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Ceramide Promotes Calpain-Mediated Proteolysis of Protein Kinase C β in Murine Polymorphonuclear Leukocytes

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Ceramide has been recognized as an important second messenger in intracellular signaling. We demonstrate here that ceramide promotes the down-regulation of protein kinase C (PKC) activity in phorbol ester-stimulated murine polymorphonuclear leukocytes (PMNs). As reported previously, treatment of PMNs with phorbol ester caused a translocation of PKC from the cytosolic to the membrane fractions. When PMNs were pretreated with cell-permeable ceramide analogue, C_2-ceramide, the membrane-associated PKC activity was rapidly down-regulated by phorbol ester stimulation. E64-d, a potent inhibitor of calpain which proteolyzes PKC, eliminated the rapid down-regulation of PKC activity. By hydroxyapatite column chromatography and Western blotting, the predominant PKC isoform was PKC β with a small amount of PKC α in murine PMNs. We found that ceramide strikingly promoted calpain-mediated proteolysis of PKC β *in vitro*. Ceramide was also shown to inhibit [^{3}H]phorbol 12,13-dibutyrate(PDBu) binding to PKC β. Moreover, we show that ceramide stimulates PKC β autophosphorylation. These results suggest that ceramide directly activates PKC β and promotes calpain-mediated proteolysis in murine PMNs.

Protein kinase C (PKC) is a Ca^{2+}, phospholipid-dependent, serine/threonine protein kinase and plays a crucial role in the signal transduction (1). PKC was found in many tissues and organs and regulates various cell functions (2). It is known that phorbol esters such as phorbol 12- myristate 13-acetate(PMA) activate PKC and cause a translocation of PKC from the cytosolic to the membrane fractions(3,4). After the translocation, PKC is known to be proteolyzed by calpain, which is a Ca^{2+}-dependent thiol proteinase, to an inactive form (2).

Meanwhile, ceramide is produced by sphingomyeline hydrolysis and is reported to have various effects on cellular functions, such as cell differentiation, cell growth, and induction of apoptosis(5,6). Ceramide is known to activate ceramide-dependent protein kinase (7), mitogen-activated protein kinase(8), and protein phosphatase(9). It has been reported that ceramide inhibits a translocation of PKC α in murine epidermal and human skin fibroblast cells(10), or inactivates PKC α by activating protein phosphatase in Molt-4 cells(9). Moreover, ceramide was reported to bind to PKC ζ, which is an atypical PKC, and regulate its kinase activity in tumor necrosis factor-stimulated U937 cells(11). However, the effect of ceramide on PKC β has not been elucidated.

We have previously reported that the down-regulation of PKC activity in cells is associated with cellular functions such as natural killer activity and concanavalin A-induced cap formation (12-14). In the present study, we demonstrate for the first time that ceramide promotes calpain-mediated down-regulation of PKC β in murine polymorphonuclear leukocytes(PMNs).

MATERIALS AND METHODS

Materials

C57BL/6J mice were originally obtained from Jackson Laboratories(Bar Harbor, ME). Six- to ten-week old mice were used in all experiments. Hanks' balanced salt solution (HBSS), polyclonal anti-peptide antibodies against PKC isoforms, protein A agarose were purchased from Life Technologies Inc.(Tokyo, Japan). DE52 was from Whatman. [γ-^{32}P]ATP and [^{125}I]protein A were from Amersham. [^{3}H]phorbol-12,13-dibutyrate (PDBu) was from American Radiolabeled Chemicals Inc. Ceramide, C_2-ceramide, PMA, and all other chemicals were purchased from Sigma Chemicals Co.

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Abbreviations: PKC, protein kinase C; PMNs, polymorphonuclear leukocytes; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks' balanced salt solution; PMSF, phenylmethylsulfonylfluoride; PS, phosphatidylserine; DAG, 1,2-diacylglycerol.

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Methods

Isolation of murine PMNs. PMNs were obtained by lavage with 5 ml of HBSS 16-20 h after intraperitoneal injection of 1 ml of sterile thioglycolate broth (Difco) as previously described (12). The cells obtained (5-8 $\times$ 10^6 cells / mouse) were 80-85% PMNs as determined by May-Giemsa staining, the remainder being macrophages and lymphocytes.

Assay for PKC activity. After PMNs were treated with the various agents, cells were disrupted by sonication for 20 s three times at 4 °C in 20 mM Tris-HCl(pH7.5), 0.25M sucrose, 2mM EDTA, 5mM EGTA, 2mM phenylmethylsulfonylfluoride (PMSF), 0.01% leupeptin and 50mM 2-mercaptoethanol. The cytosolic and the membrane fractions were prepared and partially purified with a DE52 column as previously described (12). In some experiments, PKC from total cell extract was prepared by sonication in the homogenate buffer with 0.1% Triton X-100. PKC activity was assayed by using the PKC enzyme assay system (Amersham) according to the manufacture's protocol. The protein concentration was measured by the method of Bradford (15), using bovine serum albumin as standard.

Hydroxyapatite column chromatography of PKC. After PKC from murine PMNs (1×10^8 cells) was purified by a DE52 column as described above, samples were desalted and applied to a packed hydroxyapatite column (TSK-GEL HA-1000, Tosoh, Tokyo, Japan), which was connected to a Pharmacia FPLC system (16). The column was equilibrated with 20mM potassium phosphate buffer(pH7.5), containing 0.5mM EGTA, 0.5mM EDTA, 10mM 2-mercaptoethanol, and 10% glycerol. The protein kinase was eluted by an application of 30 ml of linear concentration gradient of potassium phosphate (20 to 250mM) at a flow rate of 0.5 ml/min. Fractions of 1 ml each were collected and enzyme activity was assayed.

Western blotting. PKC purified by hydroxyapatite column chromatography was subjected to SDS-polyacrylamide gel electrophoresis using a 7.5% acrylamide gel. The protein was transferred onto a nitrocellulose membrane. Non-specific sites were blocked by incubating the membrane with 1% bovine serum albumin and 0.05% Tween 20 for 2 h at 25 °C. The immunoreaction complex was detected with [^{125} I]protein A (0.1 μ Ci/ml) as described previously (17). The membrane was exposed to Fuji RX films with intensifying screens at −70°C for three days.

Proteolysis of PKC with calpain. Proteolysis of PKC with calpain was performed according to the method described by Kishimoto et al.(18). The reaction mixture (0.4ml) contained 20 mM Tris-HCl(pH7.5), 10mM 2-mercaptoethanol, fatty acid-free bovine serum albumin (20 μg), Triton X-100(0.015%), Tween 20(1%), PKC samples and 1 μg of μ-calpain(Chemicon International Inc., Temecula, CA). Triton X-100, Tween 20, and albumin at these concentrations did not interfere with proteolysis, but apparently stabilized PKC during incubation. Phosphatidylserine (PS) and 1,2-diacylglycerol (DAG) were added, and the reaction was started by the addition of $CaCl_2$ at a final concentration of 0.2 mM. Where indicated, ceramide was added to the incubation. After incubation at 20°C for various lengths of time, the reaction was stopped by the addition of 10 μl of 25mM EGTA. Aliquots were immediately assayed for protein kinase activity.

[^{3}H]PDBu binding assay. Binding of [^{3}H]PDBu was measured according to the method of Huang et al.(19). The reaction mixture (0.2ml) contained 30mM Tris-HCl(pH7.5), 6mM magnesium acetate, 0.25mM EGTA, 0.4mM $CaCl_2$, 40 μg/ml PS, 0.5mg/ml bovine serum albumin, 50nM [^{3}H]PDBu, and PKC samples. Ceramide was added to the mixture at various concentrations. Non-specific binding was determined by the addition of 100 μM of unlabeled PDBu. The mixture was incubated at room temperature for 30 min followed by incubation at 4 °C for 30 min after adding 0.5ml of 30% DEAE-cellulose in 20 mM Tris-HCl (pH7.5). Bound [^{3} H]PDBu was separated by filtering through a Whatman GF/C glass fiber filter. The radioactivity was determined in a scintilation counter.

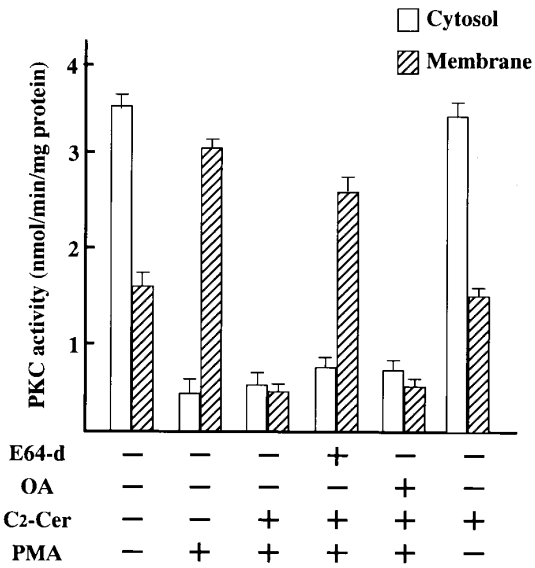

FIG. 1. Effect of C_2-ceramide on PKC activity in murine PMNs. After murine PMNs(5×10^6 cells) were treated with C_2-ceramide(3 μM) or HBSS for 20 min at 37°C, PMA(0.1 μM) was added and incubated for 20 min. In some experiments, cells were preincubated with E64-d (1 μg/ml) or 10 nM of okadaic acid (OA) for 20 min before treatment with C_2-ceramide. PKC activity was assayed in the cytosolic (open column) and the membrane (hatched column) fractions. The data are means±S.E. of five independent experiments.

Immunoprecipitation and autophosphorylation of PKC. Immunoprecipitation was performed according to the method described previously (20). The homogenate from PMNs was incubated with 2 μl of antipeptide antibodies against PKC isoforms at 0°C for 2 h, and then 100 μl of protein A agarose was added. After 1h- incubation at 4°C, the immunoprecipitates were washed three times with a washing solution (10 mM Tris-HCl,pH7.5, 500 mM NaCl, 0.5% Nonidet P-40, 1mM PMSF and 0.005% leupeptin) and once with a solution of 20 mM Tris-HCl(pH7.5), 0.5 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 10% glycerol, 0.01% leupeptin and 2 mM PMSF. The reaction for autophosphorylation was done according to the method described previously (21). The immunoprecipitates were mixed in a final volume of 20 μl in an assay mixture (20 mM Tris-HCl pH7.5, 10mM $MgCl_2$, 0.8mM $CaCl_2$, 0.25mM EDTA, 0.25mM EGTA, 1 μM of [γ-P^{32}]ATP) in the presence or absence of PS, DAG and ceramide. After the mixture was incubated for 5min at 37°C, the reaction was stopped by adding 3×sample buffer and then subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The amounts of protein loaded in each lane were approximately equal, confirmed by Coomassie Brilliant Blue staining. The phosphorylation was quantified by using an image analyzer (Fuji).

RESULTS

C_2-Ceramide Promotes the Down-regulation of PKC in PMA-Treated PMNs

We first examined the effect of C_2-ceramide on PMA-induced translocation of PKC activity in murine PMNs. As shown in Fig.1, a translocation of PKC from the cytosolic to the membrane fractions was observed after treatment with PMA(0.1 μM) for 20 min. When cells were pretreated with C_2-ceramide (3 μM) for 20 min,

the membrane-associated PKC activity had remarkably declined after PMA treatment. However, E64-d (1 μg/ml), a potent inhibitor of calpain which proteolyzes PKC, abolished the rapid decline in the membrane-associated PKC. E64-d did not affect the translocation of PKC. As it is known that ceramide activates protein phosphatase and inactivates PKC α (9), okadaic acid which is an inhibitor of protein phosphatase, was used in our experiment. However, okadaic acid(10nM) did not alter the ceramide-induced down-regulation of PKC. In addition, C_2-ceramide alone did not alter PKC activity in either the cytosolic or the membrane fractions of murine PMNs.

We next examined which isoforms of PKC were down-regulated by C_2-ceramide in PMA-treated murine PMNs. To separate PKC isoforms, we performed hydroxyapatite column chromatography and assayed for PKC activity in each fraction. As shown in Fig.2A, one major peak and a small peak of PKC were observed. By Western blotting, the first major peak reacted only to anibody against PKC β (Fig.2B). The second small peak reacted only to antibody against PKC α and, in addition, other PKC isoforms did not show detectable levels in murine PMNs (data not shown). Since we found that the predominant PKC isoform was PKC β and the amount of PKC α was very small in murine PMNs, we focused our attention on PKC β and examined the effect of ceramide on PKC β in our subsequent experiments. We then examined whether ceramide enhanced calpain-mediated proteolysis of PKC β *in vitro*. Fig.2C shows that ceramide (3 μM) strikingly enhanced the calpain-mediated proteolysis of PKC β in the presence of PS and DAG. Addition of ceramide to the incubation without DAG also enhanced the proteolysis. Parallel to the decline in PKC activity, Ca^{2+}, phospholipid-independent kinase activity was increased. We also confirmed by hydroxyapatite column chromatography that in whole PMNs, treatment of cells with C_2-ceramide followed by PMA stimulation also induced PKC β degradation (data not shown). These results demonstrate that ceramide promotes calpain-mediated down-regulation of PKC β in murine PMNs.

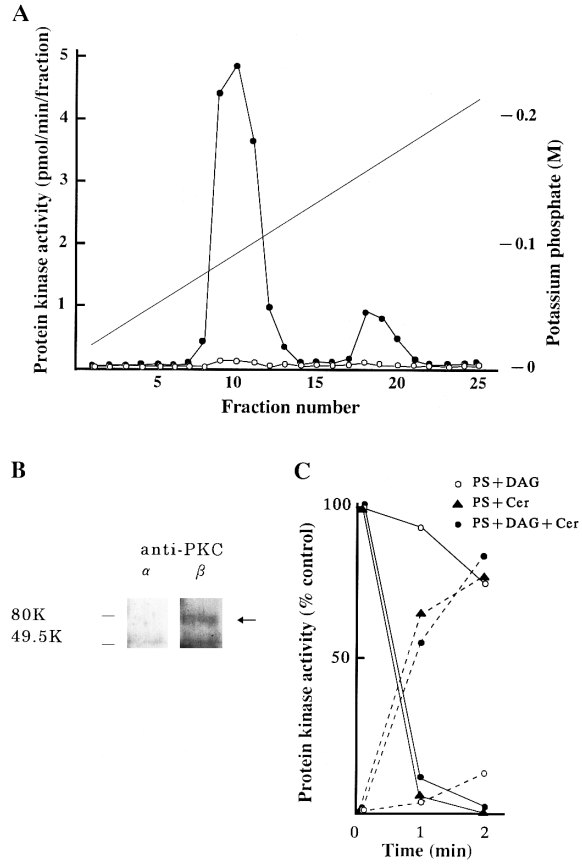

FIG. 2. Separation of PKC isoforms and the effect of ceramide on calpain-mediated proteolysis of PKC β in murine PMNs. *Panel A,* PKC from total cell extract, purified by a DE52 column, was desalted and applied to a hydroxyapatite column. Protein kinase activity was assayed in the presence(●) or absence(○) of Ca^{2+} and phospholipid. The solid line means the concentration of potassium phosphate. The data are representative of five independent experiments. *Panel B,* the major peak fractions of PKC activity by hydroxyapatite column chromatography were concentrated and analyzed by Western blotting using antibodies against PKC isoforms. Molecular sizes are shown (*left*) and the arrow indicates PKC β (*right*). *Panel C,* the peak fractions of PKC β were incubated with 0.2mM of $CaCl_2$, 20 μg/ml of PS and 1 μg of μ-calpain in the presence of 3 μM of DAG (○), 3 μM of ceramide (▲) or DAG and ceramide (●). The solid line means PKC activity (percent of control) and the dotted line means Ca^{2+}, phospholipid-independent kinase activity. The data are representative of five independent experiments with similar results.

Ceramide Inhibits Phorbol Ester Binding to PKC β

It is known that DAG, which is an activator of PKC, inhibits phorbol ester binding to PKC (21). To investigate the mechanism of ceramide-induced down-regulation of PKC β, the effect of ceramide on phorbol ester binding to PKC β was examined. As shown in Fig. 3, ceramide inhibited [^{3}H]PDBu binding to PKC β with 50% inhibition occurring at 3 μM. However, ceramide slightly enhanced PKC β activity when added to the PKC assay mixture (Fig.3). These results indicate that ceramide competes for phorbol ester binding sites of PKC β and activates its kinase activity.

Ceramide Stimulates Autophosphorylation of PKC β

To confirm the fact that ceramide directly activates PKC β, we examined the effect of ceramide on the autophosphorylation of PKC β *in vitro*. As shown in Fig. 4, ceramide (3 μM) markedly stimulated autophosphorylation of immunoprecipitated PKC β in the presence of PS. It is noted that the levels of autophosphorylation stimulated by ceramide were higher (2.26 times) than phosphorylation induced by DAG at the same concentration, when phosphorylation was quantified by an image analyzer. Ceramide also stimulated the auto-

phosphorylation in the presence of PS and DAG. It was found that ceramide directly stimulated PKC β autophosphorylation.

DISCUSSION

In the present study, we demonstrate for the first time that ceramide strikingly enhances calpain-mediated down-regulation of PKC β in murine PMNs. PKC β is a classical PKC which is a Ca^{2+}, phospholipid-dependent protein kinase (21). PKC β is related to immunological function, since PKC β-deficient mice develop an immunodeficiency characterized by impaired humoral immune responses and reduced cellular responses of B cells (22). Recently, Lee et al. (9) have reported that ceramide inactivates PKC α by activating protein phosphatase in Molt-4 cells (9). They mentioned in that report that ceramide also inactivates PKC β_{II}, by *in vitro* kinase assay using immunoprecipitated PKC and histone H1 as substrate. In our experiments, however, pretreatment of murine PMNs with okadaic acid, a potent protein phosphatase inhibitor, did not alter the ceramide-induced down-regulation of PKC measured by using specific substrate peptide for PKC. The discrepancy may be due to the difference between human cell line and murine PMNs, or the difference in the experimental condition of kinase assay.

Phorbol ester is known to bind to cystein-rich repeat of C1 region of PKC β (21). In our study, ceramide was found to inhibit phorbol ester binding to PKC β. It is thus considered that ceramide binds to phorbol ester binding sites of PKC β, similar to DAG (21). Although ceramide inhibited phorbol ester binding, it inhibited neither PKC β activity nor PMA-induced translocation of PKC from the cytosolic to the membrane fractions. Despite the inhibition of phorbol ester binding, ceramide slightly enhanced PKC activity when added to the assay mixture for PKC. In addition, ceramide did not directly affect calpain activity, confirmed by calpain assay (23) using casein as substrate(data not shown). These findings suggest that ceramide directly activates PKC β.

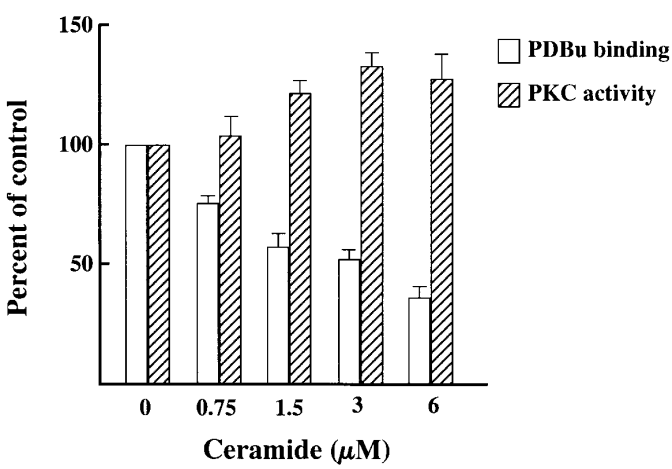

FIG. 3. Effects of ceramide on phorbol ester binding and protein kinase activity of PKC β. PKC β purified by hydroxyapatite column chromatography was measured for [^{3}H]PDBu binding activity (open column) and PKC activity (hatched column) in the presence of various concentrations of ceramide. The activities are shown as percent of control and are means±S.E. of three experiments.

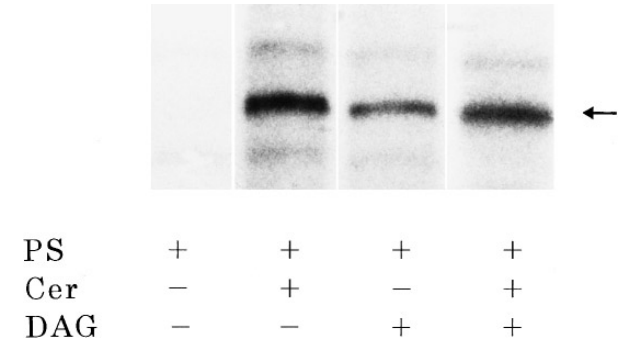

FIG. 4. Effect of ceramide on PKC β autophosphorylation. Immunoprecipitated PKC β was assayed for autophosphorylation. Incubation was performed in the presence or absence of PS (10 μg/ml), DAG (3 μM) and ceramide (3 μM). Autophosphorylated PKC β is indicated with an arrow (*right*). Data are representative of three independent experiments with similar results.

Ceramide was also shown to stimulate autophosphorylation of PKC β. It is known that the PMA-induced down-regulation of PKC α requires its autophosphorylation (24). In that paper, the mutant PKC α which lacks kinase activity, is resistant to down-regulation by phorbor ester. Moreover, other reports have demonstrated that the autophosphorylation of residues in the hinge region, close to the site of cleavage by calpain, may alter the susceptibility of PKC β to proteolysis (21,25). In our study, the levels of PKC β autophosphorylation stimulated by ceramide were more than two times higher than that induced by the same concentration of DAG. While DAG slightly increases the proteolysis of PKC β, ceramide dramatically promotes the proteolysis probably because it strongly stimulates PKC β autophosphorylation. Ceramide may alter the conformation of PKC β, resulting in the enhancement of the susceptibility of calpain-mediated proteolysis. However, the precise mechanism of ceramide-promoted proteolysis of PKC β remains to be resolved. Further study is in progress to elucidate the mechanism and its role in the intracellular signal transduction.

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