

Negative Regulation of Class IA Phosphoinositide 3-kinase by Protein Kinase C δ Limits Fc γ Receptor-Mediated Phagocytosis in Macrophages

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Stimulation of macrophages by various ligands results in the activation of both phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC). Here, we showed that PKC δ selectively inhibits class IA PI3K. Prior exposure of macrophages to a PKC activator, phorbol 12-myristate 13-acetate (PMA) inhibited the PI3K activation induced by the Fc γ receptor (Fc γ R) ligation but not that induced by C5a. Prolonged PKC inhibition by GF109203X increased the basal PI3K activity of quiescent macrophages. The effect of the PKC inhibitor can be observed in macrophages from mice lacking class IB PI3K (p110 γ). Thus PKC was suggested to selectively attenuate the class IA activity. Chronic PKC activation by PMA induced PKC δ degradation and Akt activation. Enhancement of the basal Akt activity was also observed in cells stably deficient in PKC δ prepared by shRNA technique. Fc γ R-mediated phagocytosis was dramatically increased in these cells. Thus it is suggested that inactivation of class IA PI3K by PKC δ is functioning in regulation of Fc γ R-mediated phagocytosis.

Key words: Akt, Fc γ receptor, Phagocytosis, Phosphoinositide 3-kinase, PKC δ .

Abbreviations: PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; Fc γ R, Fc γ Receptor; EA, IgG coated-sheep red blood cell; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide.

INTRODUCTION

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that catalyses the transfer of the γ -phosphate group of ATP to the D-3 position of phosphoinositides. Its product, PtdIns (3,4,5)P $_3$ (PIP $_3$), targets Akt/PKB, Bruton's tyrosine kinase (Btk), PDK, atypical PKCs, phospholipase C γ and others (1). The PI3K pathway is commonly activated through surface receptors in response to hormones and growth factors. In macrophages, this pathway is activated by the immune complex (2), TLR ligands, cytokines and chemotactic peptides (3,4).

Mammalian PI3K can be grouped into three major classes (I, II and III), based on their primary sequences, mechanisms of regulation and substrate specificities (5). Of the class I PI3Ks, class IA subtypes are heterodimers that consist of a catalytic subunit (p110) and a regulatory subunit (p85). These subtypes are thought to be the major *in vivo* source of PIP $_3$ upon activation of the receptors possessing protein-tyrosine kinase activity or the receptors coupling to Src-type protein-tyrosine kinases (5). In mammals, there are multiple isoforms of class IA PI3K. Different genes encode class IA catalytic subunits, referred to as p110 α , p110 β and p110 γ , while two genes encode the associating regulatory subunits, referred to as p85 α and p85 β (5). Class I includes another member, PI3K γ , which is mainly expressed in

hematopoietic cells (6). This subtype consists of a catalytic subunit (p110 γ) and a regulatory subunit (p101) and is classified as class IB. PI3K γ can be activated by the $\beta\gamma$ subunits of G proteins and thus mediates the signal from G protein-coupled receptors (6–8).

The PKC family consists of at least 11 isoforms that can be classified into three main subfamilies based on their homology and cofactor requirements for activation: conventional PKC α , β I, β II and γ are diacylglycerol (DAG)- and calcium-dependent; novel PKC δ , ϵ , θ and η are DAG-dependent and calcium-independent; and finally atypical PKC ζ , λ and ι are dependent only on phospholipids (9). We and several other groups have shown that PKC negatively regulate PI3K activity (10–16). However, the signaling events involved in the negative regulation are largely unknown. Furthermore, the PI3K isoforms susceptible to PKC activation is not identified. Likewise, the PKC isoforms responsible for the negative regulation have not been clarified yet. In this report, we show that PKC-dependent inhibition of PI3K was observed in PI3K γ -deficient cells as well as wild-type cells. In addition, PI3K was spontaneously activated in PKC δ -deficient cells through increased association of tyrosine phosphorylated proteins and the p85 regulatory subunit of PI3K. Thus, we present the evidence showing that PKC δ is involved in the inhibition of class IA PI3K but not class IB. Finally, we provide a finding that Fc γ R-mediated phagocytosis, which is closely related to PI3K activity, was dramatically increased in PKC δ -deficient cells. Taken together, PKC δ may limit PI3K activity to regulate Fc γ R-mediated events in normal macrophages.

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EXPERIMENTAL PROCEDURES

Material—Materials were obtained from the following sources: PMA, GF109203X, zymosan, pertussis toxin, ATP and C5a (Sigma); wortmannin (Kyowa Medex, Tokyo, Japan); RPMI 1640 medium (Invitrogen); protein assay kit (Bio-Rad); phosphatidylserine and phosphatidylinositol (Matreya, Pleasant Gap, PA); [γ - 32 P]ATP (New England Nuclear); Na $_2$ 51 CrO $_4$ (MP Biomedicals); antibodies against pAkt (Ser473 and Thr308), pErk1/2 (Thr202/Tyr204) and pIKK α/β (Ser180/Ser181) (Cell Signalling); anti-Akt1/2, anti-p110 β , anti-p110 γ , anti-cCbl and PY99 (Santa Cruz); anti-p67^{phox} (B. D. Biosciences Clontech); antibodies raised against PKCs (Transduction Laboratories, Lexington, KY); anti-p85 polyclonal antibody was prepared by immunizing rabbits with full-length GST-p85 α . Serum opsonized zymosan was prepared by incubating zymosan (Sigma) with normal mouse serum at 37°C for 30 min.

Cells—Female C57/BL6 mice, 8–12 weeks old, were purchased from Japan SLC Inc. PI3K γ $^{-/-}$ mice with a C57/BL6 background were kindly donated by Dr Takehiko Sasaki (Akita University, Akita, Japan); peritoneal macrophages were harvested from these mice. Briefly, mice were injected intra-peritoneally with 2 ml of 3% thioglycollate broth. After 3 days, the peritoneal exudate cells were harvested by washing the peritoneal cavity with ice-cold phosphate-buffered saline (PBS). The cells were seeded at about 5×10^5 cells/well in 24-well plates to adhere to dishes and incubated in humidified 5% CO $_2$ at 37°C for 1–2 h in RPMI 1640 medium supplemented with 10% FCS. Non-adherent cells were washed with PBS and the attached cells were designated as macrophages. The mouse macrophage-like cell line, Raw264.7 cells, were maintained in RPMI 1640 medium fortified with 2.5 g/l (4.5 g/l as a final concentration) glucose and 10% FCS in humidified 5% CO $_2$ at 37°C.

Stimulation of the Cells—For short-term incubation (0–60 min), culture media (RPMI 1640) of Raw264.7 cells or mouse peritoneal macrophages in 24-well plates were aspirated and replenished with incubation buffer (complete RPMI 1640 medium without NaHCO $_3$, fortified with 20 mM HEPES, pH 7.4). The cells were treated with or without GF109203X and/or PMA followed by stimulations with various ligands as indicated in the figure legends. Fc γ R stimulation was performed by sequential addition with mouse IgG (5 μ g/ml) and anti-mouse IgG (20 μ g/ml). After the cells were incubated with mouse IgG for 15 min, the ligation reaction was started by the addition of anti-mouse IgG and the cells were incubated for additional 10 min. When cells were treated with PMA or GF109203X before Fc γ R ligation, mouse IgG was added before these treatment and anti-mouse IgG was added after the treatment.

RNA Interference—Two sets of oligonucleotides were cloned into the pH1 vector to express PKC δ siRNA hairpins downstream of the human H1 RNA promoter as described (17,18). The following sequences were used: 5'-GAACGCTTCAACATCGACA-3 (sequence-1) and 5'-ggcgcgtgttatcagattg-3 (sequence-2). Raw264.7 cells (5×10^6 to 10×10^6 cells) were transfected by electroporation in a 300 μ l final volume at 250 V/950 μ F (Gene Pulser II,

Bio-Rad). Twenty-four hours after transfection, puromycin (5 μ g/ml) was added to the cells for selection and incubation was continued for several more days. The resistant colonies were replated to 96-well plates at 0.5–1.0 cell per well and cultured for an additional weeks to obtain monoclonal PKC δ -deficient cells. Colonies transfected with both sequences were used without otherwise specified. To determine the efficiency of gene silencing, total RNA was isolated with RNeasy (Qiagen) and mRNA was assessed by RT-PCR. Control cells were prepared in the same way with pH1 vector containing a 400 bp staffer sequence instead of probe sequence.

Western blotting—Cells were washed with PBS and lysed in 50 μ l of lysis buffer containing 25 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 150 mM NaCl, 1 mM sodium orthovanadate (Na $_3$ VO $_4$), 1 mM EDTA, 0.1% BSA, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μ M leupeptin, 20 μ M *p*-aminophenylmethylsulfonyl fluoride and 1 mM dithiothreitol. The cell lysates were centrifuged at 15,000 rpm for 10 min. Protein concentrations in the resultant supernatants were determined with a Bio-Rad assay kit. Total cell lysates (100 μ g of protein) were mixed with 10 μ l of $\times 5$ sample buffer [62.5 mM Tris (pH 6.8), 1% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue] and heated at 100°C for 3 min. The proteins were separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked in 5% skim milk and incubated with appropriate antibodies. The associated antibodies were detected by enhanced chemiluminescence (Perkin-Elmer).

PI3K Activity Assay—The anti-phosphotyrosine (PY99) or anti-p85 immunoprecipitates were assayed for their PI3K activities (19). The cell lysate was precleared with preimmune IgG and Protein G-Sepharose at 4°C for 1 h and subjected to immunoprecipitation with these antibodies. The immunoprecipitates were washed twice with the lysis buffer, twice with 40 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.5 M LiCl and twice with 40 mM Tris-HCl, 1 mM DTT, 100 mM NaCl. Aliquots (2×10^6 cells equivalent) of immunoprecipitates were suspended in 0.1 ml of the reaction mixture consisting of 40 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.2 mM phosphatidylinositol, 0.2 mM phosphatidylserine, 5 mM MgCl $_2$ and 0.1 mM (1 μ Ci and 5 μ Ci for anti-p85 and PY99 immunoprecipitate, respectively) [γ - 32 P]ATP. The reaction was allowed to proceed at 37°C for 15 min before termination by the addition of 20 μ l of 8% HClO and 0.45 ml of chloroform/methanol (1:2, v/v). After vigorous stirring, the mixture was added to 0.15 ml of chloroform and 0.15 ml of 8% HClO $_4$ to separate the organic phase, which was washed with chloroform-saturated 0.5 M NaCl containing 1% HClO $_4$ and then evaporated to dryness. The extract was dissolved in 15 μ l $\times 2$ of chloroform/methanol (9:1, v/v) to be spotted on a silica gel plate (Silica Gel 60, Merck). The plate was developed in chloroform/methanol/28% NH $_4$ OH/water (70:100:25: 15, by vol.), dried and visualized for radioactivities in the phosphatidylinositol by imaging analyzer (Fuji, Bas3000).

Preparation of IgG-Coated Erythrocytes and Measurement of Phagocytosis—Sheep erythrocytes were

labelled with ^{51}Cr as described previously (2). IgG-coated erythrocytes (EA) were prepared by incubating the labelled cells with rabbit anti-sheep erythrocyte antibody at 37°C for 10 min in 5 mM Verona1 buffer (pH 7.5) supplemented with 0.1% gelatin, 75 mM NaCl, 0.15 mM CaCl_2 , 0.5 mM MgCl_2 (GVB) and 10 mM EDTA followed by incubation on ice for 15 min. EA were washed three times with GVB and finally suspended in the short-term incubation buffer. Binding and phagocytosis of EA were measured as reported earlier (2). Monolayers of Raw 264.7 cells (5×10^5 cells/well in 24-well plate) were incubated with ^{51}Cr -labelled EA (5×10^7 cells) at 37°C for the indicated period of time. The monolayers were washed three times with phosphate-buffered saline (PBS), to remove free EA, before a brief exposure to 0.1 ml of hypotonic PBS (five times diluted). The radioactivity released to the supernatant during this procedure of hypotonic shock was measured for the amount of EA bound on the surface of the phagocytes. The monolayers were washed further three times with PBS and finally solubilized in 0.5% Triton X-100. The radioactivity in the solution was measured for the amount of engulfed EA. The number of EA was calculated from the radioactivity and expressed as incorporated or bound EA/one Raw 264.7 cell.

RESULTS

Activation of PKC Inhibited PI3K Activation Induced by Fc γ R Ligation But Not by C5a—Akt phosphorylation, which is closely related to the activation level of PI3K, was increased by Fc γ R ligation in mouse macrophage cell line Raw264.7 (Fig. 1A). When the cells were treated with increasing concentrations of PMA, a PKC activator, for 5 min before the Fc γ R-ligation, Fc γ R-dependent Akt phosphorylation was inhibited in a manner dependent on the PMA concentration. A GTP-binding protein-coupled receptor ligand, C5a, also increased the Akt phosphorylation, which was not susceptible to PMA at all (Fig. 1A). The amounts of Akt (total Akt) were not affected by the PMA treatment (Fig. 1A). Exposure of the cells to a pan-PKC inhibitor, GF109203X, for 5 min prior to the PMA addition completely blocked the PMA-induced inhibition of Akt phosphorylation (Fig. 1B). Since Fc γ R-ligation activates class IA PI3K while C5a activates Class IB, PKC is suggested to control the activity of class IA but not that of class IB PI3K.

Activation of class IA PI3K is known to accompany the increased PI3K activity in the anti-phosphotyrosine immunoprecipitate. Raw264.7 cells were treated with PMA in the presence or absence of GF109203X before Fc γ R ligation. The cell lysates were subjected to immunoprecipitation with anti-phosphotyrosine and the immune complexes were assayed for the PI3K activity *in vitro* with phosphatidylinositol as a substrate. Phosphotyrosine-associated PI3K activity appeared after Fc γ R ligation, which was inhibited by PMA treatment in a dose-dependent manner (Fig. 2A). As expected, the inhibition by PMA was completely abolished by the prior treatment of the cells with GF109203X (Fig. 2A). PI3K activity in the anti-p85 immunoprecipitate, which is considered to reflect the total cellular PI3K activity, was

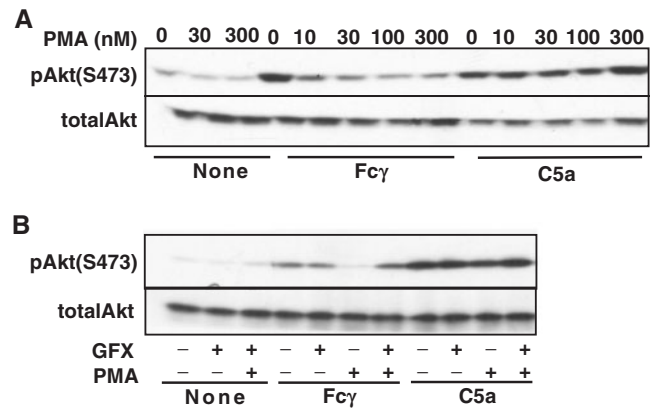


Fig. 1. Activation of PKC by PMA inhibited Akt phosphorylation induced by Fc γ R ligation but not by C5a. Raw264.7 cells were treated as indicated in each figure. Whole cell lysates were analysed by western blotting with the antibody against the phosphorylated Akt (pAkt). The membranes used for the blotting were re-probed with anti-Akt (total Akt). (A) Cells were treated with the increasing concentration of PMA for 5 min and stimulated by Fc γ R ligation (5 $\mu\text{g}/\text{ml}$ mouse IgG and 20 $\mu\text{g}/\text{ml}$ anti-mouse IgG; see 'EXPERIMENTAL PROCEDURES') for 10 min or by C5a (50 nM) for 5 min. (B) Cells were preincubated with (+) or without (-) GF109203X (10 μM) for 5 min before the treatment with (+) or without (-) PMA (0.1 μM) for 5 min and then stimulated with anti-mouse IgG (Fc γ) for 10 min or C5a for 5 min. Similar results were obtained at least three times and representative data are shown.

not affected by the PMA treatment (Fig. 2B). Wortmannin, a pan-PI3K inhibitor, completely inhibited the PI3K activity when added directly to the assay mixture, while the direct addition of PMA did not cause the inhibition (Fig. 2B). Thus, the inhibition of PI3K in phosphotyrosine-associated fraction by PMA observed in Fig. 2A was not due to its direct effect on PI3K.

Prolonged Inhibition of PKC by GF109203X Resulted in Akt Activation Both in Wild-Type and p110 γ -/- Macrophage—Although GF109203X by itself did not cause Akt activation up to 20 min incubation (as shown in Fig. 1B), prolonged treatment (60 min) of Raw264.7 cells with the PKC inhibitor gradually increased the pAkt level in the quiescent state (Fig. 3A). The similar activation of Akt by the PKC inhibitor was observed in mouse peritoneal macrophages (Fig. 3B). In the macrophages from p110 γ -deficient mouse, C5a did not activate Akt in agreement with a preferred role of the class IB PI3K in mediating the GPCR signal (6–8). In the p110 γ (-/-) cells, the pAkt level in the quiescent state was lower than that of the wild-type cells. GF109203X increased the pAkt level both in the p110 γ -/- and the wild-type cells (Fig. 3B). Accordingly, prolonged inhibition of PKC appeared to activate the class IA members of PI3K.

Prolonged Exposure of Macrophages to PMA Resulted in PKC δ Degradation and Akt Phosphorylation—It has been well documented that conventional and novel PKC isoforms are down regulated in response to chronic activation by PMA (20). Although the mechanisms are not fully understood, one of the mechanisms is ubiquitin/proteasome-dependent degradation of PKC isoforms (21). We next examined the effect of PKC down regulation on

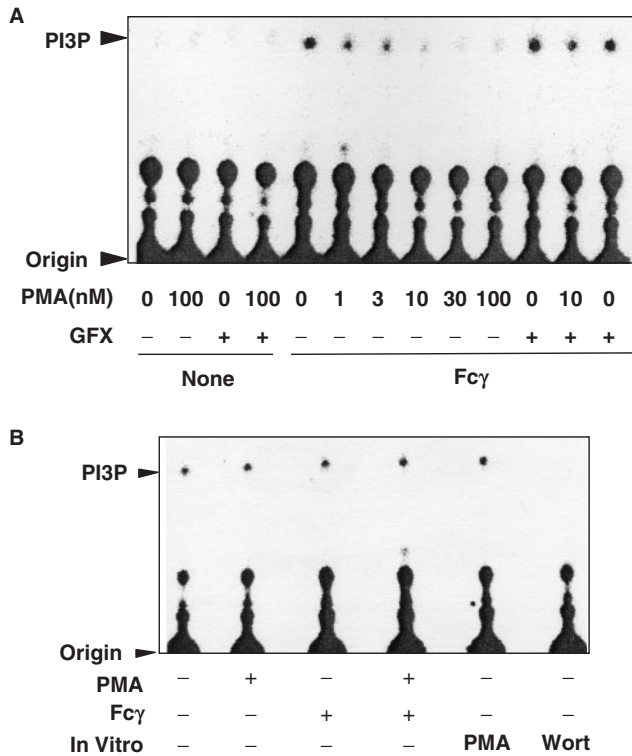


Fig. 2. Activation of PKC by PMA inhibited PI3K activation induced by Fc γ R ligation. Raw264.7 cells were treated as indicated in each figure. Anti-phosphotyrosine (A) or anti-p85 (B) immunoprecipitates prepared from the cell lysates were assayed for PI3K as described under 'EXPERIMENTAL PROCEDURES'. (A) Cells were preincubated with (+) or without (-) GF109203X (10 μ M) for 5 min, treated with PMA for 5 min and stimulated by Fc γ R ligation for 10 min. (B) Cells were treated with (+) or without (-) PMA (0.1 μ M) for 5 min and then stimulated with (+) or without (-) anti-mouse IgG (Fc γ) 10 min. Aliquots of the precipitate from non-treated cells were assayed in the presence of PMA (0.1 μ M) or wortmannin (0.1 μ M, wort) *in vitro*. Similar results were obtained at least three times and representative data are shown.

Akt phosphorylation. The 30 min exposure of macrophages to PMA increased the Akt phosphorylation in a manner dependent on PMA concentration (Fig. 4). We have previously reported that dominant PKC isoforms in macrophages are PKC δ , PKC ϵ , PKC λ and although a lesser extent, PKC α and PKC β as far as detected by western blotting with the antibodies used (18). Among these, PKC δ , a novel PKC was remarkably decreased by the PMA treatment. PKC α , a conventional PKC, was only a little decreased, while the amount of PKC λ , atypical PKC, was not changed at all under the same conditions (Fig. 4). Hence, we speculated that the decrease of PKC δ might be responsible for the increased Akt phosphorylation.

Akt Phosphorylation in the Quiescent State Was Increased in PKC δ -Deficient Cells—To investigate the role of PKC δ in the Akt phosphorylation, we prepared PKC δ -deficient cells using an shRNA technique. Akt phosphorylation in the quiescent state was dramatically enhanced in these cells (Fig. 5). Stimulations of the

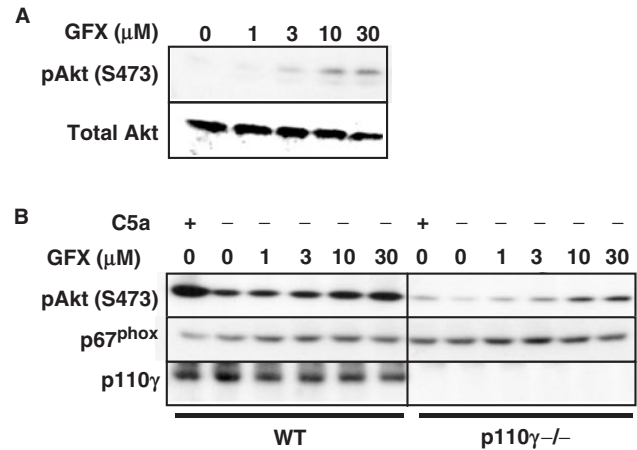


Fig. 3. Prolonged inhibition of PKC by GF109203X resulted in Akt activation both in wild type and p110 γ -/- macrophage. (A) Raw264.7 cells were treated with the increasing concentrations of GF109203X for 60 min. (B) Macrophages prepared from wild type (WT) or p110 γ -/- mouse were incubated with the increasing concentrations of GF109203X for 60 min or with C5a (50 nM, +) for 5 min. Whole cell lysates were analysed by western blotting with the antibody against the phosphorylated Akt (pAkt). The membranes used for the blotting were re-probed with anti-Akt (total Akt in A), anti-p67^{phox} (for invariable control in B) or anti-PI3K p110 γ (B). Similar results were obtained at least three times and representative data are shown.

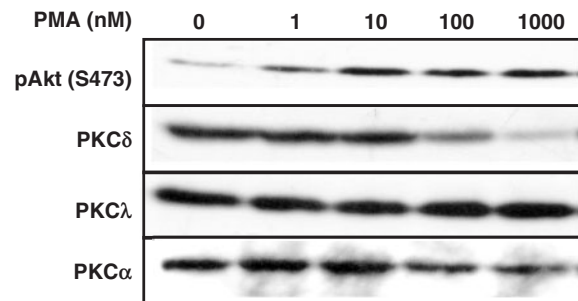


Fig. 4. Prolonged exposure of macrophages to PMA resulted in PKC δ degradation and Akt phosphorylation. Raw 264.7 cells were incubated with the increasing concentration of PMA for 30 min. Whole cell lysates were analysed by western blotting with the anti-phospho-Akt (pAkt). The membranes used for the blotting were re-probed with antibodies against PKCs. Similar results were obtained at least three times and representative data are shown.

PKC δ -deficient cells with C5a, immunocomplex or serum-opsinized zymosan resulted in the additive increase of the phosphorylation (Fig. 5). Basal phosphorylation levels of Erk1/2 or IKK were not changed in the PKC δ -deficient cells indicating that the increase of Akt phosphorylation was not due to a non-specific augmentation of phosphorylation or a non-specific inhibition of phosphatases.

Increased Phosphorylation of Akt in the PKC δ -Deficient Cells was Susceptible to Wortmannin But Not to Pertussis Toxin—A pan-PI3K inhibitor, wortmannin decreased the basal Akt phosphorylation observed in

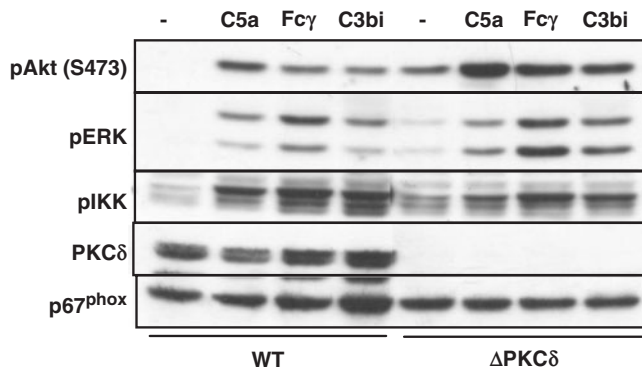


Fig. 5. Akt phosphorylation in the quiescent state was increased in PKC δ -deficient cells. Wild-type (WT) or PKC δ -deficient cells (Δ PKC δ) were stimulated with C5a (50 nM) for 2 min, anti-mouse IgG (20 μ g/ml, Fc γ) for 10 min or serum-treated zymosan (1 mg/ml, C3bi) for 15 min. Whole cell lysates were analysed by western blotting with the antibody against the phosphorylated Akt (pAkt). The membranes used for the blotting were re-probed with antibodies against phospho-Erk1/2 (pERK), phospho-IKK (pIKK), PKC γ and p67^{phox} (for invariable control). The results with a clone expressing both of two silencing sequences (see EXPERIMENTAL PROCEDURES) are shown. Similar results were obtained with another clone.

the PKC δ -deficient cells (Fig. 6A). Wortmannin inhibited the C5a-induced increase in the Akt phosphorylation with the same concentration dependency. Since the Akt phosphorylation is induced by PI3K activation, the enhancement of basal Akt phosphorylation in the PKC δ -deficient cells appeared to be due to the basal PI3K activation.

Activation of PI3K is known to induce the phosphorylation of Akt on Thr308 as well as Ser473. In PKC δ -deficient cells, the basal phosphorylation of Akt on Thr308 was observed (Fig. 6B). C5a-induced increase in the Thr308 phosphorylation was attenuated by pertussis toxin, which possesses ADP ribosyl transferase activity and inhibits the functions of a heterotrimeric GTP-binding protein, Gi (Fig. 5B). In contrast, the basal level of Akt phosphorylation in the absence of C5a was not decreased by the pertussis toxin treatment (Fig. 6B). Thus, it was likely that the increased level of basal Akt phosphorylation observed in the PKC δ -deficient cells was dependent on class IA PI3K activation.

Increased Association of PI3K Activity with Tyrosine-Phosphorylated Peptides in PKC δ -Deficient Cells—The PI3K activity in the anti-p85 immunoprecipitate was not increased in the PKC δ -deficient cells (Fig. 7), suggesting that the increase in the total PI3K activity was not the basis of the effect of PKC-deficiency. On the other hand, PI3K activity associated with anti-phosphotyrosine immunoprecipitate was increased, although very slightly (Fig. 7). It is intriguing to speculate that PKC δ in the wild-type cells may phosphorylate some proteins on serine/threonine residues, thereby making them poor substrate of tyrosine kinase and preventing the activation of class IA PI3K. Alternatively, the phosphorylation of some peptides on serine/threonine residues might prevent the association of their tyrosine-phosphorylated forms with PI3K.

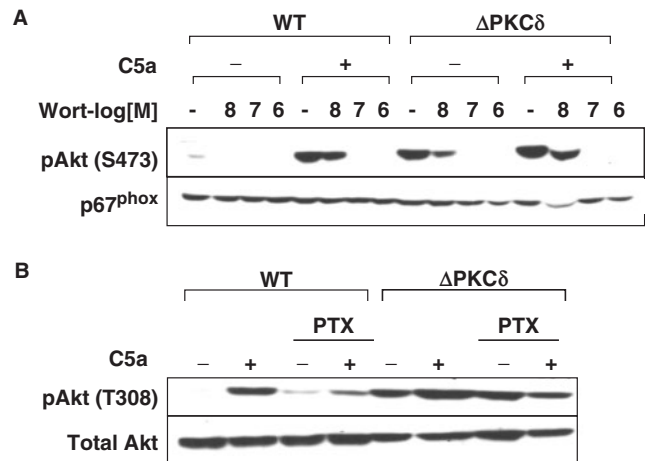


Fig. 6. Increased phosphorylation of Akt in the PKC δ -deficient cells was susceptible to wortmannin but not to pertussis toxin. Wild-type (WT) or PKC δ -deficient cells (Δ PKC δ) were treated with wortmannin (Wort, in A) for 10 min or pertussis toxin (50 ng/ml, pertussis toxin in B) for 16 h and then stimulated with (+) or without (–) C5a (50 nM) for 5 min. Whole cell lysates were analysed by western blotting with the antibody against phosphorylated Akt (pAkt) (Ser 473 in A and Thr 308 in B). The membranes used for the blotting were re-probed with antibody against p67^{phox} (for invariable control in A) or Akt (total Akt in B). The results with a clone expressing both of two silencing sequences (see EXPERIMENTAL PROCEDURES) are shown. Similar results were obtained with another clone.

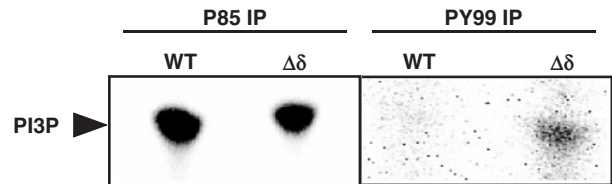


Fig. 7. Increased association of PI3K activity with tyrosine phosphorylated peptides in PKC δ -deficient cells. Anti-p85 or Anti-phosphotyrosine immunoprecipitates were prepared from wild-type (WT) or PKC δ -deficient cells (Δ δ). PI3K activities were assayed with the precipitates as described under 'EXPERIMENTAL PROCEDURES'. The results with a clone expressing both of two silencing sequences (see EXPERIMENTAL PROCEDURES) are shown. Similar results were obtained with another clone.

Enhancement of Fc γ R-Mediated Phagocytosis in PKC δ -Deficient cells—We have previously shown that PI3K activity is indispensable for Fc γ R-mediated phagocytosis in guinea pig neutrophils (2). As shown in Fig. 8A, the phagocytosis in Raw 264.7 cells was inhibited by wortmannin in a dose-dependent manner indicating that Fc γ R-mediated phagocytosis is closely related to PI3K activity also in these cells (Fig. 8B). In contrast, binding of EA to Fc γ R was not affected by the wortmannin treatment up to 90 min (Fig. 8B). In PKC δ -deficient cells, where PI3K activity was higher than that in normal cells, the phagocytosis was extremely increased (Fig. 8C). Since the binding of EA was comparable to the control cells (Fig. 8C), the number of Fc γ R was unchanged in PKC δ -deficient cells. Similar results were obtained when

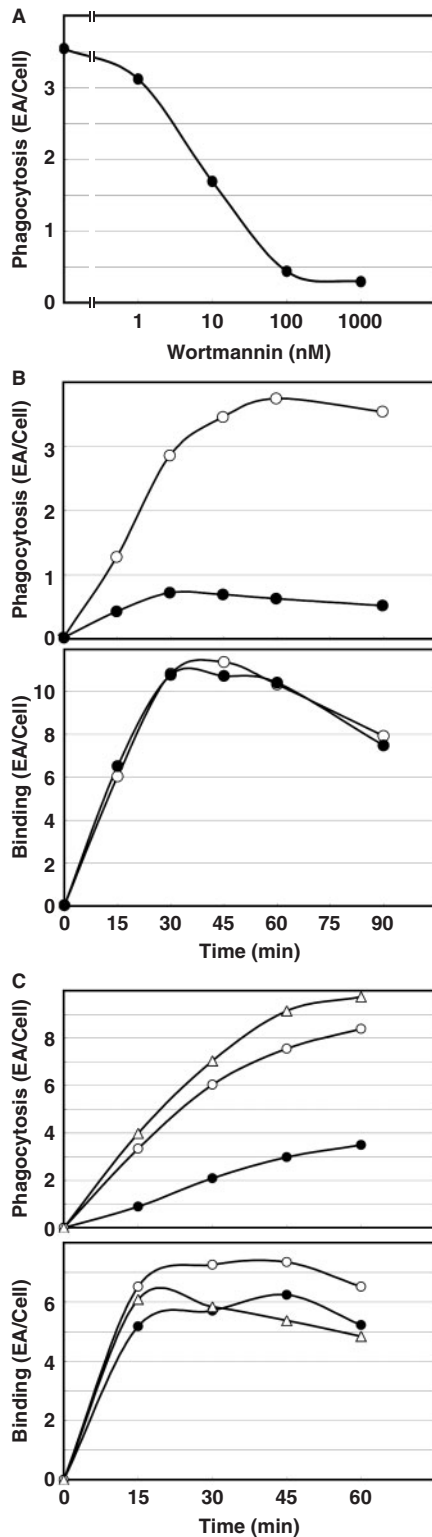


Fig. 8. Enhancement of Fc γ R-mediated phagocytosis in PKC δ -deficient cells. Phagocytosis and binding of ^{51}Cr -labelled and IgG-coated erythrocytes (EA) were determined as described under 'EXPERIMENTAL PROCEDURES'. (A) Wild-type Raw 264.7 cells were treated with the increasing concentrations of wortmannin for 5 min. The cells were incubated for further 60 min with the addition of EA. (B) Wild-type cells were treated with (closed circle) or without (open circle) $0.1\ \mu\text{M}$

shRNA probes with different target sequences were used to obtain the PKC-deficient cells. In normal cells, PKC δ may limit PI3K activity thereby suppress the Fc γ R-mediated phagocytosis.

DISCUSSION

Our present study has indicated that activation of PKC reduces phosphorylation of Akt in both basal and Fc γ R-stimulated conditions. The inhibition of Akt was mediated through the inhibition of PI3K. The PI3K susceptible to the PKC activation is class IA, because (i) PI3K activation induced by Fc γ R ligation but not by C5a stimulation was attenuated by PMA (Fig. 1), (ii) Akt activation by PKC inhibition was observed in PI3K γ -/- cells as well as wild type (Fig. 3), (iii) PKC δ depletion-induced augmentation of Akt phosphorylation was not changed by pertussis toxin treatment (Fig. 6B) and (iv) PI3K activity associated with tyrosine phosphorylated peptides was inhibited by the activation of PKC (Fig. 2A). PKC δ was considered to be the major PKC isoform responsible for the PI3K inhibition, because the PKC δ -deficient cells exhibited the increased level of Akt phosphorylation in their quiescent state (Figs 5 and 6). Furthermore, PI3K activity associated with tyrosine phosphorylated proteins was increased in the PKC δ -deficient cells (Fig. 7). Thus these results indicated that PKC δ is a negative regulator of class IA PI3K.

Both positive and negative regulation of Akt phosphorylation by PKC have been reported previously. Classical PKCs directly phosphorylates Akt only on Ser473 in a cell type- and stimulus-specific fashion (14,22). In contrast, numerous studies have reported that PMA-induced activation of PKC results in inhibition of Akt phosphorylation in a variety of cell types (10–16). However, the PKC isoforms involved in mediating the PMA effect were different in these studies. For instance, activation of PKC α by PMA was shown to inhibit insulin-induced Akt phosphorylation in vascular smooth muscle cells (23), whereas PKC ϵ but not PKC α , β I or δ was a major player in PMA-induced inhibition of Akt in GM-CSF-activated 32D cells (11). Involvement of PKC δ in the negative regulation of Akt was suggested in IGF-1-stimulated PC12 cells (24) and IGF-1- or EGF-stimulated mouse keratinocytes (13). In the latter case, however, both PKC δ and ϵ play the regulatory role in the Akt phosphorylation. Thus the PKC isoforms involved in the negative regulation may be different depending on cell types.

The use of 'isoform-specific' inhibitors is a powerful approach to determine the specific functions of the isoforms in cells. Some works have employed such inhibitors to determine the PKC isoforms responsible for the negative regulation of PI3K. In the case of PKC δ ,

wortmannin for 5 min, added with EA and then incubated for the indicated periods of time. (C) Wild-type (closed circle), PKC δ -deficient cell (prepared with the silencing sequence-1, open circle), or another clone of PKC δ -deficient cell (prepared with the silencing sequence-2, open triangle) were incubated with EA for the indicated times. Each experiment was performed at least three times with similar results. Similar results were obtained with a clone expressing both silencing sequences (see EXPERIMENTAL PROCEDURES).

rottlerin is often used to elucidate the function of this subtype, because it has been reported as a specific inhibitor of PKC δ (25). However, Davies *et al.* (26) have reported that the chemical has no effect on PKC δ , even when assayed at a very low ATP concentration in the absence of phospholipid, the conditions used in the original report. We ourselves observed that it has no effect on purified PKC δ even at 100 μ M *in vitro* (data not shown). The failure of rottlerin to inhibit PKC δ has also been described in a homepage of the providing company (<http://www.lclabs.com/PROFILE/P-R/R-9630.php4>).

Because of the lack of alternative inhibitors, we prepared the stable clones of PKC δ -deficient cells using shRNA technique. Using these cells, we were able to demonstrate the negative regulatory role of PKC δ in PI3K activation. However, it should be commented that the present study does not exclude the possibility that the other subtypes have potential ability to inhibit PI3K and play the similar negative regulatory role in some cell types, since PKC δ is the dominant PKC isoform in macrophages.

It is not surprising that the PI3K isoform susceptible to PKC is class IA but not class IB because we have previously reported that Fc γ R-coupled tyrosine phosphorylation of Cbl, which is a major p85-binding peptide, is abrogated by PKC activation in guinea pig neutrophils (10). In these cells, PMA causes the serine-phosphorylation of Cbl. The phosphorylated Cbl obtains a resistance to tyrosine-phosphorylation by Syk and thus loses its ability to bind p85 and activate PI3K. Thus it might be speculated that the deficiency of PKC δ increases the basal PI3K activity in Raw264.7 cells by decreasing the serine-dephosphorylated forms of Cbl and increasing the binding of this adaptor to PI3K.

In a B lymphocyte cell line, BCR-mediated activation of PKC μ induces the serine phosphorylation of Syk, which in turn inhibits the ability of Syk to phosphorylate PLC γ (27). This mechanism functions in a negative feedback loop regulation of BCR-initiated signalling cascades. It is also reported that GM-CSF-induced activation of Lyn, a Src family tyrosine kinase is inhibited by PMA treatment (15). Since both Lyn and Syk play indispensable roles in Fc γ R-mediated activation of macrophages, it is possible that either of these tyrosine kinase is a target of PKC δ for the inhibition of class IA PI3K.

We have previously shown that PI3K activity is indispensable for Fc γ R-mediated phagocytosis in guinea pig neutrophils (2). In agreement with this, the phagocytosis was susceptible to wortmannin also in Raw 264.7 cells (Fig. 8A). Interestingly, the phagocytosis was extremely enhanced in PKC δ -deficient cells (Fig. 8C). The increased PI3K activity in these cells may contribute to the activation of phagocytosis. This is the first observation indicating the involvement of PKC δ in the negative regulation of Fc γ R-mediated event. The effect of PMA on Fc γ R-mediated phagocytosis has been controversial. PMA markedly enhanced complement receptor (CR3)- but not Fc γ R-mediated phagocytosis in human monocyte-derived macrophages (28). In contrast, Fc γ R-mediated phagocytosis is enhanced by PMA in human neutrophils (29). In the present study, we showed that the effect of PMA on the phagocytosis may vary depending on the concentration of PMA and on the

incubation periods, because short-term exposure (5 min) of Raw 264.7 cells to PMA resulted in the inhibition of PI3K while 30-min exposure to PMA resulted in the activation of PI3K due to PKC δ degradation (Fig. 4).

In the present study, we have shown a functional role of PKC δ in regulation of class IA PI3K. Activation of class IA PI3K has been known as a common event observed after various TLR ligation in macrophages and dendritic cells. Because PI3K plays a negative regulatory role in TLR-induced cytokine productions (30–34), activation of class IA PI3K by an inhibitor of PKC δ is expected to decrease the innate immune response. In addition, we have observed that the deficiency of PKC δ inhibits the PI3K-independent mechanisms in various TLR signaling systems (18). In the cases of TLR2 and TLR4, the association of PKC δ with TIRAP/Mal, a TLR2 and TLR4-specific adaptor, is considered to be a key event leading to the cytokine responses (18). Because PKC δ is expressed dominantly in immune cells, PKC δ -specific inhibitors are expected to be useful in manipulating the functions of these cells *in vivo*.

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CONFLICT OF INTEREST

None declared.

REFERENCES

- Engelman, J.A., Luo, J., and Cantley, L.C. (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat. Rev. Genet.* **7**, 606–619
- Ninomiya, N., Hazeki, K., Fukui, Y., Seya, T., Okada, T., Hazeki, O., and Ui, M. (1994) Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. *J. Biol. Chem.* **269**, 22732–22737
- Tsukamoto, K., Hazeki, K., Hoshi, M., Nigorikawa, K., Inoue, N., Sasaki, T., and Hazeki, O. (2008) Critical roles of the p110 beta subtype of phosphoinositide 3-kinase in lipopolysaccharide-induced Akt activation and negative regulation of nitrite production in RAW 264.7 cells. *J. Immunol.* **180**, 2054–2061
- Hazeki, K., Nigorikawa, K., and Hazeki, O. (2007) Role of phosphoinositide 3-kinase in innate immunity. *Biol. Pharm. Bull.* **30**, 1617–1623
- Wymann, M.P. and Pirola, L. (1998) Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta.* **1436**, 127–150
- Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R., and Wymann, M.P. (1998) Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science.* **282**, 293–296
- Hirsch, E., Katanaev, V.L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F., and Wymann, M.P. (2000) Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science.* **287**, 1049–1053

8. Sasaki, T., Irie-Sasaki, J., Jones, R.G., Oliveira-dos-Santos, A.J., Stanford, W.L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Joza, N., Mak, T.W., Ohashi, P.S., Suzuki, A., and Penninger, J.M. (2000) Function of PI3Kgamma in thymocyte development, T cell activation and neutrophil migration. *Science*. **287**, 1040–1046
9. Mellor, H. and Parker, P.J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332 (Pt 2)**, 281–292
10. Hazeki, K., Hazeki, O., Matsuo, T., Seya, T., Yamashita, T., Nagasawa, S., Band, H., and Ui, M. (1999) Role of Syk in Fc gamma receptor-coupled tyrosine phosphorylation of Cbl in a manner susceptible to inhibition by protein kinase C. *Eur. J. Immunol.* **29**, 3302–3312
11. Liu, H., Qiu, Y., Xiao, L., and Dong, F. (2006) Involvement of protein kinase Cepsilon in the negative regulation of Akt activation stimulated by granulocyte colony-stimulating factor. *J. Immunol.* **176**, 2407–2413
12. Doornbos, R.P., Theelen, M., van der Hoeven, P.C., van Blitterswijk, W.J., Verkleij, A.J., and van Bergen en Henegouwen, P.M. (1999) Protein kinase Czeta is a negative regulator of protein kinase B activity. *J. Biol. Chem.* **274**, 8589–8596
13. Li, L., Sampat, K., Hu, N., Zakari, J., and Yuspa, S.H. (2006) Protein kinase C negatively regulates Akt activity and modifies UVC-induced apoptosis in mouse keratinocytes. *J. Biol. Chem.* **281**, 3237–3243
14. Matsumoto, M., Ogawa, W., Hino, Y., Furukawa, K., Ono, Y., Takahashi, M., Ohba, M., Kuroki, T., and Kasuga, M. (2001) Inhibition of insulin-induced activation of Akt by a kinase-deficient mutant of the epsilon isozyme of protein kinase C. *J. Biol. Chem.* **276**, 14400–14406
15. al-Shami, A., Bourgoin, S.G., and Naccache, P.H. (1997) Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. I. Tyrosine phosphorylation-dependent stimulation of phosphatidylinositol 3-kinase and inhibition by phorbol esters. *Blood*. **89**, 1035–1044
16. Wen, H.C., Huang, W.C., Ali, A., Woodgett, J.R., and Lin, W.W. (2003) Negative regulation of phosphatidylinositol 3-kinase and Akt signalling pathway by PKC. *Cell Signal.* **15**, 37–45
17. Kubo, H., Hazeki, K., Takasuga, S., and Hazeki, O. (2005) Specific role for p85/p110beta in GTP-binding-protein-mediated activation of Akt. *Biochem. J.* **392**, 607–614
18. Kubo-Murai, M., Hazeki, K., Sukenobu, N., Yoshikawa, K., Nigorikawa, K., Inoue, K., Yamamoto, T., Matsumoto, M., Seya, T., Inoue, N., and Hazeki, O. (2007) Protein kinase Cdelta binds TIRAP/Mal to participate in TLR signaling. *Mol. Immunol.* **44**, 2257–2264
19. Matsuo, T., Hazeki, K., Hazeki, O., Katada, T., and Ui, M. (1996) Activation of phosphatidylinositol 3-kinase by concanavalin A through dual signaling pathways, G-protein-coupled and phosphotyrosine-related and an essential role of the G-protein-coupled signals for the lectin-induced respiratory burst in human monocytic THP-1 cells. *Biochem. J.* **315 (Pt 2)**, 505–512
20. Liu, W.S. and Heckman, C.A. (1998) The sevenfold way of PKC regulation. *Cell Signal.* **10**, 529–542
21. Srivastava, J., Procyk, K.J., Iturriz, X., and Parker, P.J. (2002) Phosphorylation is required for PMA- and cell-cycle-induced degradation of protein kinase Cdelta. *Biochem. J.* **368**, 349–355
22. Kroner, C., Eybrechts, K., and Akkerman, J.W. (2000) Dual regulation of platelet protein kinase B. *J. Biol. Chem.* **275**, 27790–27798
23. Motley, E.D., Eguchi, K., Gardner, C., Hicks, A.L., Reynolds, C.M., Frank, G.D., Mifune, M., Ohba, M., and Eguchi, S. (2003) Insulin-induced Akt activation is inhibited by angiotensin II in the vasculature through protein kinase C-alpha. *Hypertension* **41**, 775–780
24. Zheng, W.H., Kar, S., and Quirion, R. (2000) Stimulation of protein kinase C modulates insulin-like growth factor-1-induced akt activation in PC12 cells. *J. Biol. Chem.* **275**, 13377–13385
25. Gschwendt, M., Muller, H.J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G., and Marks, F. (1994) Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Commun.* **199**, 93–98
26. Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
27. Sidorenko, S.P., Law, C.L., Klaus, S.J., Chandran, K.A., Takata, M., Kurosaki, T., and Clark, E.A. (1996) Protein kinase C mu (PKC mu) associates with the B cell antigen receptor complex and regulates lymphocyte signaling. *Immunity*. **5**, 353–363
28. Newman, S.L., Mikus, L.K., and Tucci, M.A. (1991) Differential requirements for cellular cytoskeleton in human macrophage complement receptor- and Fc receptor-mediated phagocytosis. *J. Immunol.* **146**, 967–974
29. Gresham, H.D., Zheleznyak, A., Mormol, J.S., and Brown, E.J. (1990) Studies on the molecular mechanisms of human neutrophil Fc receptor-mediated phagocytosis. Evidence that a distinct pathway for activation of the respiratory burst results in reactive oxygen metabolite-dependent amplification of ingestion. *J. Biol. Chem.* **265**, 7819–7826
30. Aksoy, E., Amraoui, Z., Goriely, S., Goldman, M., and Willems, F. (2002) Critical role of protein kinase C epsilon for lipopolysaccharide-induced IL-12 synthesis in monocyte-derived dendritic cells. *Eur. J. Immunol.* **32**, 3040–3049
31. Chen, C.C., Wang, J.K., Chen, W.C., and Lin, S.B. (1998) Protein kinase C eta mediates lipopolysaccharide-induced nitric-oxide synthase expression in primary astrocytes. *J. Biol. Chem.* **273**, 19424–19430
32. Castrillo, A., Pennington, D.J., Otto, F., Parker, P.J., Owen, M.J., and Bosca, L. (2001) Protein kinase Cepsilon is required for macrophage activation and defense against bacterial infection. *J. Exp. Med.* **194**, 1231–1242
33. Monick, M.M., Carter, A.B., Flaherty, D.M., Peterson, M.W., and Hunninghake, G.W. (2000) Protein kinase C zeta plays a central role in activation of the p42/44 mitogen-activated protein kinase by endotoxin in alveolar macrophages. *J. Immunol.* **165**, 4632–4639
34. Platten, M., Eitel, K., Wischhusen, J., Dichgans, J., and Weller, M. (2003) Involvement of protein kinase Cdelta and extracellular signal-regulated kinase-2 in the suppression of microglial inducible nitric oxide synthase expression by N-[3,4-dimethoxycinnamoyl]-anthranilic acid (traniLAST). *Biochem. Pharmacol.* **66**, 1263–1270