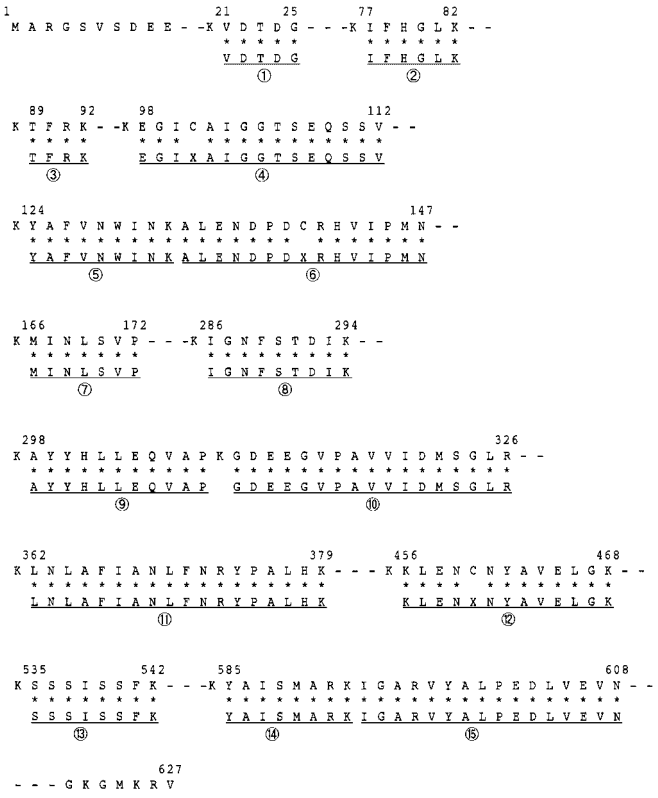

Fig. 7. **Alignment of the sequences of 15 peptides derived from the 67 kDa protein with the amino acid sequence of human L-plastin.** The asterisks indicate the identical amino acids in the peptides and human L-plastin. X denotes an unidentified amino acid.

vated neutrophils treated with calyculin A alone, and with both calyculin A and KT5926 (data not shown).

The phosphorylation of $p47^{phox}$ is known to be a key step in the activation of NADPH oxidase. The effect of calyculin A on the phosphorylation of $p47^{phox}$ in the membrane fraction of PMA-activated neutrophils was compared in the presence and absence of calyculin A. In the presence of calyculin A, the phosphorylation state of $p47^{phox}$ in the membrane fraction was not correlated with the activation of NADPH oxidase (data not shown).

Protein Sequence Analysis of the 67 kDa Protein—To clarify whether or not the p67 kDa protein is $p67^{phox}$, we examined the anti-$p67^{phox}$ antibody reactive spot by means of Western botting after two-dimensional gel electrophoresis. The 67 kDa protein was well separated from the immunoreactive spot of $p67^{phox}$ (data not shown).

To identify the 67 kDa protein, we determined its amino acid sequence. Proteolytic fragments of the 67 kDa protein were prepared by *in gel*–digestion with lysyl endopeptidase and subsequent fractionation by high performance liquid chromatography. The peptides obtained (Fig. 7) were analyzed by the automated Edman degradation method. Fifteen individual peptides were found to align with human L-plastin (*36*). It was noted that no other sequence except for that of L-plastin was obtained, indicating that the 67 kDa protein spot contained only L-plastin.

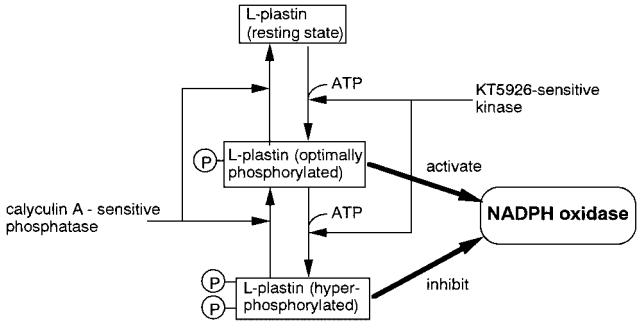

Fig. 8. **Schematic representation of the role of L-plastin in the activation of NADPH oxidase.** Whereas L-plastin is not phosphorylated in resting cells, PMA activates directly or indirectly a KT5926-sensitive kinase, which causes the phosphorylation of L-plastin. Optimally phosphorylated L-plastin may play some important role in the PMA-dependent activation of NADPH oxidase. The addition of calyculin A results in the inhibition of L-plastin phosphatase, which causes the hyperphosphorylation of L-plastin. Hyperphosphorylated L-plastin may be inactive in the activation of NADPH oxidase.

DISCUSSION

The phosphorylation and dephosphorylation cycles for key proteins are very important in many signal transduction pathways. In order to clarify the role of the phosphorylation and dephosphorylation cycles in the activation of NADPH oxidase we examined the effect of calyculin A on the OZ- and PMA-dependent activation of human neutrophils. Calyculin A inhibited the PMA- and OZ-induced activation of NADPH oxidase in a dose-dependent manner. We hypothesized that some protein(s), which plays a key role in the activation of NADPH oxidase, may be hyperphosphorylated by calyculin A through the inhibition of phosphatase, type 1 or 2A. Therefore, using various kinase inhibitors, we antagonized the calyculin A-induced inhibition of O_2^- generation by PMA-stimulated neutrophils. K-252a and KT5926 effectively restored the inhibition by calyculin A. While K-252a can inhibit a variety of protein kinases (*33*), KT5926 is reported to inhibit myosin light chain kinase and Ca^{2+}/calmodulin-dependent protein kinase II selectively (*31*, *32*). The finding that KT5926 inhibited the PMA-stimulated activation of NADPH oxidase in the absence of calyculin A also suggests that KT5926-sensitive kinase or its downstream kinase may phosphorylate some protein that plays a key role in PMA-induced activation of NADPH oxidase.

We tried to identify the proteins that were hyperphosphorylated by calyculin A, but were not hyperphosphorylated in the presence of both calyculin A and KT5926. Two-dimensional gel electrophoresis of phosphoproteins revealed that many proteins were hyperphosphorylated on the addition of calyculin A. However, the calyculin A–induced hyperphosphorylation state of the 67 kDa cytosolic protein of PMA-activated neutrophils was clearly reduced by KT5926. In addition to the 67 kDa protein, the calyculin A-induced hyperphosphorylation of several other cytosolic proteins was slightly reduced on the addition of KT5926. Whereas dephosphorylation of these proteins was also observed on the addition of wortmannin, dephosphorylation of the 67 kDa protein was

only observed in the presence of KT5926. In the absence of calyculin A, PMA rapidly phosphorylated this 67 kDa protein, but the extent of phosphorylation was markedly lower than that in the presence of calyculin A. In the absence of calyculin A, KT5926 markedly reduced the PMA-induced phosphorylation of the 67 kDa protein, coinciding with the KT5926-dependent inhibition of O_2^- generation. These results suggest that PMA-induced phosphorylation of the 67 kDa protein, which is catalyzed by a KT5926-sensitive kinase, is required for the activation of NADPH oxidase. The hyperphosphorylated 67 kDa protein, which is provoked by the inhibition of calyculin A–sensitive phosphatase, prevents the PMA-induced activation of NADPH oxidase. KT5926 may reduce the calyculin A–induced hyperphosphorylation of the 67 kDa protein to the same level as that induced by PMA alone, resulting in the restoration of calyculin A–induced inhibition of NADPH oxidase. Therefore, the optimal phosphorylation of the 67 kDa protein may be indispensable for the PMA-stimulated activation of NADPH oxidase (Fig. 8).

Analysis of the amino acid sequences of 15 peptides derived from the 67 kDa protein clearly indicated that the 67 kDa protein is L-plastin, an actin-bundling protein (*37*). A variety of agents are capable of stimulating L-plastin phosphorylation in leukocytes, including IL-1, IL-2, tumor necrosis factor, lipopolysaccharide, IL-8, immune complexes and PMA (*20*, *36*, *38–43*). Jones and Brown (*44*) proposed that L-plastin plays an important role in cell adhesion and phagocytosis. In the present paper, we propose that L-plastin plays a key role in the PMA-dependent activation of NADPH oxidase. This is the first report suggesting a role for L-plastin on the activation of NADPH oxidase. It has been reported that the PMA-induced activation of NADPH oxidase is coupled with phosphorylation of the 67 kDa protein, which is not $p67^{phox}$ (*45*). Judging from our present data, this 67 kDa protein may be identical to L-plastin. On the other hand, several reports (*46–51*) suggested that NADPH oxidase activity could be modulated by cytoskeletal elements. Shinomiya *et al*. (*40*) reported that the phosphorylation site(s) of mouse L-plastin may be located in the N-terminal region(s) (serine 5 or serine 7), but they failed to determine which serine residue (Ser-5 or Ser-7) was phosphorylated. Jones *et al*. reported that the phosphoserine at position 5 is the major site of phosphorylation in L-plastin (*52*). We failed to determine the N-terminal sequence of the 67 kDa protein, so it remains unclear which sites of L-plastin were phosphorylated in the presence or absence of calyculin A. We are now going to explore the hyperphosphorylation site(s) in L-plastin by tandem mass spectrometry.

Furthermore, it will be of interest to determine how phosphorylated L-plastin interacts with NADPH oxidase components or the upstream components of the NADPH oxidase activation pathway. Immunoprecipitates prepared with anti-$p47^{phox}$ polyclonal antibodies contained $p67^{phox}$, but not L-plastin (data not shown). We failed to prove any interaction with $p67^{phox}$ or $p47^{phox}$ with L-plastin in neutrophils treated with PMA or various inhibitors. Therefore, we suspect that L-plastin plays an important role in the PMA-dependent activation of NADPH oxidase through the modulation of other molecule(s).

REFERENCES

1. Babior, B.M. (1999) NADPH oxidase: an update. *Blood* **93**, 1464–1476
2. Segal, A.W. and Abo, A. (1993) The biochemical basis of the NADPH oxidase of phagocytes. *Trends Biochem. Sci.* **18**, 43–47
3. Fuchs, A., Bouin, A.P., Rabilloud, T., and Vignais, P.V. (1997) The 40-kDa component of the phagocyte NADPH oxidase ($p40^{phox}$) is phosphorylated during activation in differentiated HL60 cells. *Eur. J. Biochem.* **249**, 531–539
4. El Benna, J., Dang, P.M., Gaudry, M., Fay, M., Morel, F., Hakim, J., and Gougerot-Pocidalo, M.A. (1997) Phosphorylation of the respiratory burst oxidase subunit $p67^{phox}$ during human neutrophil activation. Regulation by protein kinase C-dependent and independent pathways. *J. Biol. Chem.* **272**, 17204–17208
5. El Benna, J., Han, J., Park, J.W., Schmid, E., Ulevitch, R.J., and Babior, B.M. (1996) Activation of p38 in stimulated human neutrophils: phosphorylation of the oxidase component $p47^{phox}$ by p38 and ERK but not by JNK. *Arch. Biochem. Biophys.* **334**, 395–400
6. Inanami, O., Johnson, J.L., McAdara, J.K., Benna, J.E., Faust, L.R., Newburger, P.E., and Babior, B.M. (1998) Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of $p47^{PHOX}$ on serine 303 or 304. *J. Biol. Chem.* **273**, 9539–9543
7. Dewas, C., Fay, M., Gougerot-Pocidalo, M.A., and El-Benna, J. (2000) The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine-induced $p47^{phox}$ phosphorylation in human neutrophils. *J. Immunol.* **165**, 5238–5244
8. Dang, P.M., Fontayne, A., Hakim, J., El Benna, J., and Perianin, A. (2001) Protein kinase C zeta phosphorylates a subset of selective sites of the NADPH oxidase component $p47^{phox}$ and participates in formyl peptide-mediated neutrophil respiratory burst. *J. Immunol.* **166**, 1206–1213
9. Chen, Q., Powell, D.W., Rane, M.J., Singh, S., Butt, W., Klein, J.B., and McLeish, K.R. (2003) Akt phosphorylates $p47^{phox}$ and mediates respiratory burst activity in human neutrophils. *J. Immunol.* **170**, 5302–5308
10. Hoyal, C.R., Gutierrez, A., Young, B.M., Catz, S.D., Lin, J.H., Tsichlis, P.N., and Babior, B.M. (2003) Modulation of $p47^{PHOX}$ activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase. *Proc. Natl Acad. Sci. USA* **100**, 5130–5135
11. Heyworth, P.G., Curnutte, J.T., Nauseef, W.M., Volpp, B.D., Pearson, D.W., Rosen, H., and Clark, R.A. (1991) Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. *J. Clin. Invest.* **87**, 352–356
12. Sumimoto, H., Kage, Y., Nunoi, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, M., Minakami, S., and Takeshige, K. (1994) Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase. *Proc. Natl Acad. Sci. USA* **91**, 5345–5349
13. Groemping, Y., Lapouge, K., Smerdon, S.J., and Rittinger, K. (2003) Molecular basis of phosphorylation-induced activation of the NADPH oxidase. *Cell* **113**, 343–355
14. Janssens, V. and Goris, J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* **353**, 417–439
15. Cohen, P.T. (2002) Protein phosphatase 1–targeted in many directions. *J. Cell Sci.* **115**, 241–256
16. Garcia, R.C., Whitaker, M., Heyworth, P.G., and Segal, A.W. (1992) Okadaic acid produces changes in phosphorylation and translocation of proteins and in intracellular calcium in human neutrophils. Relationship with the activation of the NADPH oxidase by different stimuli. *Biochem. J.* **286**, 687–692
17. Yamaguchi, M., Sasaki, J., Kuwana, M., Sakai, M., Okamura, N., and Ishibashi, S. (1993) Cytosolic protein phosphatase may

turn off activated NADPH oxidase in guinea pig neutrophils. *Arch. Biochem. Biophys.* **306**, 209–214
18. Djerdjouri, B., Combadiere, C., Pedruzzi, E., Hakim, J., and Perianin, A. (1995) Contrasting effects of calyculin A and okadaic acid on the respiratory burst of human neutrophils. *Eur. J. Pharmacol.* **288**, 193–200
19. Gay, J.C., Raddassi, K., Truett, A.P., 3rd, and Murray, J.J. (1997) Phosphatase activity regulates superoxide anion generation and intracellular signaling in human neutrophils. *Biochim. Biophys. Acta* **1336**, 243–253
20. Shibata, M., Ohoka, T., Mizuno, S., and Suzuki, K. (1993) Characterization of a 64-kd protein phosphorylated during chemotactic activation with IL-8 and fMLP of human polymorphonuclear leukocytes. I. Phosphorylation of a 64-kd protein and other proteins. *J. Leukoc. Biol.* **54**, 1–9
21. Ding, J. and Badwey, J.A. (1992) Effects of antagonists of protein phosphatases on superoxide release by neutrophils. *J. Biol. Chem.* **267**, 6442–6448
22. Suzuki, K., Yamaguchi, T., Tanaka, T., Kawanishi, T., Nishimaki-Mogami, T., Yamamoto, K., Tsuji, T., Irimura, T., Hayakawa, T., and Takahashi, A. (1995) Activation induces dephosphorylation of cofilin and its translocation to plasma membranes in neutrophil-like differentiated HL-60 cells. *J. Biol. Chem.* **270**, 19551–19556
23. Suzuki, K., Yamaguchi, T., Oshizawa, T., Yamamoto, Y., Nishimaki-Mogami, T., Hayakawa, T., and Takahashi, A. (1995) Okadaic acid induces both augmentation and inhibition of opsonized zymosan-stimulated superoxide production by differentiated HL-60 cells. Possible involvement of dephosphorylation of a cytosolic 21K protein in respiratory burst. *Biochim. Biophys. Acta* **1266**, 261–267
24. Nagaishi, K., Adachi, R., Kawanishi, T., Yamaguchi, T., Kasahara, T., Hayakawa, T., and Suzuki, K. (1999) Participation of cofilin in opsonized zymosan-triggered activation of neutrophil-like HL-60 cells through rapid dephosphorylation and translocation to plasma membranes. *J. Biochem.* **125**, 891–898
25. Matsui, S., Matsumoto, S., Adachi, R., Kusui, K., Hirayama, A., Watanabe, H., Ohashi, K., Mizuno, K., Yamaguchi, T., Kasahara, T., and Suzuki, K. (2002) LIM kinase 1 modulates opsonized zymosan-triggered activation of macrophage-like U937 cells. Possible involvement of phosphorylation of cofilin and reorganization of actin cytoskeleton. *J. Biol. Chem.* **277**, 544–549
26. Yamaguchi, T., Kaneda, M., and Kakinuma, K. (1986) Effect of saturated and unsaturated fatty acids on the oxidative metabolism of human neutrophils. The role of calcium ion in the extracellular medium. *Biochim. Biophys. Acta* **861**, 440–446
27. Kakinuma, K. and Minakami, S. (1978) Effects of fatty acids on superoxide radical generation in leukocytes. *Biochim. Biophys. Acta* **538**, 50–59
28. Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P.C., and Babior, B.M. (1986) A possible role for protein phosphorylation in the activation of the respiratory burst in human neutrophils. Evidence from studies with cells from patients with chronic granulomatous disease. *J. Biol. Chem.* **261**, 9109–9115
29. Kawasaki, H., Emori, Y., and Suzuki, K. (1990) Production and separation of peptides from proteins stained with Coomassie brilliant blue R-250 after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **191**, 332–336
30. Badwey, J.A., Heyworth, P.G., and Karnovsky, M.L. (1989) Phosphorylation of both 47 and 49 kDa proteins accompanies superoxide release by neutrophils. *Biochem. Biophys. Res. Commun.* **158**, 1029–1035
31. Nakanishi, S., Yamada, K., Iwahashi, K., Kuroda, K., and Kase, H. (1990) KT5926, a potent and selective inhibitor of myosin light chain kinase. *Mol. Pharmacol.* **37**, 482–488
32. Hashimoto, Y., Nakayama, T., Teramoto, T., Kato, H., Watanabe, T., Kinoshita, M., Tsukamoto, K., Tokunaga, K., Kurokawa, K., Nakanishi, S., and *et al*. (1991) Potent and preferential inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by K252a and its derivative, KT5926. *Biochem. Biophys. Res. Commun.* **181**, 423–429
33. Ruegg, U.T. and Burgess, G.M. (1989) Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol. Sci.* **10**, 218–220
34. Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., and Ui, M. (1994) Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **269**, 3563–3567
35. Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y., and *et al*. (1992) Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J. Biol. Chem.* **267**, 2157–2163
36. Zu, Y.L., Shigesada, K., Nishida, E., Kubota, I., Kohno, M., Hanaoka, M., and Namba, Y. (1990) 65-kilodalton protein phosphorylated by interleukin 2 stimulation bears two putative actin-binding sites and two calcium-binding sites. *Biochemistry* **29**, 8319–8324
37. Namba, Y., Ito, M., Zu, Y., Shigesada, K., and Maruyama, K. (1992) Human T cell L-plastin bundles actin filaments in a calcium-dependent manner. *J. Biochem.* **112**, 503–507
38. Anderson, N.L., Gemmell, M.A., Coussens, P.M., Murao, S., and Huberman, E. (1985) Specific protein phosphorylation in human promyelocytic HL-60 leukemia cells susceptible or resistant to induction of cell differentiation by phorbol-12-myristate-13-acetate. *Cancer Res.* **45**, 4955–4962
39. Matsushima, K., Shiroo, M., Kung, H.F., and Copeland, T.D. (1988) Purification and characterization of a cytosolic 65-kilodalton phosphoprotein in human leukocytes whose phosphorylation is augmented by stimulation with interleukin 1. *Biochemistry* **27**, 3765–3770
40. Shinomiya, H., Hagi, A., Fukuzumi, M., Mizobuchi, M., Hirata, H., and Utsumi, S. (1995) Complete primary structure and phosphorylation site of the 65-kDa macrophage protein phosphorylated by stimulation with bacterial lipopolysaccharide. *J. Immunol.* **154**, 3471–3478
41. Shiroo, M. and Matsushima, K. (1990) Enhanced phosphorylation of 65 and 74 kDa proteins by tumor necrosis factor and interleukin-1 in human peripheral blood mononuclear cells. *Cytokine* **2**, 13–20
42. Messier, J.M., Shaw, L.M., Chafel, M., Matsudaira, P., and Mercurio, A.M. (1993) Fimbrin localized to an insoluble cytoskeletal fraction is constitutively phosphorylated on its headpiece domain in adherent macrophages. *Cell Motil. Cytoskeleton* **25**, 223–233
43. Wang, J. and Brown, E.J. (1999) Immune complex-induced integrin activation and L-plastin phosphorylation require protein kinase A. *J. Biol. Chem.* **274**, 24349–24356
44. Jones, S.L. and Brown, E.J. (1996) FcgammaRII-mediated adhesion and phagocytosis induce L-plastin phosphorylation in human neutrophils. *J. Biol. Chem.* **271**, 14623–14630
45. Heyworth, P.G., Ding, J., Erickson, R.W., Lu, D.J., Curnutte, J.T., and Badwey, J.A. (1996) Protein phosphorylation in neutrophils from patients with p67-phox-deficient chronic granulomatous disease. *Blood* **87**, 4404–4410
46. Adachi, R., Takeuchi, K., and Suzuki, K. (2002) Antisense oligonucleotide to cofilin enhances respiratory burst and phagocytosis in opsonized zymosan-stimulated mouse macrophage J774.1 cells. *J. Biol. Chem.* **277**, 45566–45571
47. Wientjes, F.B., Reeves, E.P., Soskic, V., Furthmayr, H., and Segal, A.W. (2001) The NADPH oxidase components $p47^{phox}$ and $p40^{phox}$ bind to moesin through their PX domain. *Biochem. Biophys. Res. Commun.* **289**, 382–388
48. Tamura, M., Kanno, M., and Endo, Y. (2000) Deactivation of neutrophil NADPH oxidase by actin-depolymerizing agents in a cell-free system. *Biochem. J.* **349**, 369–375
49. Tamura, M., Kai, T., Tsunawaki, S., Lambeth, J.D., and Kameda, K. (2000) Direct interaction of actin with p47(phox) of neutrophil NADPH oxidase. *Biochem. Biophys. Res. Commun.* **276**, 1186–1190

50. Grogan, A., Reeves, E., Keep, N., Wientjes, F., Totty, N.F., Burlingame, A.L., Hsuan, J.J., and Segal, A.W. (1997) Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *J. Cell Sci.* **110**, 3071–3081
51. Nunoi, H., Yamazaki, T., Tsuchiya, H., Kato, S., Malech, H.L., Matsuda, I., and Kanegasaki, S. (1999) A heterozygous mutation of beta-actin associated with neutrophil dysfunction and recurrent infection. *Proc. Natl Acad. Sci. USA* **96**, 8693–8698
52. Jones, S.L., Wang, J., Turck, C.W., and Brown, E.J. (1998) A role for the actin-bundling protein L-plastin in the regulation of leukocyte integrin function. *Proc. Natl Acad. Sci. USA* **95**, 9331–9336