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Involvement of protein kinase Cδ in the activation of NADPH oxidase and the phagocytosis of neutrophils

KENJI WAKI[1,†], OSAMU INANAMI[1,‡], TOHRU YAMAMORI[1,¶], HAJIME NAGAHATA[2,§] & MIKINORI KUWABARA[1]

[1]*Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060 0818, Japan, and* [2]*Department of Animal Health, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069 8501, Japan*

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Abstract

This experiment was performed to clarify the role of protein kinase C (PKC) δ in NADPH oxidase-dependent O_2^- production and actin polymerization followed by phagocytosis in neutrophils. Bovine neutrophils and human neutrophil-like differentiated HL-60 (dHL-60) cells were stimulated with serum-opsonized zymosan (OZ) and fMet–Leu–Phe (fMLP), respectively. Rottlerin, a specific inhibitor of PKCδ, attenuated the production of O_2^- from NADPH oxidase in both neutrophils and dHL-60 cells. However, it did not inhibit the translocation of $p47^{phox}$ from the cytosol to the membrane in either type of cell or the phosphorylation of $p47^{phox}$ in dHL-60 cells. GF109203X (GFX), an inhibitor of cPKC, attenuated not only the production of O_2^- but also the translocation of $p47^{phox}$ in both cells. Furthermore, rottlerin significantly attenuated the ingestion of opsonized particles and the formation of F-actin in OZ-stimulated neutrophils, whereas, GFX did not affect those phagocytic processes. These results suggest that both PKCδ and cPKC regulate NADPH oxidase through different pathways, but only PKCδ regulates the phagocytic function in neutrophils.

Keywords: *cPKC, NADPH oxidase, neutrophil, phagocytosis, PKCδ*

Introduction

Generation of reactive oxygen species (ROS) and phagocytosis by neutrophils play pivotal roles in the first stage of the host defense against invading microorganisms. In response to various stimuli such as bacteria and phagocytic particles, ROS generation and phagocytosis occur as important processes to ingest and to clear these infectious agents for the host defense. The ROS include O_2^- generated by NADPH oxidase and their derivatives, OCl^-, $^{\cdot}OH$ and H_2O_2 [1]. NADPH oxidase is a multicomponent enzyme generating O_2^-, which is essential for bactericidal activity [2]. This oxidase

Correspondence: M. Kuwabara, Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060 0818, Japan. Tel: 81 11 706 5235. Fax: 81 11 706 7373. E-mail: kuwabara@vetmed.hokudai.ac.jp

†Tel: 81 11 706 5237. Fax: 81 11 706 7373. E-mail: wakken@vetmed.hokudai.ac.jp
‡Tel: 81 11 706 5236. Fax: 81 11 706 7373. E-mail: inanami@vetmed.hokudai.ac.jp
¶Tel: 81 11 706 5237. Fax: 81 11 706 7373. E-mail: tohru_y@yg8.so-net.ne.jp
§Tel: 81 11 388 4844. Fax: 81 11 387 5890. E-mail: nght-h@rakuno.ac.jp

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consists of the membrane flavocytochrome b_{558} (a complex of $gp91^{phox}$ and $p22^{phox}$) and three cytosolic proteins ($p47^{phox}$, $p67^{phox}$ and Rac). When resting cells are exposed to appropriate stimuli, the cytosolic components migrate to the membrane and associate with the membrane component for the activation [2]. Key events of the activation are the phosphorylation and translocation of $p47^{phox}$. This step is regulated by several kinases, including protein kinase C (PKC) [3–9]. PKCs are categorized into three subfamilies: classical PKCs (α, β_I, β_{II} and γ), novel PKCs (δ, ϵ, η and θ)and atypical PKCs (ζ and ι/λ). This classification is based on the existence of conserved C1 and C2 domains [10–12]. These PKCs have been reported to play important roles in many bactericidal functions of phagocytes [13–18]. A recent *in vivo* study of Dekker et al. [19] has shown that neutrophils of PKCβ-knockout mouse have a 50% decrease in the level of O_2^- production compared to that in neutrophils of the normal mouse. The application of a PKCβ antisense oligonucleotide to differentiated HL-60 (dHL-60) cells also suggested that PKCβ was involved in the phosphorylation of $p47^{phox}$ and the production of O_2^- [20]. Thus, several reports showed that cPKC, especially PKCβ, was responsible for the activation of NADPH oxidase by directly phosphorylating $p47^{phox}$. However, the role of PKCδ in the activation of NADPH oxidase is still unknown.

In phagocytosis by neutrophils, it has been reported that the particle internalization is initiated by the interaction of specific receptors of phagocytes with ligands on the surface of the particle and is strongly associated with polymerization of actin at the ingestion site [21]. We recently demonstrated that the ingestion of latex particles opsonized with serum was inhibited by SB203580, a p38 MAPK inhibitor, but not GF109203X (GFX), a PKC inhibitor, suggesting that p38 MAPK, but not PKC, participated in the signaling pathways of phagocytosis [4]. However, in phagocytosis by LPS-treated peritoneal macrophages, it was reported that the myristoylated alanine-rich C kinase substrate (MARCKS), which is known as a major substrate for the PKC family, was rapidly phosphorylated in the formation of zymosan phagosomes with kinetics similar to the formation of F-actin and that the treatment with PKC inhibitors attenuated the formation of zymosan phagosomes [22]. Thus, the involvement of the PKC family in phagocytosis is still controversial. PKCδ was reported to regulate the redistribution of F-actin [23], but the involvement of PKCδ in the phagocytic process has not been reported yet.

In this study, we focused on the role of PKCδ in the signaling pathway leading to the bactericidal action, the activation of NADPH oxidase and the phagocytosis with polymerization of F-actin by employing rottlerin as a PKCδ inhibitor [24]. Effects of this inhibitor on these functions in bovine neutrophils and dHL-60 cells were examined.

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Materials and methods

Reagents

DMSO, zymosan A, horseradish peroxidase (HRP), ficoll and fMet–Leu–Phe (fMLP) were purchased from Sigma Chemical Co. (St Louis, MO). Luminol and *N*6,2′-*O*-dibutyryladenosine-3′,5′-cyclic AMP (dbcAMP) were from Wako Pure Chemicals Industries, Ltd (Osaka, Japan). Rottlerin and GFX were from Calbiochem (La Jolla, CA). [^{32}P] Orthophosphoric acid was from MP Biomedicals, Inc. (Irvine, CA). Protein G-Sepharose was from Amersham-Pharmacia Biotech (Buckinghamshire, UK). Alexa Fluor 488 Phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR). Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS(−)) was from Invitrogen Co., (Carlsbad, CA). Fluorescent polystylene latex particles (1.0 μm diameter) were obtained from Polysciences, Inc. (Warrington, PA). The anti-$p47^{phox}$ antibody was a kind gift from Babior, The Scripps Research Institute, CA.

Isolation of bovine peripheral neutrophils

Peripheral blood was obtained from clinically healthy cows and suspended in HBSS(−). The suspension was layered on Ficoll–Conray solution and centrifuged at 1500*g* for 30 min at 20°C. After removal of the supernatant, the pellet was suspended in 0.8% NH_4Cl and incubated for 10 min at 4°C to lyse erythrocytes. Cells were collected by centrifugation at 500*g* for 5 min at 4°C and washed with HBSS(−). Isolated cells were suspended in HBSS(−). Cell viability was always more than 95% by the dye-exclusion test with trypan blue.

Cell culture and differentiation

Human promyelocytic HL-60 cells were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. For differentiation, HL-60 cells were centrifuged and suspended at 1×10^6 cells/ml in fresh medium and then exposed to 0.5 mM dbcAMP for 72 h. The cell viability of dHL-60 cells determined by trypan blue exclusion was more than 90% after differentiation.

Preparation of serum-opsonized zymosan (OZ)

Zymosan was suspended in fresh bovine serum at a concentration of 10 mg/ml and incubated for 30 min at 37°C. After incubation, the suspension was washed twice with HBSS(−) and resuspended in HBSS(−) at a concentration of 10 mg/ml.

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Assay of the production of O_2^-

The production of O_2^- was measured by chemiluminescence with luminol. HBSS with 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS(+)) containing 3×10^6 neutrophils, 10 μM luminol and 50 μg/ml HRP was prepared in each well of a 96-well microplate. The suspension, with a volume of 315 μl, was incubated for the indicated time at 37°C. After incubation, neutrophils were activated by adding 35 μl of the stimulant, OZ (10 mg/ml) or fMLP (1 μM), for bovine neutrophils or dHL-60 cells, respectively, and then chemiluminescence from each cell was measured with a luminometer (Luminescencer-JNR; ATTO Co., Tokyo, Japan) for 0.5 s at 37°C.

Preparation of membrane fraction

After stimulation with OZ or fMLP, neutrophils or dHL-60 cells (2.5×10^7) were suspended in homogenization buffer (0.34 M sucrose, 7 mM $MgSO_4$, 1 mM PMSF, 10 μg/ml leupeptin in 20 mM K^+/Na^+ phosphate buffer, pH 7.4) and then sonicated (10 s × 3). Nuclei, granules and debris were removed by centrifugation at 10,000*g* for 10 min at 4°C. Supernatants were ultracentrifuged at 175,000*g* for 90 min at 4°C and pellets (membrane fraction) were resuspended in homogenization buffer. Proteins in the fraction were quantified with Bio-Rad protein assay reagent using BSA as a standard. The protein concentration was adjusted and Laemmli sample buffer was added to each sample, which was then boiled for 5 min. Proteins were subjected to SDS-PAGE and western blotting. The amounts of $p47^{phox}$ detected on the membranes fraction were quantified by densitometry.

Phosphorylaion of $p47^{phox}$

dHL-60 cells were harvested and washed once with phosphate-free DMEM. Cells (4×10^7 cells/ml) were incubated for 3 h at 37°C in phosphate-free DMEM supplemented with 0.25 mCi/ml ^{32}Pi (orthophosphoric acid). After incubation, they were washed once with HBSS(−) and stimulated with fMLP for 1 min at 37°C. Stimulation was stopped by addition of ice-cold HBSS(−) followed by brief centrifugation. Cell pellets were suspended with 800 μl of IP buffer (20 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 0.25 mM sucrose, 5 mM EDTA, 25 mM NaF, 5 mM β-glycerophosphate, 1 mM *p*-nitrophenylphosphate, 5 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM PMSF) and incubated on ice for 30 min. After centrifugation at 16,000*g* for 30 min at 4°C, supernatants were collected. Cell lysates were rotated with protein G-Sepharose for 1 h at 4°C and precleared lysates were then collected. $p47^{phox}$ was immunoprecipitated with a $p47^{phox}$ antibody complexed with protein G-Sepharose beads by overnight rotation at 4°C. Beads were washed three times with IP buffer and 1 × Laemmli sample buffer was added to each sample. Proteins were subjected to SDS-PAGE and phosphorylated $p47^{phox}$ was visualized and quantified with a FUJIX BAS2500 imaging analyzer (Fuji Photo Film, Tokyo, Japan).

Measurement of phagocytic activity

Phagocytosis of fluorescent latex particles by neutrophils was measured by the following flow cytometric technique. Serum-opsonized fluorescent particles (1.0 μm diameter) were prepared by a procedure similar to that used for OZ preparation. After washing, particles were resuspended in HBSS(−) at a concentration 1×10^8 particles/ml. Two hundred microliters of HBSS(+) containing 2×10^6 neutrophils was preincubated for 5 min at 37°C. Then, 100 μl of suspension containing opsonized particles was added to the preincubated cell suspension (cell:particle = 1:5) and the mixture was further incubated for 30 min at 4 or 37°C. After centrifugation at 500*g* for 10 min at 4°C, the cells were washed and resuspended in HBSS(−). The fluorescence of the particles in neutrophils was analyzed by flow cytometry (EPICS XL; Beckman Coulter, Fullerton, CA). The neutrophils that ingested the particles were gated by excluding the counts of free particles.

Flow cytometric analysis of the formation of F-actin

Two hundreds microliters of HBSS(+) containing 2×10^6 cells was preincubated for the indicated time at 37°C. Cells were stimulated with OZ or fMLP for the indicated time at 37°C and then the reaction was stopped by adding 1 ml of ice-cold 4.5% paraformaldehyde, followed by fixation incubation for 10 min at 4°C and subsequently for 30 min at room temperature. The cells were washed twice and resuspended in the staining solution (10 U/ml Alexa Fluor 488 Phalloidin in 100 μg/ml lysophosphatidylcholine/PBS) and incubated in the dark for 30 min at room temperature. After the unbound phalloidin was removed by washing twice with PBS, the cells were analyzed by flow cytometry.

Results

Effects of PKC inhibitors on the production of O_2^-

We first examined the production of O_2^- evoked by the activation of NADPH oxidase in bovine neutrophils exposed to OZ by chemiluminescence with luminol. Effects of inhibitors were estimated by comparing peak values of the production of O_2^-. As shown in Figure 1(a), the treatment of cells with rottlerin, a specific inhibitor of PKCδ, resulted in partial but significant inhibition of the production of O_2^-. On the other hand, GFX efficiently attenuated the production of O_2^- elicited

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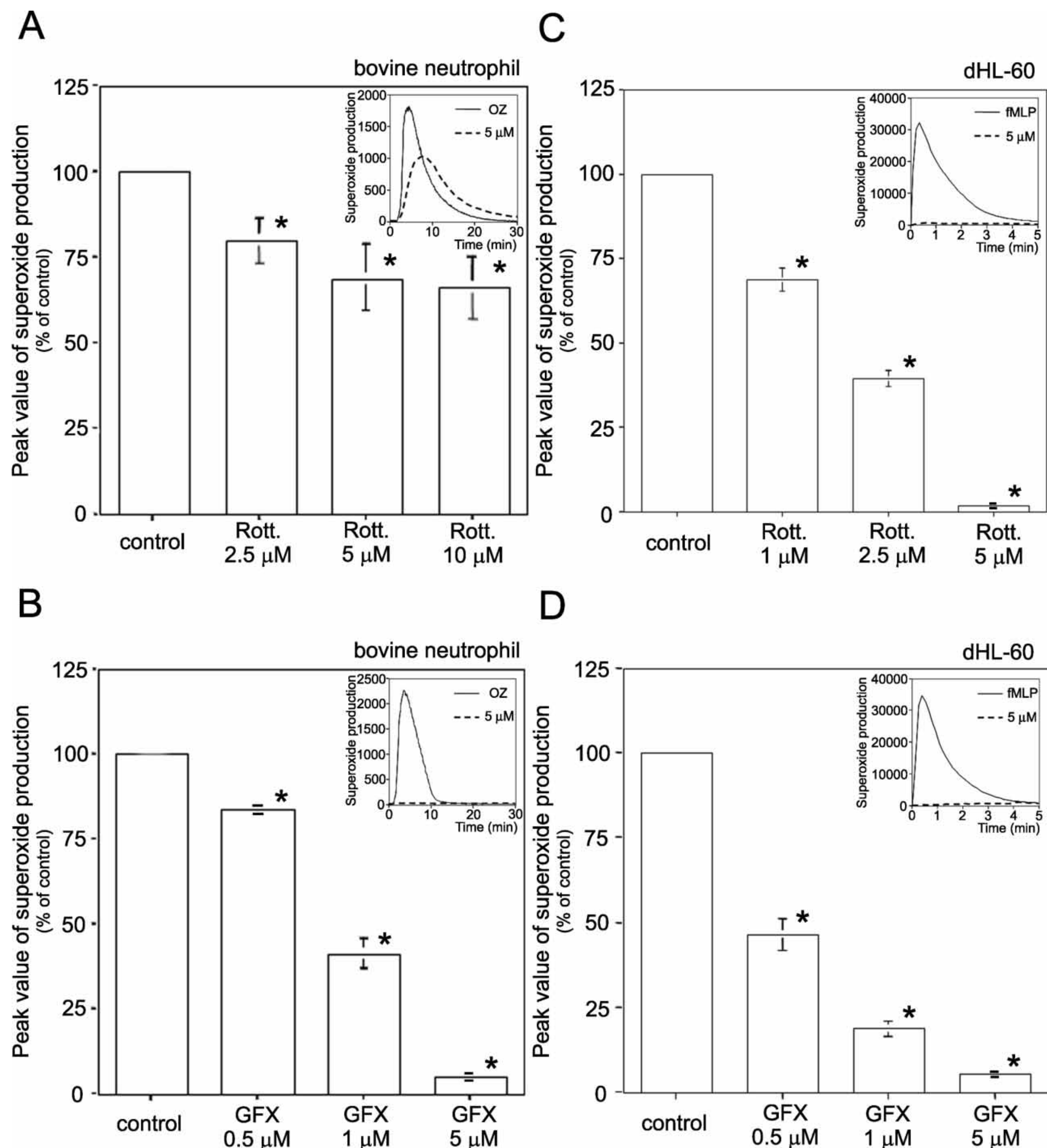

Figure 1. Effects of PKC inhibitors on the production of O_2^- in OZ-stimulated bovine neutrophils and fMLP-stimulated dbcAMP-differentiated HL-60 cells (dHL-60). The production of O_2^- was measured by chemiluminescence as described in the text. After preincubation for 5 min (bovine neutrophil) or 3 h (dHL-60) at 37°C with or without an inhibitor, cells were stimulated with 1 mg/ml OZ or 100 nM fMLP at 37°C. The peak value of O_2^- production induced by OZ in the absence of inhibitors was defined as 100%. The results are expressed as the mean ± SD of three independent measurements. (a) Rottlerin in bovine neutrophils, (b) GFX in bovine neutrophils, (c) rottlerin in dHL-60 cells and (d) GFX in dHL-60 cells. Inset: Representative time course of the O_2^- production of the control (solid line) and 5 μM inhibitor (broken line). Statistical significance was assessed by Student's *t*-test and is indicated by $*p < 0.01$, compared to the control.

by OZ and GFX almost completely abolished it at the concentration of 5 μM (Figure 1(b)). In the experiment with dHL-60 cells, chemotactic peptide fMLP was used as a stimulus. fMLP elicited more rapid and massive production of O_2^- than OZ stimulation did (insets of Figure 1). The treatment of cells with rottlerin or GFX attenuated the production of O_2^- in a concentration-dependent manner (Figure 1(c) and (d)) and each drug at 5 μM completely inhibited it. These results suggested that PKCδ was essential for the activation of NADPH oxidase irrespective of the cell type and stimulus.

Translocation of p47^{phox} to membrane and the phosphorylation of p47^{phox} proceeding it

Since the activation of NADPH oxidase was known to be triggered by the translocation of p47^{phox} to the membrane fraction as described above, we investigated the relationship between the translocation

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of p47^{phox} and PKCδ. The upper panels of Figures 2(a) and (b) show the time course of p47^{phox} translocation to the membrane fraction in OZ-stimulated bovine neutrophils and fMLP-stimulated dHL-60cells, respectively. These results were similar to those for the production of O_2^-. To determine whether PKCs were involved in the translocation of p47^{phox}, we examined the effects of PKC inhibitors on it. The treatment of cells with 5 μM GFX significantly inhibited the translocation of p47^{phox}, but that with 5 μM rottlerin did not in either neutrophils or dHL-60 cells (bottom panel of Figures 2(a) and (b)). Since the translocation of p47^{phox} is initiated by its phosphorylation, we further examined the phosphorylation of p47^{phox} in fMLP-stimulated dHL-60 cells, because OZ-stimulation was too weak to detect clear phosphorylation. As shown in Figure 2(c), the treatment of cells with rottlerin did not substantially affect the fMLP-induced phosphorylation of p47^{phox} but that with GFX reduced it to the control level.

Effects of PKC inhibitors on phagocytosis

To examine phagocytosis in bovine neutrophils, we used fluorescent particles opsonized by bovine serum for phagocytic stimulation. When neutrophils were incubated in the presence of fluorescent particles for 30 min at 4°C, a flow cytometric profile similar to that without fluorescent latex particles was observed (Figures 3(a) and (b)), indicating the absence of non-specific adherent particles. In neutrophils incubated in the presence of fluorescent latex particles for 30 min at 37°C, a fluorescent population denoted in gray appeared, indicating the presence of phagocytic neutrophils (Figure 3(c)). When neutrophils were treated with 5 μM rottlerin, a phagocytic population was diminished (Figure 3(d)), indicating the presence of the PKCδ-dependent regulation in phagocytosis. By contrast, the treatment of neutrophils with 5 μM GFX did not inhibit the phagocytic activity (Figure 3(e)). The results quantitatively represented were shown in Figure 3(f), suggesting that PKCδ, but not cPKC, was closely associated with reorganization of the cytoskeleton.

Effects of PKC inhibitors on the formation of F-actin

Cytoskeletal reorganization is a crucial step in the expression of bactericidal abilities such as chemotaxis and phagocytosis. It has been reported that phorbol 12-myristate 13-acetate (PMA), a direct activator of PKCs, stimulates actin assembly, suggesting that PKCs are involved in cytoskeletal reorganization [16]. Therefore, we examined the participation of PKCs in the formation of F-actin. Actin exists as globular actin (G-actin) in resting cells and turns into fibrous actin (F-actin) when activated. By flow cytometry using fluorescent-dye-conjugated phalloidin, we detected

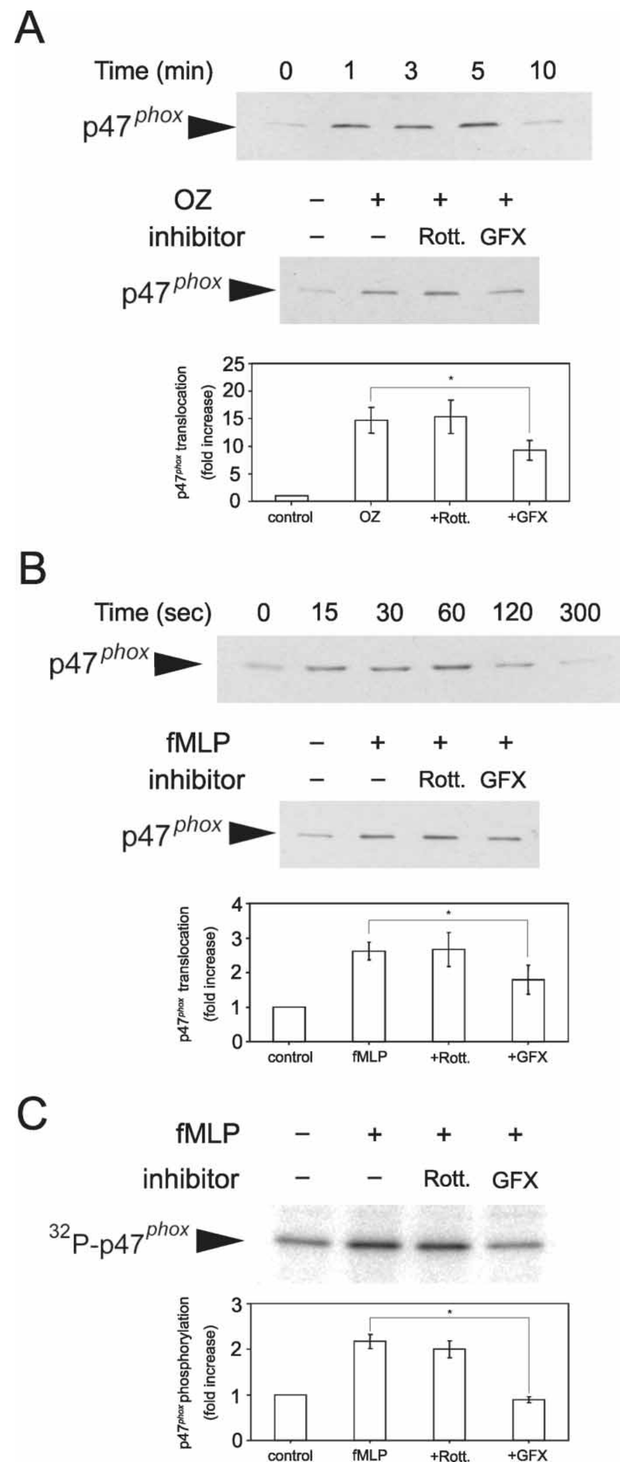

Figure 2. Effects of PKC inhibitors on the translocation and phosphorylation of p47^{phox}. (a) The translocation of p47^{phox} in OZ-stimulated bovine neutrophils. Upper panel: the time-dependent translocation of p47^{phox} on plasma membrane. Bottom panel: the effects of PKC inhibitors on the translocation of p47^{phox}. Neutrophils were incubated with or without an inhibitor for 5 min at 37°C, followed by incubation with OZ for 5 min for stimulation. (b) The translocation of p47^{phox} in fMLP-stimulated dHL-60 cells. Upper panel: the time-dependent translocation of p47^{phox} on plasma membrane. Bottom panel: the effects of PKC inhibitors on the translocation of p47^{phox}. dHL-60 cells were incubated with or without an inhibitor for 3 h at 37°C, followed by incubation with fMLP for 1 min for stimulation. (c) The effects of PKC inhibitors on the phosphorylation of p47^{phox}.

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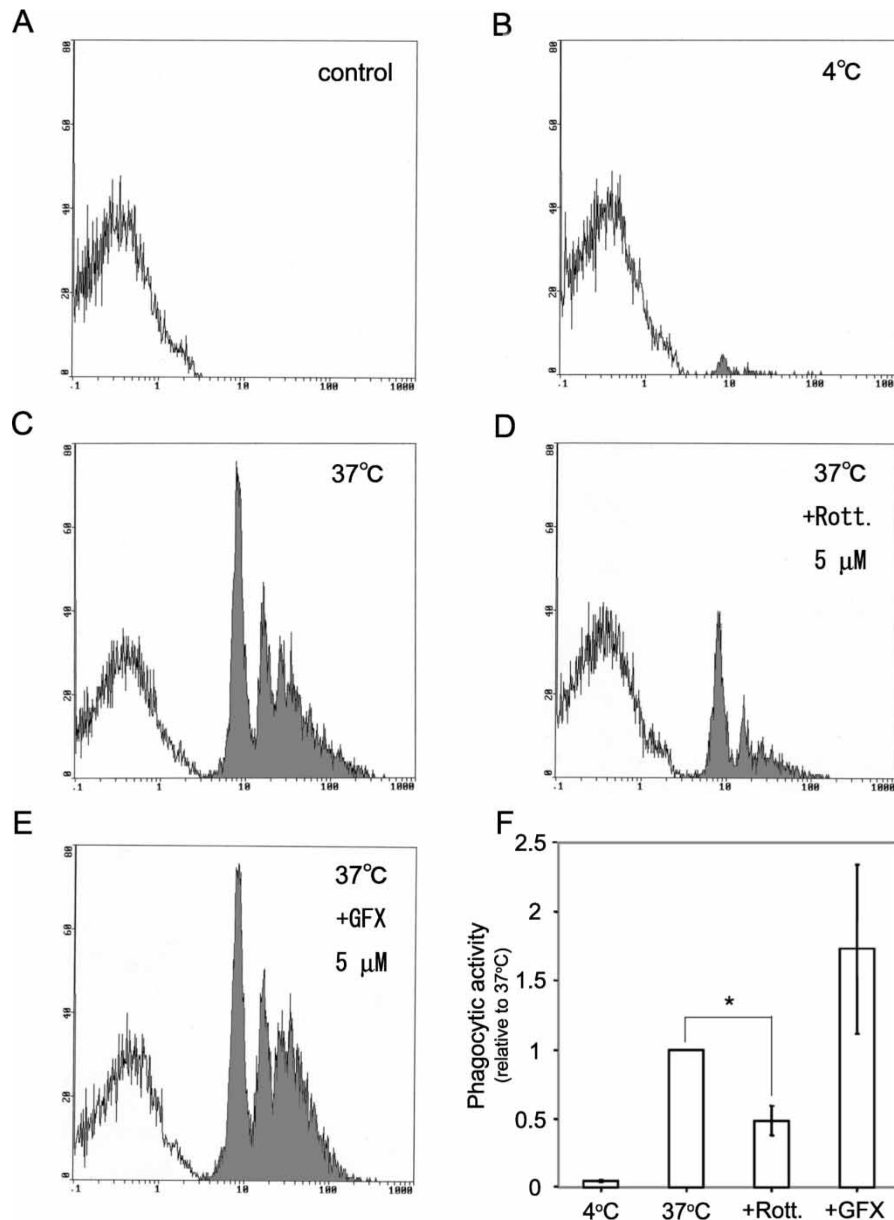

Figure 3. Effects of PKC inhibitors on the phagocytosis in serum-opsonized fluorescent latex particle-stimulated bovine neutrophils. The phagocytosis was estimated by flow cytometric analysis with fluorescent particles. Cells were preincubated with or without an inhibitor for 5 min at 37°C prior to stimulation. Representative flow cytometric profiles of (a) neutrophils without latex particles (control), (b) neutrophils incubated at 4°C for 30 min without an inhibitor, (c) neutrophils incubated at 37°C for 30 min without an inhibitor, (d) neutrophils incubated at 37°C for 30 min with 5 μM rottlerin and (e) neutrophils incubated at 37°C for 30 min with 5 μM GFX. Gray areas in profiles indicate phagocytic cells. Representative profiles from three experiments are shown. (f) Graphic representation of phagocytic activity in bovine neutrophils. The phagocytic activities are shown relative to responses at 37°C without inhibitor. The results are expressed as the mean ± SD of three independent measurements. Statistical significance was assessed by Student's *t*-test and is indicated by $*p < 0.05$, compared to responses at 37°C without inhibitor.

dHL60 cells were incubated with or without an inhibitor in the presence of 0.25 mCi/ml ^{32}Pi for 3 h at 37°C prior to stimulation with fMLP for 1 min. After stimulation, p47^{phox} was immunoprecipitated and analyzed by SDS-PAGE, followed by autoradiography as described in the text. Relative radioactivity was analyzed using a BAS2500. Data are representative of three experiments. Each graph in a, b and c shows the fold increase in the translocation and phosphorylation of p47^{phox}. The results are expressed as the mean ± SD of three independent measurements. Statistical significance was assessed by Student's *t*-test and is indicated by $*p < 0.05$, compared to OZ- or fMLP-induced responses without inhibitor.

the formation of F-actin in bovine neutrophils stimulated with OZ. The maximum formation was observed at 3 min after stimulation (Figure 4(a)) and, therefore, the effect of inhibitors was evaluated at this maximum point. The treatment of cells with rottlerin inhibited the OZ-induced formation of F-actin in a concentration-dependent manner. The treatment with 5 μM GFX had no effect on the F-actin formation (Figure 4(b)), although, this concentration of GFX completely abolished the production of O_2^- as shown in Figure 1(b).

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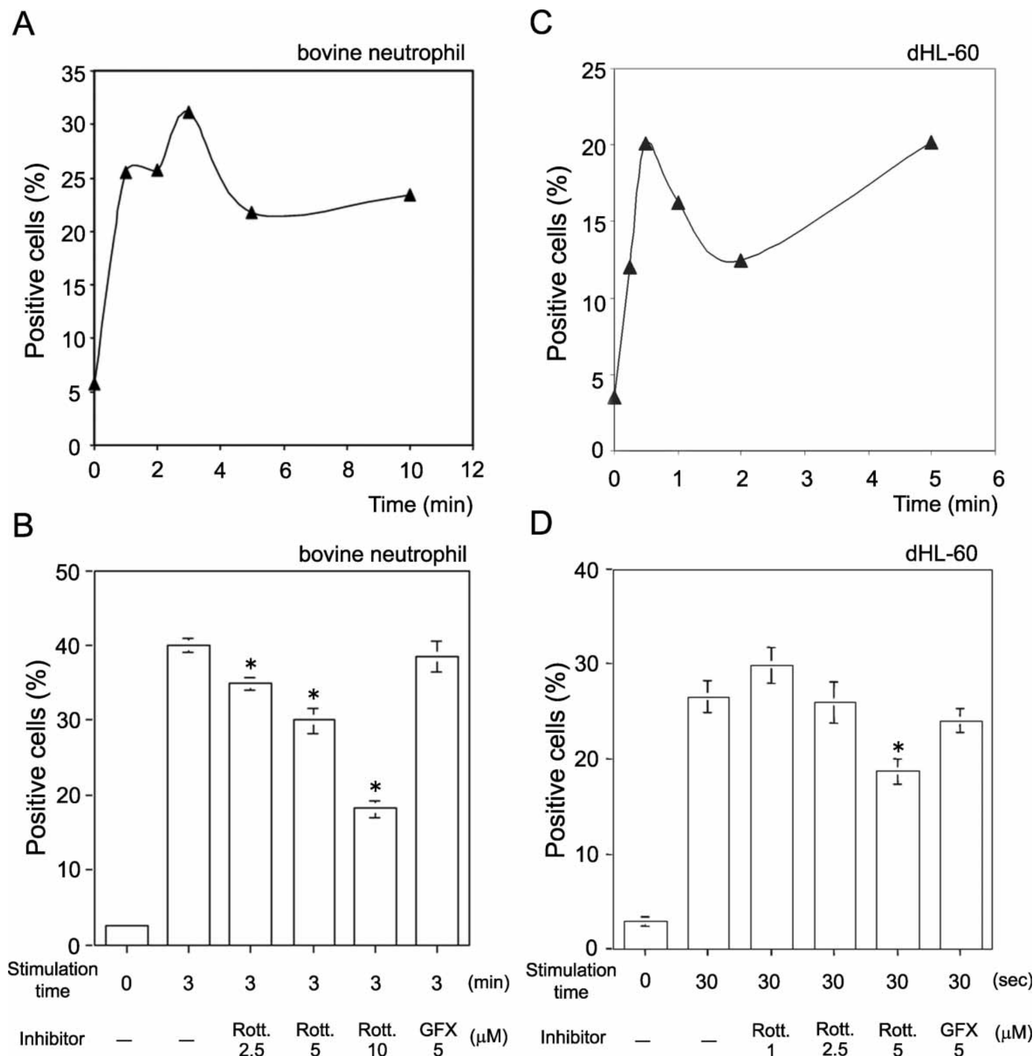

Figure 4. Effects of PKC inhibitors on the formation of F-actin in OZ-stimulated bovine neutrophils and fMLP-stimulated dHL-60 cells. The formation of F-actin was estimated by flow cytometric analysis with fluorescent-dye (Alexa Fluor 488) conjugated with phalloidin. (a) Time course of the formation of F-actin in bovine neutrophils stimulated by OZ. (b) Effects of PKC inhibitors on the formation of F-actin in OZ-stimulated bovine neutrophils. Cells were incubated with or without an inhibitor for 5 min at 37°C, followed by the incubation with OZ for 3 min for stimulation. (c) Time course of the formation of F-actin in dHL-60 cells stimulated by fMLP. (d) Effects of PKC inhibitors on the formation of F-actin in fMLP-stimulated dHL-60 cells. Cells were incubated with or without an inhibitor for 30 min at 37°C, followed by the incubation with fMLP for 30 s for stimulation. The results are expressed as the mean ± SD of three independent measurements. Statistical significance was assessed by Student's *t*-test and is indicated by $*p < 0.01$, compared to the 3 min or 30 s stimulated response in the absence of inhibitors.

When dHL-60 cells were challenged with fMLP, rapid formation of F-actin was observed (Figure 4(c)). This response was quicker than that of bovine neutrophils stimulated with OZ and the peak time was 30 s. A moderate inhibitory effect of rottlerin on the formation of F-actin was observed (Figure 4(d)). The inhibitory effect of rottlerinon the F-actin formation in OZ-treated bovine neutrophils and fMLP-treated dHL-60 cells was observed over 0–10 and 0–5 min, respectively (data not shown), indicating that this effect was due to the attenuation of overall F-actin formation but not the simple delay of it. These results suggested that PKCδ was involved in reorganization of the cytoskeleton in both bovine neutrophils stimulated with OZ and dHL-60 cells stimulated with fMLP.

Discussion

In the present study, we focused on the role of PKCδ in the activation of NADPH oxidase and phagocytosis. Many past studies employed PMA, a direct and potent activator of PKCs, to activate them for phagocytes [16,25]. To clarify the relationship between receptor-mediated functions and the signal transduction pathways associated with cPKC and PKCδ in neutrophils, we used two physiological agents, zymosan and latex particles opsonized by bovine serum, as stimuli

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for bovine neutrophils. These particles were mainly coated with IgG and iC3b and these opsonins were considered to stimulate FcγR and CR3 receptors in neutrophils, respectively, to activate a number of signaling molecules and to induce bactericidal ability [26]. Our previous study demonstrated that the OZ-induced activation of p38 MAPK, PKCs and PI3-K participated in the signaling upstream of $p47^{phox}$ and the Rac activation in bovine neutrophils [4,27]. In this study, we observed the opsonized particle-elicited production of O_2^-, phagocytosis and the formation of F-actin in bovine neutrophils. We also examined dHL-60 cells stimulated by the chemotactic peptide fMLP, which activates neutrophils via an fMLP receptor known as a seven transmembrane-spanning G-protein-linked receptor [28]. In this system, our previous reports have shown that fMLP evokes the activation of NADPH oxidase by ERK1/2 and cPKC in rat neutrophils [6] and the formation of F-actin by PI3K in dHL-60 cells [29].

In this study, we employed two pharmacological inhibitors of PKC, rottlerin, a specific inhibitor of PKCδ [24] and GFX, a broad spectrum inhibitor of PKCs, except for PKCδ, in phagocytes [30]. Rottlerin and GFX each significantly inhibited the production of O_2^- (Figure 1), whereas, only 5 μM GFX inhibited the translocation of $p47^{phox}$ to the membrane in both OZ-stimulated bovine neutrophils and fMLP-stimulated dHL-60 cells (Figure 2). Furthermore, GFX also inhibited the phosphorylation of $p47^{phox}$ in dHL-60 cells. Our previous studies reported that cPKC participated in the regulation of NADPH oxidase activity [4–6]. Korchak et al. demonstrated that depletion of PKCβ by an antisense oligonucleotide inhibited the production of O_2^- in dHL-60 cells by attenuating the phosphorylation and translocation of $p47^{phox}$ [20]. Since GFX is known to efficiently inhibit the cPKC family [31] and attenuated the activity of NADPH oxidase through the inhibition of phosphorylation and translocation of $p47^{phox}$ (Figures 1 and 2), the present data seem to support these previous studies. On the other hand, the treatment of neutrophils or dHL-60 cells with rottlerin inhibited the production of O_2^- but not the translocation of $p47^{phox}$, indicating that the inhibitory effect of rottlerin on the activation of NADPH oxidase was not due to inhibition of the translocation of $p47^{phox}$.

As shown in Figure 3, the phagocytic activity of bovine neutrophils was significantly inhibited by 5 μM rottlerin, but not by 5 μM GFX. Polymerization of actin is known to be essential for some steps of phagocytosis such as pseudopod extension and particle internalization [21]. When the formation of F-actin was examined, it was found that the treatment with rottlerin significantly attenuated the formation of F-actin not only in OZ-stimulated bovine neutrophils but also in fMLP-stimulated dHL-60 cells. Lopez-Lluch et al. [23] reported that a C2-like domain in the *N*-terminal of PKCδ interacted with F-actin and regulated the polarization of F-actin in neutrophils. Taken together, these data led us to speculate that the opsonized particle-induced phagocytic function in neutrophils was partly associated with PKCδ-mediated regulation for the formation of F-actin.

Brown et al. [32] have shown that the PMA-induced assembly of NADPH oxidase occurs primarily on intracellular granules in human neutrophils and PKCδ is involved in this process. Although, those data seem to support the present results concerning the involvement of PKCδ in the activation of NADPH oxidase as shown in Figure 1, it is difficult to explain the precise mechanisms for the regulation of NADPH oxidase by PKCδ. Quinn et al. [33] reported that the activity of NADPH oxidase in PMA-stimulated neutrophils was observed in the fraction cosedimented with the heavy plasma-membrane fraction including actin and fodrin. Woodman et al. [34] found that the activated oxidase was restricted to the membrane cytoskeleton. In a cell-free system of NADPH oxidase derived from the activated human neutrophil, actin-depolymerizing agents, DNase I and latrunculin, markedly facilitated the deactivation of NADPH oxidase [35], suggesting that F-actin has a role to prolong the lifetime of NADPH oxidase. As described above, it was demonstrated that PKCδ partly regulated the formation of F-actin in bovine neutrophils. Therefore, the inhibition of O_2^- production by rottlerin may be due to the instability of NADPH oxidase through inhibition of polymerization of F-actin.

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