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Negative Regulation of Class IA Phosphoinositide 3-kinase by Protein Kinase Cδ Limits Fcγ Receptor-mediated Phagocytosis in Macrophages

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Running title: Inhibition of class IA PI3K by PKCδ

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

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Summary

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 PKCδ 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 Fcγ receptor (FcγR) 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 (p110γ). Thus PKC was suggested to selectively attenuate the class IA activity. Chronic PKC activation by PMA induced PKCδ degradation and Akt activation. Enhancement of the basal Akt actvity was also observed in cells stably deficient in PKCδ prepared by shRNA technique. FcγR-mediated phagocytosis was dramatically increased in these cells. Thus it is suggested that inactivation of class IA PI3K by PKCδ is functioning in regulation of FcγR-mediated phagocytosis.

Key words: Akt , Fcγ receptor, Phagocytosis, Phosphoinositide 3-kinase, PKCδ

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that catalyzes 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 Cγ, 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 p110α, p110β, and p1110δ, while two genes encode the associating regulatory subunits, referred to as p85α and p85β (*5*). Class I includes another member, PI3Kγ, which is mainly expressed in hematopoietic cells (*6*). This subtype consists of a catalytic subunit (p110γ) and a regulatory subunit (p101), and is classified as class IB. PI3Kγ can be activated by the βγ 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 PKCα, βI, βII and γ are diacylglycerol (DAG)- and calcium-dependent; novel PKCδ, ε, θ and η are DAG-dependent and calcium-independent; and finally atypical PKCζ, 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 PI3Kγ-deficient cells as well as wild type cells. In addition, PI3K was spontaneously activated in PKCδ-deficient cells through increased association of tyrosine phosphorylated proteins and the p85 regulatory subunit of PI3K. Thus, we present the evidence showing that PKCδ is involved in the inhibition of class IA PI3K but not class IB. Finally, we provide a finding that FcγR-mediated phagocytosis, which is closely related to PI3K activity, was dramatically increased in PKCδ-deficient cells. Taken together, PKCδ may limit PI3K activity

to regulate FcγR-mediated events in normal macrophages.

EXPERIMENTAL PROCEDURES

Materials---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 pIKKα/β (Ser180/Ser181) (Cell Signalling); anti-Akt1/2, anti-p110β, anti-p110γ, anti-cCbl and PY99 (Santa Cruz); anti-p67^{phox} (B. D. Biosciences Clontech); antibodies raised against PKCs (Transduction Laboratories, Lexington, KY); anti-p85α polyclonal antibody was prepared by immunizing rabbits with full-length GST-p85α. 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. PI3Kγ-/- 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 x 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 2.5g/L (4.5g/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, pH7.4). The cells were treated with or without GF109203X and/or PMA followed by stimulations with various ligands as indicated in the figure legends. FcγR

stimulation was performed by sequential addition with mouse IgG (5 μg/mL) and anti-mouse IgG (20 μg/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 FcγR 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 PKCδ 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'-GGCCGTGTTATCCAGATTG-3 (sequence-2). Raw264.7 cells (5-10 x 10^6 cells) were transfected by electroporation in a 300 μL final volume at 250 V/950μF (Gene Pulser II, Bio-Rad). Twenty-four hours after transfection, puromycin (5 μg/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/well and cultured for an additional weeks to obtain monoclonal PKCδ-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 μl of lysis buffer containing 25 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 150 mM NaCl, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM EDTA, 0.1% BSA, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 20 μM p-amidinophenylmethylsulfonyl 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 μg of protein) were mixed with 10 μl of x 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 (pH7.4), 1 mM DTT, 0.5 M LiCl, and twice with 40 mM Tris-HCl, 1 mM DTT, 100 mM NaCl. Aliquots ($2x10^6$ 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 μCi and 5 μCi 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 μl 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 μl x2 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_4OH/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 labeled with ^{51}Cr as described previously (*2*). IgG-coated erythrocytes (EA) were prepared by incubating the labeled 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-labeled 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 (5 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 FcγR ligation but not by C5a---Akt phosphorylation, which is closely related to the activation level of PI3K, was increased by FcγR 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 FcγR-ligation, FcγR-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 FcγR-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 FcγR 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 FcγR 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 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 p110γ-/- 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 p110γ-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 p110γ(-/-) 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 p110γ-/- 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 PKCδ 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 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 PKCδ, PKCι, PKCλ and although a lesser extent, PKCα and PKCβ as far as detected by Western blotting with the antibodies used (*18*). Among these, PKCδ, a novel PKC was remarkably decreased by the PMA treatment. PKCα, a conventional PKC, was only a little decreased, while the amount of PKCλ, atypical PKC, was not changed at all under the same conditions (Fig. 4). Hence, we speculated that the decrease of PKCδ might be responsible for the increased Akt phosphorylation.

Akt phosphorylation in the quiescent state was increased in PKCδ deficient cells ---To investigate the role of PKCδ in the Akt phosphorylation, we prepared PKCδ-deficient cells using an shRNA technique. Akt phosphorylation in the quiescent state was dramatically enhanced in these cells (Fig. 5). Stimulations of the PKCδ-deficient cells with C5a, immunocomplex or serum-opsonized zymosan resulted in the additive increase of the phosphorylation (Fig. 5). Basal phosphorylation levels of Erk1/2 or IKK were not changed in the PKCδ-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 PKCδ deficient cells was susceptible to wortmannin but not to pertussis toxin---A pan-PI3K inhibitor, wortmannin decreased the basal Akt phosphorylation observed in the PKCδ-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 PKCδ-deficient cells appeared to be due to the basal PI3K activation.

Activation of PI3K is known to induce the phosporylation of Akt on Thr308 as well as Ser473. In PKCδ-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, Giα (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 PKCδ-deficient cells was dependent on class IA PI3K activation.

Increased association of PI3K activity with tyrosine-phosphorylated peptides in PKCδ–deficient cells---The PI3K activity in the anti-p85 immunoprecipitate was not increased in the PKCδ-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 PKCδ 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.

Enhancement of FcγR-mediated phagocytosis in PKCδ-deficient cells---We have previously shown that PI3K activity is indispensable for FcγR-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 FcγR-mediated phagocytosis is closely related to PI3K activity also in these cells (Fig. 8B). In contrast, binding of EA to FcγR was not affected by the wortmannin treatment up to 90 min (Fig. 8B). In PKCδ-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 FcγR was unchanged in PKCδ-deficient cells. Similar results were obtained when shRNA probes with different target sequences were used to obtain the PKC-deficient cells. In normal cells, PKCδ may limit PI3K activity thereby suppress the FcγR-mediated phagocytosis.

DISCUSSION

Our present study has indicated that activation of PKC reduces phosphorylation of Akt in both basal and FcγR-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 FcγR ligation but not by C5a stimulation was attenuated by PMA (Fig. 1), ii) Akt activation by PKC inhibition was observed in PI3Kγ-/- cells as well as wild type (Fig. 3), iii) PKCδ depletion-induced augmentation of Akt phosphorylation was not changed by pertussis toxin treatment (Fig. 6B), iv) PI3K activity associated with tyrosine phosphorylated peptides was inhibited by the activation of PKC (Fig. 2A). PKCδ was considered to be the major PKC isoform responsible for the PI3K inhibition, because the PKCδ-deficient cells exhibited the increased level of Akt phosphorylation in their quiescent state (Fig. 5 and 6). Furthermore, PI3K activity associated with tyrosine phosphorylated proteins was increased in the PKCδ-deficient cells (Fig. 7). Thus these results indicated that PKCδ 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 PKCα by PMA was shown to inhibit insulin-induced Akt phosphorylation in vascular smooth muscle cells (*23*), whereas PKCε but not PKCα, βI or δ was a major player in PMA-induced inhibition of Akt in GM-CSF-activated 32D cells (*11*). Involvement of PKCδ 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 PKCδ 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 PKCδ, rottlerin is often used to elucidate the function of this subtype, because it has been reported as a specific inhibitor of PKCδ (*25*). However, Davies, S. *et al.* have reported that the chemical has no effect on PKCδ, even when assayed at a very low ATP concentration in the absence of phospholipid, the conditions used in the original report (*26*). We ourselves observed that it has no effect on purified PKCδ even at 100 μM *in vitro* (data not shown). The failure of rottlerin to inhibit PKCδ has also been described in a homepage of the providing company

(http://www.lclabs.com/PRODFILE/P-R/R-9630.php4). Because of the lack of alternative inhibitors, we prepared the stable clones of PKCδ-deficient cells using shRNA technique. Using these cells, we were able to demonstrate the negative regulatory role of PKCδ 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 PKCδ 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 FcγR-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 PKCδ 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 PKCμ induces the serine phosphorylation of Syk, which in turn inhibits the ability of Syk to phosphorylate PLCγ (*27*). This mechanism functions in a negative feedback loop regulation of BCR-initiated signaling 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 FcγR-mediated activation of macrophages, it is possible that either of these tyrosine kinase is a target of PKCδ for the inhibition of class IA PI3K.

We have previously shown that PI3K activity is indispensable for FcγR-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 PKCδ-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 PKCδ in the negative regulation of FcγR-mediated event. The effect of PMA on FcγR-mediated phagocytosis has been controversial. PMA markedly enhanced complement receptor (CR3)- but not FcγR-mediated phagocytosis in human monocyte-derived macrophages (*28*). In contrast, FcγR-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 PKCδ degradation (Fig. 4).

In the present study, we have shown a functional role of PKCδ 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 PKCδ is expected to decrease the innate immune response. In addition, we have observed that the deficiency of PKCδ inhibits the PI3K-independent mechanisms in various TLR signaling systems (*18*). In the cases of TLR2 and TLR4, the association of PKCδ with TIRAP/Mal, a TLR2 and TLR4-specific adaptor, is considered to be a key event leading to the cytokine responses (*18*). Because PKCδ is expressed dominantly in immune cells, PKCδ-specific inhibitors are expected to be useful in manipulating the functions of these cells *in vivo*.

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Legend to figures

Fig. 1. **Activation of PKC by PMA inhibited Akt phosphorylation induced by FcγR ligation but not by C5a.** Raw264.7 cells were treated as indicated in each figure. Whole cell lysates were analyzed 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 FcγR ligation (5 μg/mL mouse IgG and 20 μg/mL anti-mouse IgG; see "materials and methods 2. 3") 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 (Fcγ) for 10 min or C5a for 5 min. Similar results were obtained at least three times and representative data are shown.

Fig. 2. **Activation of PKC by PMA inhibited PI3K activation induced by FcγR 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 "Materials and methods". (A) Cells were preincubated with (+) or without (-) GF109203X (10 μM) for 5 min, treated with PMA for 5 min and stimulated by FcγR ligation for 10 min. (B) Cells were treated with (+) or without (-) PMA (0.1 μM) for 5 min and then stimulated with (+) or without (-) anti-mouse IgG (Fcγ) 10 min. Aliquots of the precipitate from non-treated cells were assayed in the presence of PMA (0.1 μM) or wortmannin (0.1 μM, wort) *in vitro*. Similar results were obtained at least three times and representative data are shown.

Fig. 3. **Prolonged inhibition of PKC by GF109203X resulted in Akt activation both in wild type and p110γ-/- macrophage.** (A) Raw264.7 cells were treated with the increasing concentrations of GF109203X for 60 min. (B) Macrophages prepared from wild type (WT) or p110γ -/- mouse were incubated with the increasing concentrations of GX109203X for 60 min or with C5a (50 nM, +) for 5 min. Whole cell lysates were analyzed 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 p110γ (B). Similar results were obtained at least three times and representative data are shown.

Fig. 4. **Prolonged exposure of macrophages to PMA resulted in PKCδ degradation and**

Akt phosphorylation. Raw 264.7 cells were incubated with the increasing concentration of PMA for 30 min. Whole cell lysates were analyzed by Western blotting with the anti-phospho-Akt (pAkt). The membranes used for the blotting were re-probed with antibodies agaist PKCs. Similar results were obtained at least three times and representative data are shown.

Fig. 5. **Akt phosphorylation in the quiescent state was increased in PKCδ-deficient cells.** Wild type (WT) or PKCδ-deficient cells (ΔPKCδ) were stimulated with C5a (50 nM) for 2 min, anti-mouse IgG (20 μg/mL, Fcγ) for 10 min or serum-treated zymosan (1 mg/mL, C3bi) for 15 min. Whole cell lysates were analyzed 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), PKCδ and $p67^{phox}$ (for invariable control). Similar results were obtained with other clones with different silencing sequences and also with polyclonal PKCδ-deficient cells, whose PKCδ was completely abolished. .

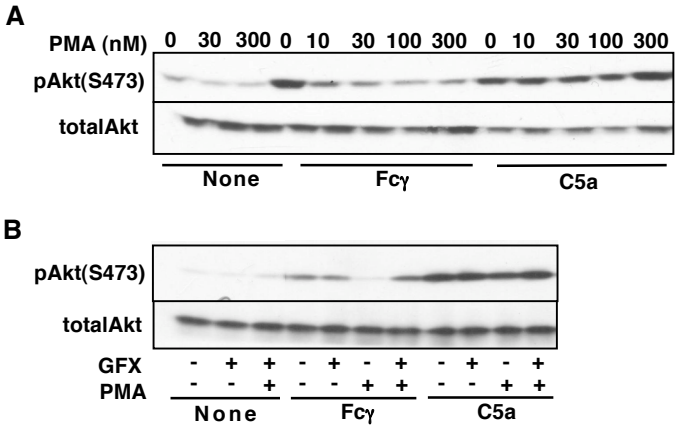
Fig. 6. **Increased phosphorylation of Akt in the PKCδ-deficient cells was susceptible to wortmannin but not to pertussis toxin.** Wild type (WT) or PKCδ-deficient cells (ΔPKCδ) were treated with wortmannin (Wort, in A) for 10 min or pertussis toxin (50 ng/mL, pertussis toxin in B) for 16 h and then stimulated with (+) or without (-) C5a (50 nM) for 5 min. Whole cell lysates were analyzed 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). Similar results were obtained with other clones with different silencing sequences.

Fig. 7. **Increased association of PI3K activity with tyrosine phosphorylated peptides in PKCδ-deficient cells.** Anti-p85 or Anti-phosphotyrosine immunoprecipitates were prepared from wild type (WT) or PKCδ-deficient cells (Δδ). PI3K activities were assayed with the precipitates as described under “Materials and methods”. Similar results were obtained with other clones with different silencing sequences.

Fig. 8. **Enhacement of FcγR-mediated phagocytosis in PKCδ-deficient cells.** Phagocytosis

and binding of ^{51}Cr-labeled 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 μM wortmannin for 5 min, added with EA, and then incubated for the indicated periods of time. (C) Wild type (closed circle), PKCδ-deficient cell (prepared with the silencing sequence-1, open circle), or another clone of PKCδ-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.

Fig. 1 A, B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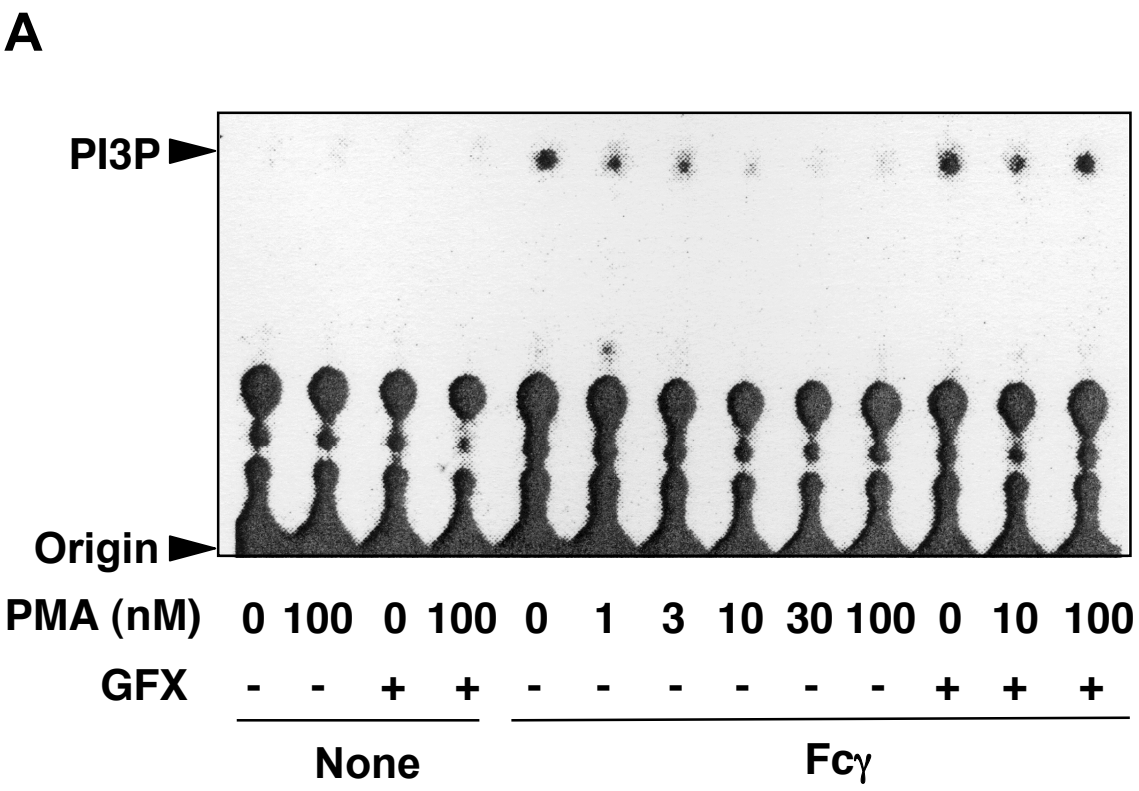
pAkt(S473)
totalAkt
None
Fcγ
C5a
B
pAkt(S473)
totalAkt
GFX - + + - + - + - + - +
PMA - - + - - + + - - + +
None
Fcγ
C5a

Fig. 2A


A
PI3P
Origin
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GFX - - + + - - - - - - + + +
None
Fcγ

Fig. 2B

B

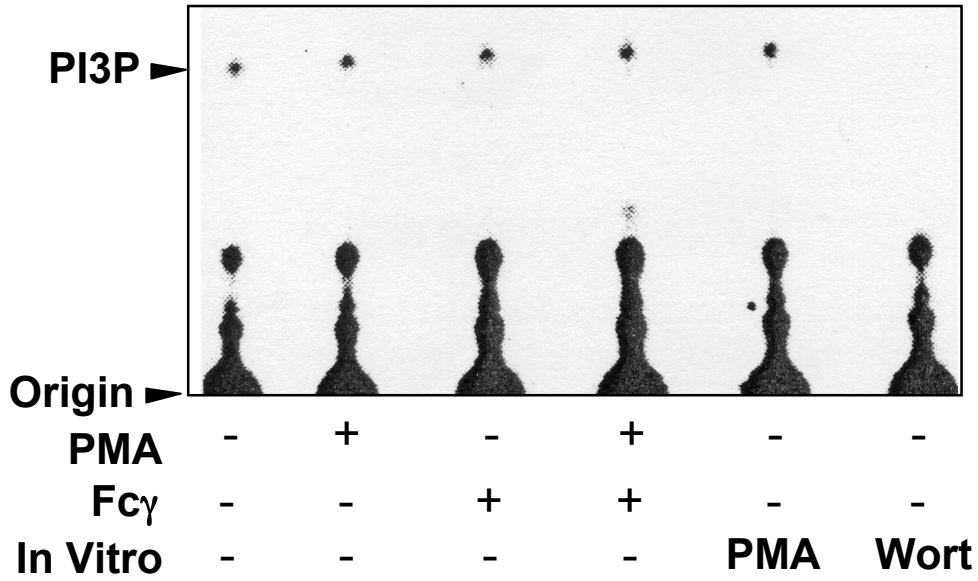
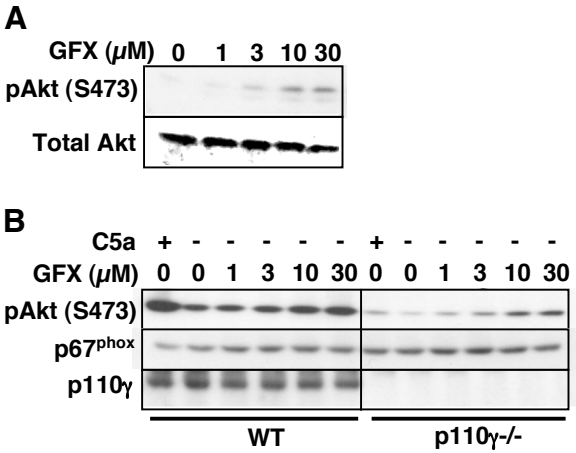


Fig. 3 A, B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pAkt (S473)
Total Akt
B
C5a + - - - - - + - - - - -
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pAkt (S473)
p67phox
p110γ
WT
p110γ-/-

Fig. 4

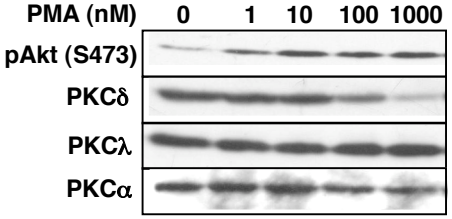
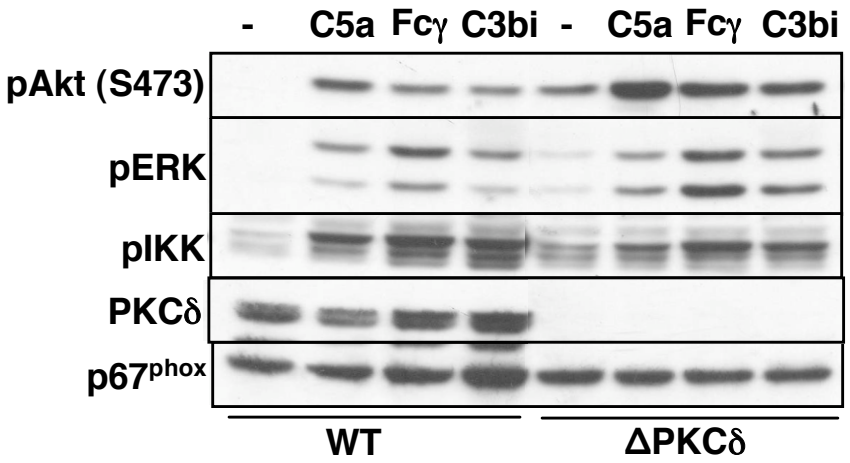


Fig. 5


-
C5a
Fcγ
C3bi
-
C5a
Fcγ
C3bi
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pERK
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PKCδ
p67phox
WT
ΔPKCδ

Fig. 6 A, B

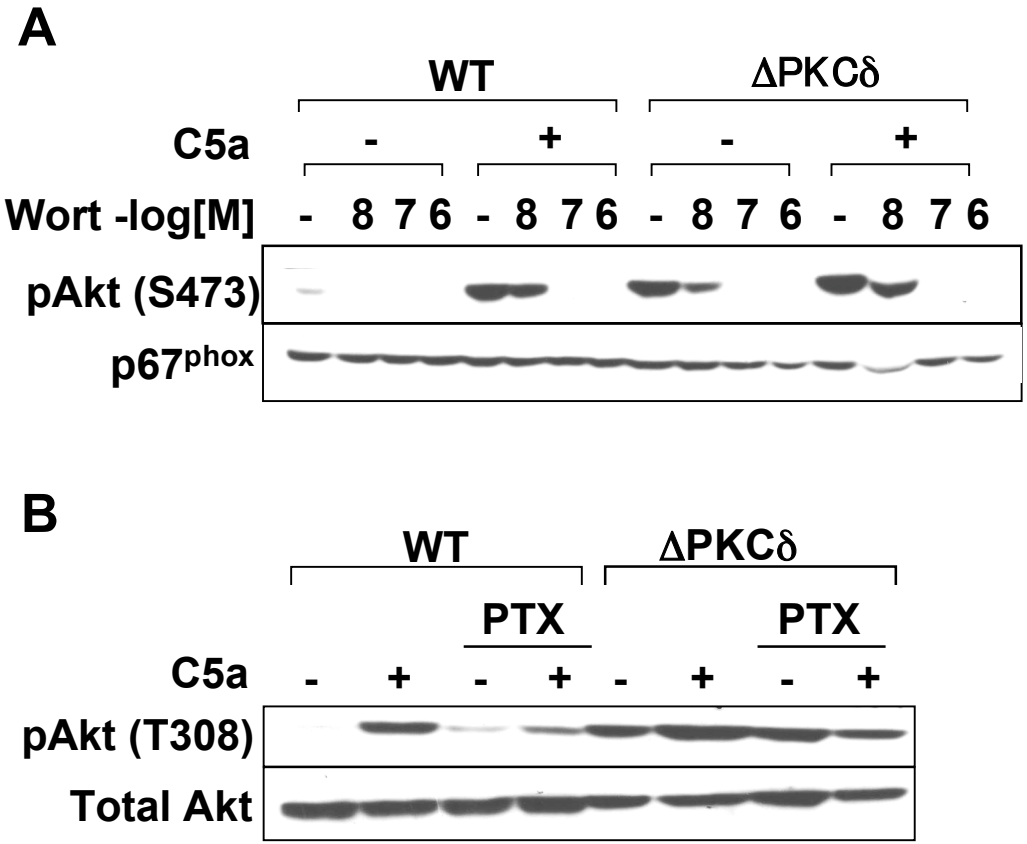

A
WT
ΔPKCδ
C5a
-
+
-
+
Wort -log[M]
- 8 7 6 - 8 7 6 - 8 7 6 - 8 7 6
pAkt (S473)
p67phox
B
WT
ΔPKCδ
PTX
PTX
C5a
- + - + - + - +
pAkt (T308)
Total Akt

Fig. 7

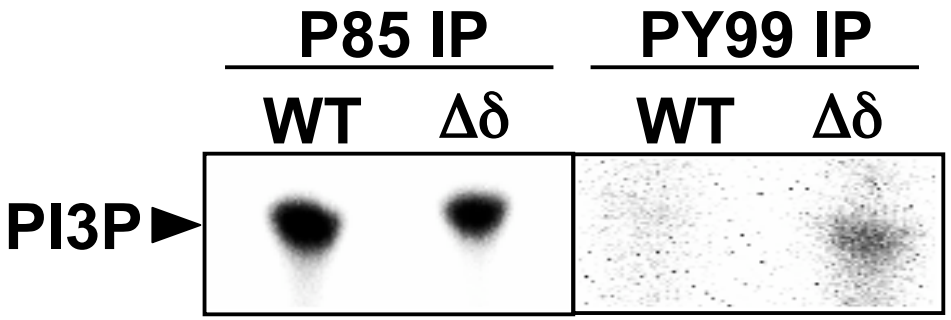


Fig. 8A

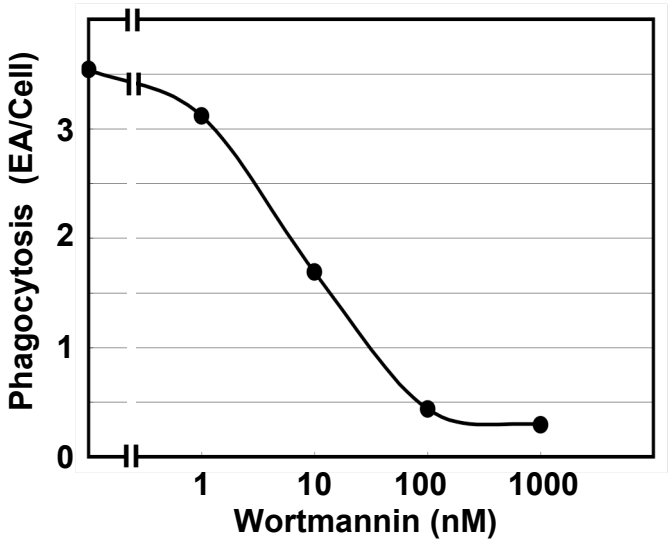
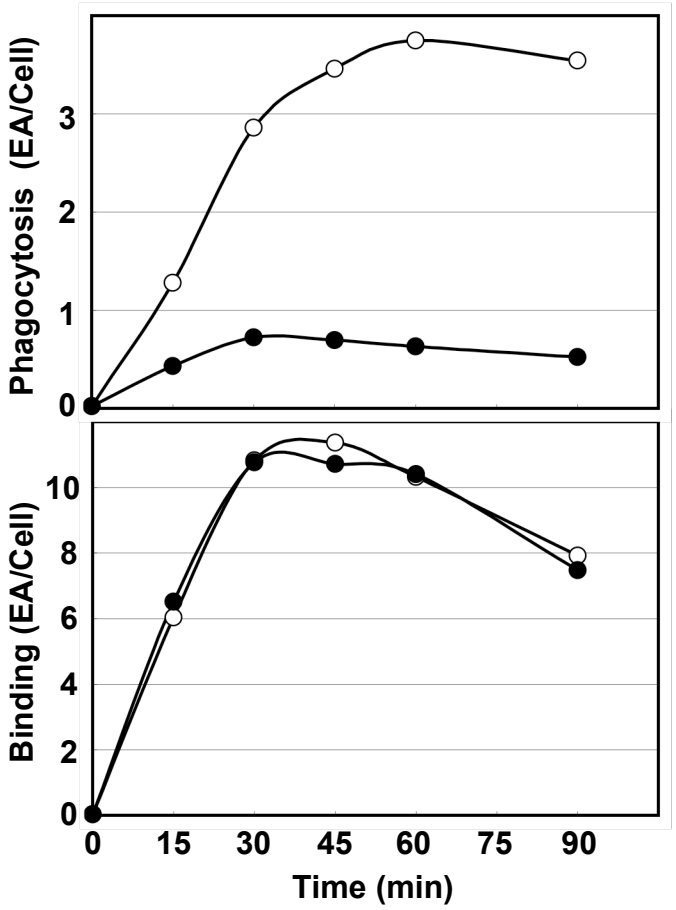


Fig. 8B


Phagocytosis (EA/Cell)
0
1
2
3
Binding (EA/Cell)
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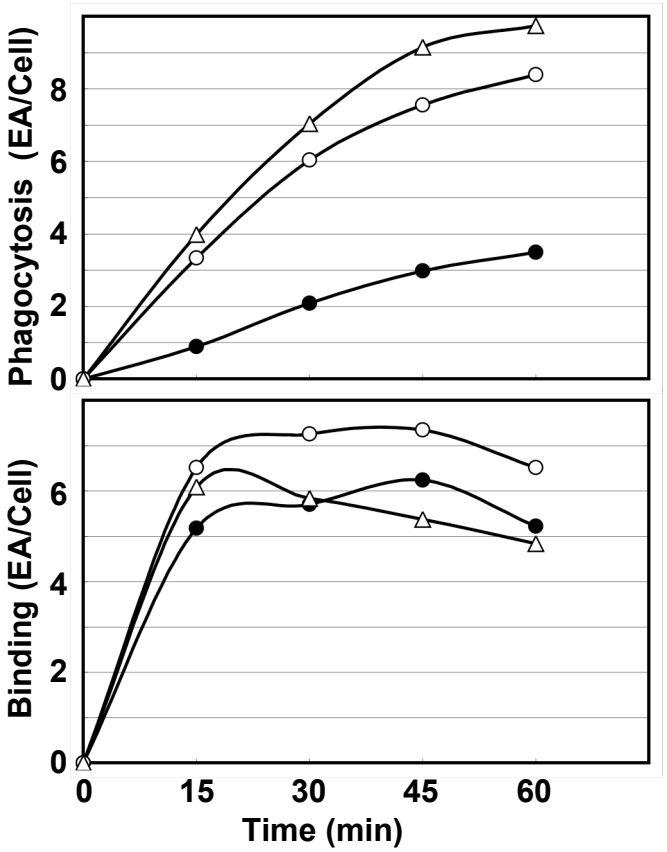
2
4
6
8
10
0
15
30
45
60
75
90
Time (min)

Fig. 8C


Phagocytosis (EA/Cell)
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Binding (EA/Cell)
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2
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15
30
45
60
Time (min)