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Calyculin A modulates activation of the NADPH-oxidase in Me_2SO-differentiated HL-60 cells

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Abbreviations: DG, diacylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; FMLP, N-formyl-methionyl-leucyl-phenylalanine; MAPK, mitogen-activated-protein kinase; Me_2SO, dimethyl sulfoxide; phox, phagocytic oxidase

Abstract

Human promyelocytic leukemia cells (HL-60) have been used as a model system in which to study the effects of protein phosphatase inhibitors on NADPH-oxidase activation. Since O_2^- is generated by NADPH-oxidase , we examined the effect of calyculin A pre-treatment on oxidase activation in response to various agonists. When Me_2SO-differentiated HL-60 cells were treated with calyculin A prior to the addition of phorbol 12-myristate 13-acetate (PMA), O_2^- production was inhibited; however, calyculin A enhanced O_2^- production by N-formyl-methionyl-leucyl-phenylala-nine (FMLP). The decreased O_2^- production seen with calyculin A pretreatment followed by PMA may be due to diminished translocation of the p47-phox and p67-phox, cytosolic components of the oxidase, and inhibition of arachidonic acid release. Interestingly calyculin A pretreatment followed by either agonist significantly enhanced mitogen-activated-protein kinase (MAPK) activity. The differential effects of pretreatment with calyculin A on subsequent oxidase stimulation elicited by FMLP or PMA provide further evidence for substantial heterogeneity in the acti-vation of the respiratory burst.

Keywords: NADPH-oxidase, calyculin A, p47-phox, p67-phox, arachidonic acid

Introduction

Stimulation of neutrophils causes a “respiratory burst” including increased oxygen consumption and the generation of reactive oxygen metabolites such as O_2^- and H_2O_2, which provide a major bactericidal mechanism in these cells (Babior, 1984a). The enzymatic activity responsible for this respiratory burst resides in membrane-bound NADPH-oxidase, which reduces molecular oxygen to O_2^- (Babior, 1984b). The NADPH-oxidase consists of integral membrane proteins (cytochrome b558) (Cross *et al.,* 1981; Gabig and Lefker, 1984) and “soluble” (cytoplasmic/ cytoskeletal) components. The latter include p47-phox, p67-phox, and rac 2, a ras-related GTP-binding protein (Nunoi *et al.,* 1988; Lomax *et al.,* 1990; Abo *et al.,* 1991a; Abo and Pick, 1991b; Uhlinger *et al.,* 1993). The oxidase is dormant in resting cells, but can be activated by both particulate and soluble stimuli (Babior *et al.,* 1975; Babior, 1984b). The mechanism for the activation of the oxidase is very complex and has been the subject of intense study. Several studies have indicated the involvement of diacylglycerol (DG) /protein kinase C (PKC) , since PKC activators, phorbol 12-myristate 13-acetate (PMA) (De Chatelet *et al.,* 1976) and some DGs (Fujita *et al.,* 1984; Cox *et al.,* 1986), activate the NADPH-oxidase system. Stimulation of neutrophils with receptor-linked agonists such as N-formyl-methionyl-leucyl-phenylalanine (FMLP) results in the activation of PLC β_2 and hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to the generation of inositol 1,4,5-triphosphate, a calcium messenger, and DG (Volp *et al.,* 1983; Ohta *et al.,* 1985; Smith *et al.,* 1986).

Activation of the oxidase involves the translocation of a fraction (<10%) of p47-phox and p67-phox to the plasmalemma where the functional complex is assembled (Clark *et al.,* 1990; Nauseef *et al.,* 1991; Tyagi *et al.,* 1992). One route of stimulation that occurs with activators of PKC, such as PMA, involves multisite phosphorylation of p47-phox (Heyworth and Badwey, 1990). PKC can catalyze the phosphorylation of p47-phox *in vitro* (Kramer *et al.,* 1988; Pilloud-Dagher *et al.,* 1992; Uhlinger and Perry, 1992b) and the predicted sequence of p47-phox contains several potential phosphorylation sites for this kinase (Lomax *et al.,* 1989). It has been suggested that the NADPH-oxidase activity in neutrophils may be regulated by the phosphorylation/dephosphorylation state of p47-phox which is a function of PKC and possibly other kinases, and protein phosphatases. The respiratory burst may be initiated not only by activation of kinases but also by the

inhibition of phosphatases and it is believed that the latter enzymes may regulate the magnitude and duration of neutrophil activation (Babior, 1988; Ding and Badwey, 1992; Lu *et al.,* 1992; Berkow and Dodson, 1993; Yama-guchi *et al.,* 1993).

The human promyelocytic leukemia cell line, HL-60, can be differentiated into either monocyte/macrophage-like cells or granulocytes by various chemical agents (Koeffler, 1983; Collins, 1987). HL-60 cells have been used as a model system in which to study the effects of protein phosphatase inhibitors on NADPH-oxidase activation. In differentiated HL-60 cells, but not in undifferentiated cells, NADPH-oxidase activity is stimulated by phorbol esters and other agonists. To further investigate the roles of serine/threonine phosphatases in the activation of NADPH-oxidase, the effects of calyculin A on the O_2^- production of dimethyl sulfoxide (Me_2SO)-differentiated HL-60 cells were examined.

Although there has been much investigation of the role of PKC in the activation of neutrophils, very little is known about the role of other protein kinases. It has recently been shown that stimulation of neutrophils with FMLP, granulocyte-monocyte colony stimulating factor (GM-CSF), or PMA activates the mitogen-activated protein kinase (MAPK) (Gomez-Cambronero *et al.,* 1992; Ohta *et al.,* 1992; Okuda *et al.,* 1992; Raines *et al.,* 1992; Thompson *et al.,* 1993; Torres *et al.,* 1993). The MAPK cascade is a signaling pathway common to many neutrophil agonists, which may be activated either transiently or in a sustained manner. We therefore investigated the effects of calyculin A on MAPK activity in response to FMLP or PMA.

Materials and Methods

Materials

RPMI 1640 media, horse heart cytochrome c (type III), bovine erythrocyte superoxide dismutase, PMA, FMLP, Me_2SO, calyculin A, penicillin, streptomycin, myelin basic protein (MBP), leupeptin, pepstatin, and protein G-sepharose were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum was from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibodies to p47-phox and to p67-phox were generous gifts of H. Nunoi (Tokyo University, Japan) . Monoclonal antibody to MAPK was obtained from Zymed Lab (South San Francisco, USA). ECL kit and [γ-^{32}P]ATP were obtained from Amersham (Little Chalfont, England). Other reagents were obtained from commercial sources.

Cell and cell culture

HL-60 cells were obtained from ATCC (Rockville, MD) and grown in a suspension culture in RPMI 1640 supplemented with 20% heat inactivated fetal bovine serum, 60 µg/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37˚C in 5% CO_2. To induce myeloid differentiation, cells were seeded to a density of 1×10^6 cells/ml and were cultivated for 6 days in medium containing 1.25% (v/v) Me_2SO.

Assay of O_2^- production

O_2^- production of the cells stimulated with PMA or FMLP was measured by determining the maximal rate of superoxide dismutase inhibitable cytochrome c reduction at 550 nm (Kwak *et al.,* 1991). Cells (2×10^6/ml) were incubated in an assay mixture containing 135 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM KCl, 5 mM glucose, and 50 µM cytochrome c at 37˚C for 10 min and the reactions were initiated by adding 100 nM PMA or 1 µM FMLP.

Translocation of proteins

Me_2SO-differentiated HL-60 cell supensions (4×10^6/ml) in reaction buffer (135 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM KCl, and 5 mM glucose) were preincubated with or without calyculin A (100 nM) for 10 min, then incubated at 37˚C for 10 min with 0.1% Me_2SO, 100 nM PMA, or 1 µM FMLP in a total volume of 1 ml. Incubations were terminated by adding 2 volumes of ice-cold phosphate buffered saline (PBS). Cell pellets were resuspended at 4×10^6 cells/ml in the HEPES buffered saline which was composed of 130 mM NaCl, 1 mM EGTA, 30 ng/ml leupeptin, 5 ng/ml pepstatin, 1 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 10 mM HEPES, pH 7.4 (Uhlinger *et al.,* 1992a). The cells were disrupted by sonication using the microtip of a Branson sonifier (model 185) of 10 W at 2˚C with three 15 sec bursts. The suspensions of disrupted cells were centrifuged at 1,000 *g* for 5 min and the post-nuclear supernatants were recentrifuged at 100,000 *g* for 30 min at 2˚C using a Beckman model L8 80 M ultracentrifuge. The cytosol and membrane which was suspended in HEPES buffered saline containing 0.34 M sucrose, 30 ng/ml leupeptin, 5 ng/ml pepstatin, and 1 µg/ml PMSF were divided into aliquots and stored at -70˚C. Equivalent amounts of the membrane fractions were analyzed for p47-phox and p67-phox by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/ PAGE) and Western blotting as described below.

Electrophoresis and Western blots

SDS/PAGE was performed as described by Laemmli (1970) on 7.5% (w/v) acrylamide gels. Gels were transferred onto nylon membranes (Hybond N). The membranes were then probed with antibodies to p47-phox (1:5,000 dilution) or p67-phox (1:5,000 dilution) and anti-mouse immunoglobulin. P47-phox or p67-phox was visualized using the enhanced chemiluminescence system (Amersham).

Measurements of [^{3}H]arachidonic acid release

Incorporation and release of [^{3}H]arachidonic acid were performed as previously reported (Tarsi-Tsuk and Levy, 1990). Me_2SO-differentiated HL-60 cells (5×10^7/ml) were incubated for 30 min at 37˚C in Ca^{2+}/Mg^{2+}-free PBS containing 1 mCi of [^{3}H]arachidonic acid (100 mCi/mmol) (Amersham). The cells were washed once with PBS containing 0.1% fatty acid-free human serum albumin (HSA) and twice with PBS. The pellet was resuspended to 4×10^6 cells/ml in incubation buffer (137 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, and 10 mM Hepes, pH 7.4) with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ containing 0.1% HSA. The incorporation of [^{3}H]arachidonic acid was 80% of the total radioactivity added to the cells after incubation for 30 min. The cells (4×10^6/ml) were activated in 37˚C with PMA (100 nM) or FMLP (1 μM) in the presence or absence of calyculin A (100 nM) for 3 min (FMLP) and 10 min (PMA). The reaction was terminated by centri-fugation at 4˚C and samples of the supernatants were counted for radioactivity by liquid scintillation.

Immune complex kinase assay for MAPK

Assays were carried out on lysates to test for activation of MAPK. The enzyme was detected by use of MBP (0.1 mg/ml) as a substrate for MAPK. Lysates were added to 100 μl of lysis buffer (20 mM Tris, pH 8.0, 1% Triton X-100, 10% glycerol, 131 mM NaCl, 15 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 1 mM PMSF, 20 mM leupeptin, and 10 mg/ml aprotinin). Then 1 μl of the antibody against MAPK and 5 μl of protein G-sepharose were added. The mixture was incubated at 4˚C for 2~24 h. The mixture was washed 3 times with kinase buffer (20 mM Tris, pH 7.4 and 10 mM $MgCl_2$). The immune complexes were resuspended in 30 μl of kinase buffer containing 7 μg of MBP, 2 μM cold ATP, and 5 μCi of [γ–^{32}P]ATP and incubated at 30˚C for 30 min. The reaction was stopped by adding 30 μl of SDS sample buffer. The samples were then boiled for 5 min and electrophoresed on an SDS/12% polyacrylamide gel. Gels were stained with Coomassie blue, dried and subjected to autoradiography with intensifying screens at -70˚C.

Results

Effect of calyculin A on O_2^- production in differentiated HL-60 cells

Previous studies have shown that serine/threonine phosphatases as well as protein kinases were involved in the regulation of the respiratory burst in neutrophils (Babior, 1988; Ding and Badwey, 1992; Lu *et al.,* 1992; Berkow and Dodson, 1993; Yamaguchi *et al.,* 1993). We therefore examined the effect of a serine/threonine phosphatase inhibitor on the activation of the NADPH-oxidase in response to different agonists in differentiated HL-60 cells. HL-60 cells were incubated with 100 nM calyculin A for 10 min at 37˚C. Calyculin A itself did not induce O_2^- production and did not affect cell viability (data not shown). When the differentiated HL-60 cells were treated with calyculin A for 10 min prior to the addition of PMA, the rate of O_2^- production of the calyculin A-treated cells was significantly lower than that of the cells treated with PMA alone (Table 1, Figure 1).

Because FMLP activates O_2^- production in an ATP-dependent manner and PKC has been proposed to mediate the effects of this chemoattractant (Benna *et al.,* 1994), we expected calyculin A to have a similar effect on the FMLP-induced respiratory burst as it did on the PMA response. This prediction was tested. In contrast to the inhibitory effects of calyculin A on PMA activation, the phosphatase inhibitor potentiated the respiratory burst triggered by FMLP. This was due primarily to an increase

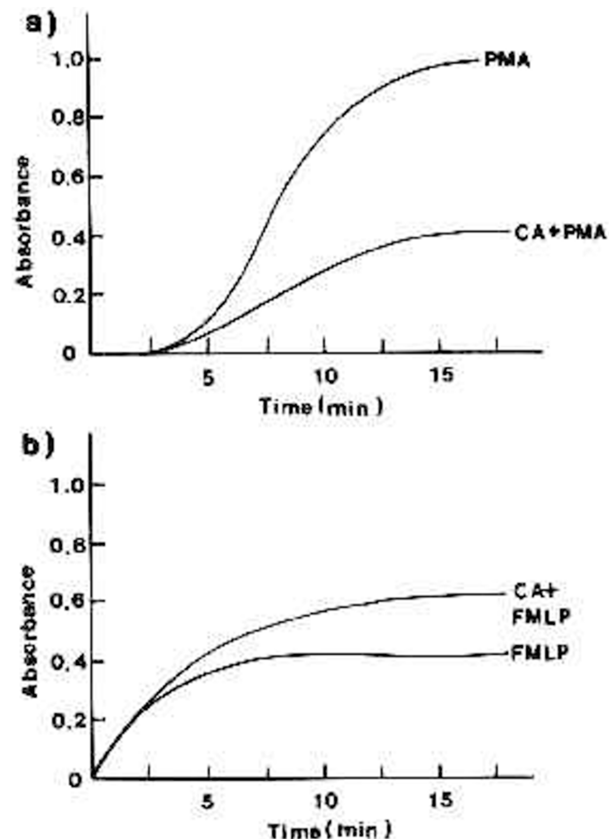

Figure 1. Differential effects of calyculin A on O_2^- production of Me_2SO-differentiated HL-60 cells stimulated with PMA or FMLP. Me_2SO-differentiated HL-60 cells (2×10^6/ml) were treated with or without 100 nM calyculin A for 10 min prior to the addition of 100 nM PMA (a) and 1 μM FMLP (b) to the reference (containing superoxide dismutase) or sample cuvettes. The O_2^- mediated reduction of ferricytochrome c was determined as the change in the absorbance at 550 nm. Chromatograms show the representative of three experiments.

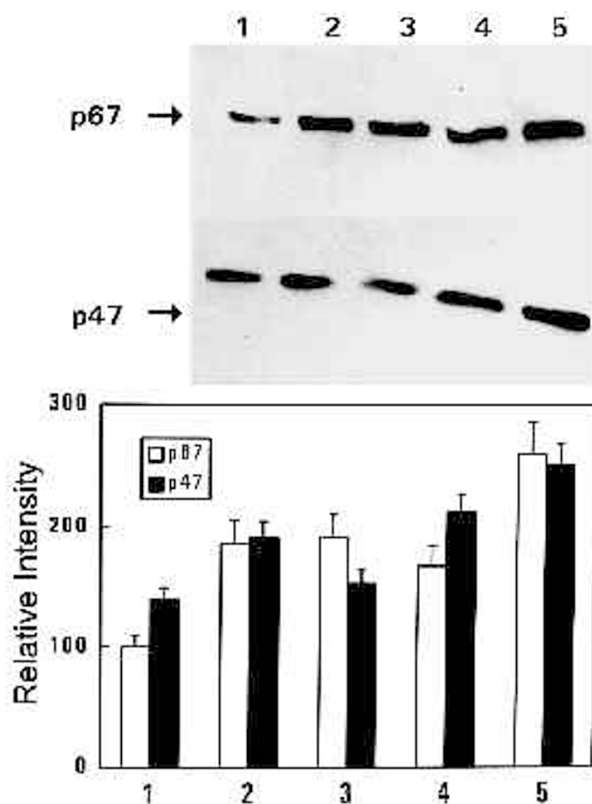

Figure 2. Translocation of p47-phox and p67-phox after pretreatment with calyculin A. Upper panel, equivalent amount of membrane fractions of HL-60 cells (30 µg / lane) were immunoblotted with anti-p47-phox and anti-p67-phox antibodies as described in "Materials and Methods". Lower panel, each band of the upper panel was quantitated by densitometric analysis. Me_2SO-differentiated HL-60 cells were treated with: 0.1% Me_2SO (lane 1), 100 nM PMA (lane 2), 100 nM calyculin A prior to the addition of 100 nM PMA (lane 3), 1 µM FMLP (lane 4), 100 nM calyculin A prior to the addition of 1 µM FMLP (lane 5). Results are representatives (upper panel) and expressed as the mean ± SE of three experiments (lower panel).

Table 1. Effect of calyculin A on the O_2^- production of Me_2SO-differen-tiated HL-60 cells. Me_2SO-differentiated HL-60 cells (2×10^6/ml) were treated with or without 100 nM calyculin A for 10 min prior to the addition of 100 nM PMA and 1 µM FMLP to the reference (containing superoxide dismutase) or sample cuvettes. The O_2^- mediated reduction of ferricyto-chrome c was determined as the maximal change in the absorbance at 550 nm as described. The optical density was determined at a 15 min endpoint. Results are expressed as a mean ± SD of three experiments.

Treatment	Cyt. c reduction (nmol/min/2×10^6cells)	O.D. at 15 min endpoint
PMA	16.6 ± 1.45	1.00 ± 0.09
Calyculin A+PMA	4.0 ± 0.43	0.42 ± 0.04
FMLP	9.2 ± 0.65	0.42 ± 0.02
Calyculin A+FMLP	9.2 ± 0.65	0.62 ± 0.05

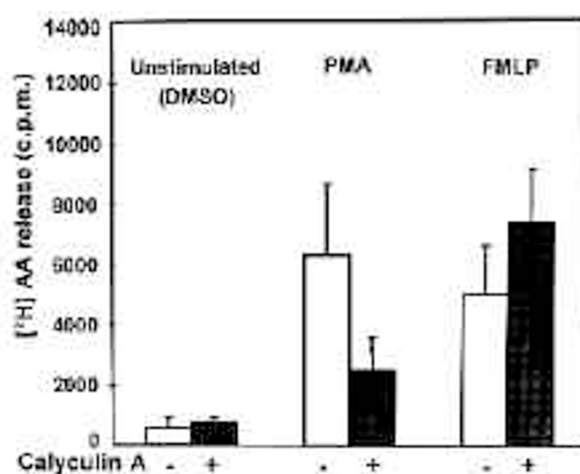

Figure 3. Effect of calyculin A on [^{3}H]arachidonic acid release. Me_2SO-differentiated HL-60 cells were preincubated without (0.1% Me_2SO) or with 100 nM calyculin A for 10 min followed by stimulation with PMA (100 nM) for 10 min and FMLP (1 µM) for 3 min. [^{3}H]arachidonic acid release was quantitated by liquid scintillation as described. Results are expressed as the mean ± SE of three experiments.

in the duration and extent of the response, rather than to an elevated initial (maximal) rate (Table 1, Figure 1). The contrasting effects of calyculin A on the FMLP- and PMA-induced responses imply that stimulation of the NADPH-oxidase by phorbol esters may proceed through a pathway(s) in addition to PKC, perhaps involving other cellular kinase(s).

Translocation of proteins from cytosol to membranes

The differences we observed in O_2^- generation led us to examine the assembly of the oxidase. The cytosolic proteins p47-phox and p67-phox, essential for the activity of the NADPH-oxidase, translocate to the membrane upon stimulation of neutrophils with PMA and other agonists (Nauseef *et al.,* 1991). Treatment with calyculin A before cell stimulation with PMA decreased the amount of trans-located p47-phox (Figure 2), suggesting that this could be an explanation for the inhibition of O_2^- generation in response to PMA.

Effect of calyculin A on [^{3}H]arachidonic acid release

It has been suggested that arachidonic acid may act as a positive modulator for the NADPH-oxidase (Tarsi-Tsuk and Levy, 1990). We therefore studied the effect of calyculin A on [^{3}H]arachidonic acid release in response to an agonist. Calyculin A inhibited [^{3}H]arachidonic acid release by PMA, but augmented the release of [^{3}H]arachi-donic acid induced by FMLP (Figure 3). These data indicate that the inhibition of O_2^- production by PMA, in cells pretreated with calyculin A, may be related to the decreased release of arachidonic acid.

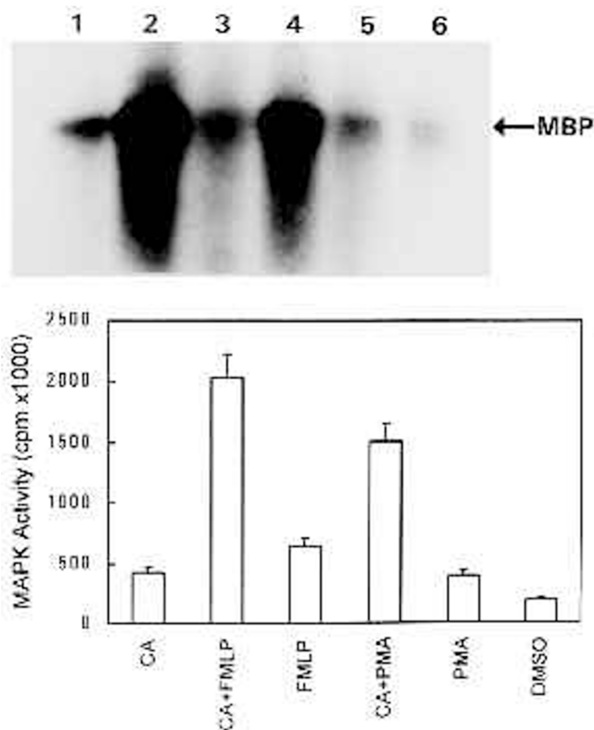

Figure 4. Effect of calyculin A on the MAPK activity. Assays were carried out on equivalent amount of lysates (30 μg / lane) to test for activation of MAPK. The enzyme was detected by using MBP (0.1 mg/ml) as substrate for MAPK. Upper panel, differentiated HL-60 cells were treated with: 100 nM calyculin A for 10 min (lane 1), 100 nM calyculin A for 10 min before addition of 1 μM FMLP (lane 2), 1 μM FMLP for 3 min (lane 3), 100 nM calyculin A for 10 min before the addition of 100 nM PMA (lane 4), 100 nM PMA for 10 min (lane 5), 0.1% Me_2SO (lane 6, control). Results are the representative of three separate experiments. Lower panel, results are measured by densitometry and expressed as the mean ± SE of three separate experiments.

Effect of calyculin A on MAPK activity

Cytosolic phospholipase A_2 (PLA_2) has been shown to be phosphorylated and activated by MAPK (Lin *et al.,* 1993). Since we observed differences in arachidonic acid release, we investigated the effect of calyculin A on MAPK activa-tion. Me_2SO-differentiated HL-60 cells were preincubated with calyculin A for 10 min and then treated with PMA or FMLP, and MAPK activity in whole cell lysates was assayed by measuring the phosphorylation of MBP. PMA and FMLP both activated MAPK. Calyculin A alone activated MAPK over control levels. The combination of PMA or FMLP and calyculin A greatly enhanced the MAPK activity (Figure 4). These results suggested that the inhibition of arachidonic acid release by calyculin A pretreatment followed by PMA stimulation is not due to the inhibition of MAPK.

Discussion

NADPH-oxidase of neutrophils is activated to produce O_2^- by a variety of agents. There is a characteristic lag phase between the addition of the stimulus and initiation of the response. This lag phase is thought to be caused by a sequence of intermediate steps, which take place after triggering, before the onset of the respiratory burst. When the stimulus is the bacterial peptide FMLP, deacti-vation occurs shortly after the maximum rate of O_2^- generation has been achieved. In contrast, when PMA elicits the respiratory burst through a presumed direct effect on PKC, the lag phase is longer and deactivation does not seem to take place. Phosphorylation of the cytosolic protein p47-phox, which is part of the NADPH-oxidase system, appears to be necessary for activation of the burst. The kinetics of activation of the burst and phosphorylation of p47-phox are coincident (Clark *et al.,* 1990), Although differences have been observed in the degree of phosphorylation in response to different stimuli, for example, PMA elicited a higher level of phosphoryla-tion than FMLP. The direct correlation between activation and phosphorylation prompted us to study the effect of the potent protein phosphatase inhibitor, calyculin A , on the NADPH-oxidase activity of Me_2SO-differentiated HL-60 cells. An enhancement of steady-state protein phos-phorylation might either trigger activity or prolong the respiratory burst. Calyculin A greatly prolonged oxidase activity, after stimulation with FMLP, although no direct activation was observed. This suggested that dephos-phorylation might be involved in the deactivation of the NADPH-oxidase. In contrast, inhibition of the response of Me_2SO-differentiated HL-60 cells to PMA after treatment with calyculin A was observed. This finding was concor-dant with results seen by another group (Lu *et al.,* 1992). The diminished translocation of p47-phox from cytosol to the membrane may be the cause of this inhibition. Calyculin A alone did not inhibit the translocation of p47-phox to the membrane.

To further investigate the differential responses elicited by FMLP and PMA, we examined the release of arachi-donic acid. The present study shows that with PMA as the stimulus, activation of the oxidase and the release of arachidonic acid was inhibited by calyculin A. These observations suggest that arachidonic acid has a role in the activation of the oxidase. Phosphorylation/ dephos-phorylation or hyperphosphorylation have been suggested to be related to inhibition of the translocation of p47-phox and O_2^- production (Yamaguchi *et al.,* 1995). However, those studies demonstrated no differences between the phosphorylation of p47-phox and O_2^- production in res-ponse to FMLP or PMA in calyculin A treated cells. Our results suggest that the decreased release of arachidonic

acid, as a result of protein phosphatase inhibition by calyculin A, may be responsible for the inhibition of O_2^- production.

Other reports have suggested that MAPK or a related proline-directed kinase may participate in the regulation of O_2^- production by activated neutrophils (Thompson *et al.,* 1993). In our experiments, calyculin A significantly augmented the MAPK activities in both PMA-stimulated cells and especially in FMLP-stimulated cells. It has been suggested that PKC plays an important role in insulin stimulation of protein phosphatase-1 via the activation of MAPK cascade (Srinivasan and Begum, 1994). It is also likely that the FMLP cascade leads to MAPK activation and subsequent phosphorylation and activation of $cPLA_2$ which releases arachidonic acid (Lin *et al.,* 1993; Nemenoff *et al.,* 1993). It is thought that the MAPK cascade may participate in the activation of the NADPH-oxidase, but the changes in MAPK activity elicited by calyculin A pre-treatment of HL-60 cells, stimulated by FMLP and PMA, is not coincident with the changes seen in NADPH-oxidase activity. The apparent anomaly between MAPK activation and the presumed inhibition of $cPLA_2$ activity, in response to PMA and calyculin A-pretreatment, is currently under investigation.

The dramatically different effects of pre-treatment with calyculin A on the subsequent stimulation by FMLP or PMA provide further evidence of significant differences in the activation pathways of the respiratory burst. Calyculin A should prove to be a very useful tool for the elucidation of the mechanisms involved in this process.

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