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Differential Requirement for Classic and Novel PKC Isoforms in Respiratory Burst and Phagocytosis in RAW 264.7 Cells

Elaine C. Larsen,* Jeannine A. DiGennaro,† Naoaki Saito,‡ Sapna Mehta,† Daniel J. Loegering,§ Joseph E. Mazurkiewicz,* and Michelle R. Lennartz*‡

The binding of Ab (IgG)-opsonized particles by FcγRs on macrophages results in phagocytosis of the particles and generation of a respiratory burst. Both IgG-stimulated phagocytosis and respiratory burst involve activation of protein kinase C (PKC). However, the specific PKC isoforms required for these responses have yet to be identified. We have studied the involvement of PKC isoforms in IgG-mediated phagocytosis and respiratory burst in the mouse macrophage-like cell line, RAW 264.7. Like primary monocyte/macrophages, their IgG-mediated phagocytosis was calcium independent and diacylglycerol sensitive, consistent with novel PKC activation. Respiratory burst in these cells was Ca2+ dependent and inhibited by staurosporine and calphostin C as well as by the classic PKC-selective inhibitors Gö 6976 and CGP 41251, suggesting that classic PKC is required. In contrast, phagocytosis was blocked by general PKC inhibitors but not by the classic PKC-specific drugs. RAW 264.7 cells expressed PKCs α, β1, δ, ε, and ζ. Subcellular fractionation demonstrated that PKCs α, δ, and ε translocate to membranes during phagocytosis. In Ca2+-depleted cells, only novel PKCs δ and ε increased in membranes, and the time course of their translocation was consistent with phagosome formation. Confocal microscopy of cells transfected with green fluorescent protein-conjugated PKC α or ε confirmed that these isoforms translocated to the forming phagosome in Ca-replete cells, but only PKC ε colocalized with phagosomes in Ca2+-depleted cells. Taken together, these results suggest that the classic PKC α mediates IgG-stimulated respiratory burst in macrophages, whereas the novel PKCs δ and/or ε are necessary for phagocytosis.

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Immunglobulin G-mediated phagocytosis is part of the normal host defense system and may also be a component of inflammatory diseases such as rheumatoid arthritis and atherosclerosis. It is initiated by the binding of IgG-opsonized particles to FcγRs on the surface of monocyte/macrophages and neutrophils. Phagocytosis requires the cells to coordinate changes in cytoskeleton and membrane structure during pseudopod extension and particle internalization. In addition to phagocytosis, FcγR ligation activates other signaling pathways, including those regulating intracellular Ca2+ flux, mitogen-activated protein kinase cascades, and the respiratory burst (1, 2). Current research has focused on identifying signaling pathways activated by FcγR. Intracellular events resulting from FcγR clustering include tyrosine phosphorylation, actin rearrangement, arachidonic acid release, and activation of signaling enzymes phosphoinositide 3-kinase, protein kinase C (PKC), phospholipase C, phospholipase D, and phospholipase A2 (1–4). However, the specific functions of these components in FcγR-mediated signaling remain unknown.

Our previous work has confirmed a role for PKC activation during IgG-mediated phagocytosis in human monocytes and the human mononcyt cell line, Mono Mac 6 (5, 6). PKC is a family of related enzymes, which is divided into three groups on the basis of structure and cofactor requirements (7). The classic PKC (cPKC) isoforms α, βII, and γ require Ca2+, diacylglycerol (DAG), and phosphatidylserine (PS) for optimal activity. The novel PKC (nPKC) isoforms δ, ε, η, and θ lack the Ca2+ requirement but are activated by DAG and PS. The atypical PKC isoforms ζ and η bind PS, but are insensitive to Ca2+ and DAG. Despite the differing cofactor requirements, there is little difference between the in vitro substrate specificities of the isoforms. Because cells usually express several PKC isoforms, in vivo function is thought to be regulated by intracellular location and binding to specific targeting proteins (7).

Although PKC activation is necessary for phagocytosis in monocyte/macrophages, it is not known which PKC isoform(s) function in FcγR-mediated signaling (5, 6, 8). Both cPKC (α and β) and nPKC (ε) isoforms have been reported to translocate to membranes during FcγR cross-linking or phagocytosis (9, 10), but there is no direct evidence that these isoforms are required for FcγR-mediated signaling. Phagocytosis proceeds normally in the absence of a Ca2+ signal (11, 12) and can be increased by the treatment of cells with the PKC activators PMA and DAG (5, 6). These characteristics are consistent with the cofactor requirements for nPKC isoforms, i.e., Ca2+ independent and DAG sensitive. Therefore, we tested the hypothesis that one or more of the nPKC isoforms is required for phagocytosis.

IgG-mediated phagocytosis is accompanied by the generation of a respiratory burst; however, the role of specific PKC isoforms in respiratory burst is not certain. Respiratory burst can be activated...
by PMA in both neutrophils and monocyte/macrophages (13–15). In neutrophils, IgG-stimulated respiratory burst is decreased by pharmacological inhibition of PKC (16) or by selective antisense down-regulation of cPKC β (14), indicating a requirement for PKC. In comparison with neutrophils, studies in monocyte/macrophages have not been as consistent. In human monocytes, respiratory burst stimulated by opsonized zymosan was reduced to background levels by selective down-regulation of cPKC α (17). However, in guinea pig macrophages, pharmacological inhibition of PKC produced only a modest decrease in FcγR-mediated respiratory burst (15). We addressed the question of whether the mouse macrophage cell line RAW 264.7 was similar to human monocytes in requiring cPKC α for FcγR-mediated respiratory burst.

We have previously demonstrated that IgG-mediated phagocytosis requires PKC and that PKC activation is upstream of mitogen-activated protein kinase and Ca2⁺,CaM-dependent phospholipase (2, 5). Phagocytes also require PKC to produce the respiratory burst that accompanies IgG-mediated phagocytosis (1). To determine the functions of PKC in these two signaling pathways, it is necessary to identify the relevant PKC isoform(s). Activation of cPKC and nPKC is associated with their translocation to specific sites (6). Therefore, we examined the translocation of cPKC and nPKC isoforms during phagocytosis.

Materials and Methods
EDTA, EGTA, BSA, thimerosal, ammonium persulfate, Triton X-100, sucrose, DTT, HRP, and NaN₃ were obtained from Sigma (St. Louis, MO). Homovanillic acid and DMSO were obtained from Aldrich (Milwaukee, WI). Tris base, SDS, CaCl₂, and MgCl₂ were purchased from Mallinckrodt Baker (Paris, KY). PBS was purchased from BioWhittaker (Walkersville, MD). Calphostin C, staurosporine, PMA, and 4a-PMA were obtained from LC Laboratories (Woburn, MA). DAG was purchased from Avanti Polar Lipids (Alabaster, AL). Gli9203X was obtained from Biomed Research (Plymouth Meeting, PA). CPG 41251 was a gift from Novartis (Basel, Switzerland). G6 6976 was purchased from Alexis Biochemicals (San Diego, CA).

Cell culture
The RAW 264.7 mouse macrophage cell line was maintained in RPMI 1640 media (Life Technologies, Grand Island, NY) plus sodium pyruvate, nonessential amino acids, glutamate (BioWhittaker), and 10% newborn bovine serum (BBSA). For confocal analysis, the BSA-coating step included 5 μg of Alexa 568 (Molecular Probes, Eugene, OR)-conjugated BSA. BSA was conjugated to the Alexa dye according to manufacturer’s directions.

Phagocytosis assay
If cells were described as Ca depleted, they were incubated (45 min, 37°C) in HBSS buffer (−Ca cells), otherwise the assay was performed in HBSS²⁺ (−Ca⁺ cells). When used, inhibitors were added for the last 30 min of the incubation. ElgG (6 × 10⁹) were added to each well for the designated time at 37°C. The cells were washed twice with 0.83% NH₄Cl, once in PBS, and solubilized in 2 M NaOH. Phagocytosis is defined as the cell-associated radioactivity after hypotonic lysis of noninternalized targets and expressed as a percentage of phagocytosis in control cells. Nonspecific binding was determined using nonopsonized SRBC and subtracted from the phagocytosis values.

Synchronized phagocytosis of BiG
To determine the time course of phagocytosis of BiG, 2 × 10⁶ cells were plated on 13-mm glass coverslips (Ernest F. Fullam, Latham, NY) in 24-well plates. The cells were incubated in buffer as described above and then chilled on ice for 20 min. The buffer was removed, and 0.5 ml of ice-cold buffer containing 1 × 10⁸ BiG was added. After 5 min on ice for target binding, the plates were transferred to a 37°C water bath, and, at each time point, phagocytosis was stopped by fixing the cells with 3.7% formaldehyde. The coverslips were washed three times with PBS and blocked with 10% sheep serum (30 min, 21°C). The following incubations were performed with Ab diluted in 10% sheep serum (30 min, 21°C): 1) rabbit anti-BSA, 1:250; 2) FITC-conjugated goat anti-rabbit (Rockland, Gilbertsville, PA), 1:200; 3) permeabilize 5 min in 0.1% Triton X-100; 4) block as above; 5) rabbit anti-BiG (6) (Texas-red conjugated goat anti-rabbit; ICN Pharmaceuticals, Aurora, OH), 1:250; and 7) Hoechst nuclear stain (Molecular Probes). Coverslips were washed three times with PBS after each incubation. After washing, the coverslips were mounted on glass slides with Prolong antifade medium (Molecular Probes) and viewed with a triple-band filter. The cell nuclei stained blue, internalized beads were red, and external beads or portions of beads were yellow/green. The number of completely internalized beads were counted in a minimum of 100 cells and expressed as the phagocytic index: (no. of red beads/number of cells counted) × 100.

H₂O₂ production
H₂O₂ production was determined as described previously (18). Briefly, 1.2 × 10⁶ cells were incubated for 1 h in 1 ml HBSS containing 100 nM homovanillic acid and 1 IU HRP, with 1 × 10⁸ BiG (see Figs. 5 and 7) or 50 ng PMA (see Fig. 6) as the stimulus. The homovanillic acid oxidation product was measured fluorometrically.

Phagocytosis time course and cell fractionation
For PMA stimulation, confluent cultures of RAW 264.7 cells in 10-cm dishes were treated with 100 nM PMA or an equivalent volume of DMSO (carrier) (10 min, 37°C), the media was aspirated, and the cells were scraped and sonicated in 0.4 ml of lysis buffer. Lysates were centrifuged 45 min at 100,000 × g. The supernatant (designated cytosol) was removed, and the pellet was solubilized in lysis buffer plus 0.2% Triton X-100. The samples were centrifuged again as above. The supernatant (designated membrane) was removed from the pellet (designated insoluble fraction). For phagocytosis experiments, the protocol for synchronized phagocytosis of BiG was followed using cells in 100-mm culture dishes and 10 × 10⁶ targets. Phagocytosis was stopped at the various time points by scraping and sonicating the cells in lysis buffer; cell lysates were processed as described above. As no PKCs were detected in the insoluble cell fractions following phagocytosis (data not shown), PKC levels were quantified only in the membrane and cytosolic fractions.

DNA assay
DNA was measured by the method of Labarca and Paigen (19). Briefly, aliquots of whole cell lysate were diluted with assay buffer and 2% (final concentration) bisbenzamide, and the fluorescence was compared with a standard curve prepared with calf thymus DNA.

Western blots
For PMA experiments, protein in cytosolic and membrane fractions was quantified by the Bradford protein assay. Different proportions of the cell fractions were loaded to obtain a measurable signal; however, the amount in each fraction was matched between treatments to allow comparison. This corresponded to ~6% of the cytosol, 25% of the membrane, and 25%...
of the insoluble fractions. For phagocytosis experiments, equal volumes of the membrane fractions were loaded. Proteins were transferred to nitrocellulose membranes, and standards were located with Ponceau S stain. The membranes were blocked with 3% BSA in TBST and probed with the appropriate primary and secondary Ab in 1% BSA in TBST. For the primary Ab, mAb against PKCs α, δ, ε, ζ, and η (Transduction Laboratories, San Diego, CA) and polyclonal Ab against PKCs βI, βII, and ε (Santa Cruz Biotechnology, Santa Cruz, CA) were used. For the secondary Ab, goat anti-rabbit HRP (Santa Cruz Biotechnology) and rabbit anti-mouse HRP (The Jackson Laboratory, Bar Harbor, ME) were used. Bands were detected with Ultra Supersignal ECL reagent (Pierce, Rockford, IL) and were quantified by densitometry. In phagocytosis experiments, the densities of the PKC bands were normalized for cell DNA before comparison by ANOVA.

**AnoVA.**

The PKC bands were normalized for cell DNA before comparison by ANOVA. Results with the majority of PKC I, like the other isoforms, is cytosolic.

**Confocal imaging**

Transfected cells were replated onto 13-mm coverslips (5 × 10⁵ cells/coverslip) for 3 h. Media were removed and the cells were washed and detected only in positive controls but not in RAW 264.7 cell lysate. Media were removed and the cells were washed and detected only in positive controls but not in RAW 264.7 cell lysate. In phagocytosis experiments, the densities of the PKC bands were normalized for cell DNA before comparison by ANOVA.

**Statistics**

Unless otherwise stated, all measurements were made in triplicate on at least three separate cell preparations. Data are expressed as the mean ± SEM. Comparisons were made by ANOVA. Results with p ≤ 0.05 were considered significant.

**Results**

**RAW 264.7 cells express multiple PKC isoforms**

We identified the PKC isoforms expressed in RAW 264.7 cells by Western blot analysis with isoform-specific Abs (Fig. 1). cPKC α, cPKC βI, nPKC δ, nPKC ε, and atypical PKC ζ were detected in RAW 264.7 cell lysates. cPKC γ, nPKC θ, and nPKC η could be detected only in positive controls but not in RAW 264.7 cell lysate (data not shown). PKC βII could not be reproducibly detected in cell fractions.

**Phagocytosis in RAW 264.7 cells is Ca independent**

Earlier studies demonstrated that IgG-mediated phagocytosis is Ca²⁺ independent in monocytes and macrophages (11, 12). Ca²⁺ depletion did not affect the extent or rate of IgG-mediated phagocytosis by RAW 264.7 cells (Fig. 2), indicating that this is an appropriate model for studying Ca-independent phagocytic signaling. The cells were Ca²⁺ depleted by incubation in Mg/EGTA buffer (45 min, 37°C). We have previously shown that this treatment virtually eliminates free intracellular calcium concentration ([Ca²⁺]) in monocytes as determined by fura-2 fluorescence in cells stimulated with IgG-opsonized particles (12). Similar results were obtained with RAW 264.7 cells stimulated with either platelet-activating factor or immune complexes (data not shown).

Ca²⁺ depletion inhibits membrane translocation of PKCs α and βI, but not PKC δ or ε

Because Ca²⁺ depletion did not alter phagocytosis, we examined its effect on PKC translocation. We tested the hypothesis that Ca²⁺ depletion would inhibit membrane localization of PKCs α and βI in response to PMA, a potent stimulus of PKC translocation. The baseline distribution of PKC isoforms was the same in +Ca and −Ca cells, indicating that Ca²⁺ depletion alone did not alter PKC location (data not shown). PKCs α, βI, δ, and ε are primarily cytosolic in unstimulated cells (Fig. 3). Although the intensity of the bands in the cytosol and membrane fractions for βI appear similar, the differences in loading must be taken into account. As ~25% of the membrane fraction vs 6% of the cytosol was loaded, the majority of PKC βI, like the other isoforms, is cytosolic.

PMA treatment of Ca-containing cells caused a >6-fold decrease in cytosolic PKC α (Fig. 3, lane 1 vs 4) and a substantial increase in the particulate (membrane + insoluble) fractions (>12-fold; lanes 2 and 3 vs lanes 5 and 6). In contrast, Ca²⁺ depletion prevented the translocation of PKC α to membrane and insoluble fractions in response to PMA, resulting in a PKC α distribution similar to that of resting cells (Fig. 3, lanes 1–3 vs lanes 7–9). PMA stimulated PKC βI translocation to the insoluble fraction in the presence, but not the absence, of Ca²⁺. In the presence of Ca²⁺, PKC βI in the cytosol and membrane fractions decreased by 9-fold.
Phagocytosis requires a Ca\textsuperscript{2+}-independent, DAG-sensitive PKC

Phagocytosis in human monocytes is increased by PMA or DAG, consistent with a role for PKC activation in phagocytosis (5, 8). Likewise, phagocytosis in RAW 264.7 cells was also PMA/DAG sensitive, increasing >60% when the cells were treated with either of these PKC activators (Fig. 4). Conversely, treatment with the PKC inhibitors calphostin C or staurosporine decreased phagocytosis in a dose-dependent fashion (Fig. 5, A and B). Thus, similar to our results in primary monocytes and MonoMac 6 cells (5, 6), phagocytosis in RAW 264.7 cells requires a PKC that is both Ca\textsuperscript{2+}-independent and PMA/DAG sensitive, matching the characteristics of the nPKC isoforms.

Inhibition of cPKC affects respiratory burst but not phagocytosis

Unlike particle uptake via complement receptors or endocytosis, IgG-mediated phagocytosis is accompanied by a respiratory burst (1, 21), which requires translocation and assembly of NADPH oxidase components at the membrane, and results in production of \( \text{O}_2\)\textsuperscript{2-} and \( \text{H}_2\text{O}_2 \) (18, 22). NADPH oxidase activation has been shown to require an increase in intracellular Ca\textsuperscript{2+} and PKC activity (22, 23). As predicted, calphostin C and staurosporine decreased \( \text{H}_2\text{O}_2 \) production by RAW 264.7 cells >75% at doses similar to those that affect phagocytosis (Fig. 5, A and B). Additionally, depletion of intracellular Ca\textsuperscript{2+} abolished \( \text{H}_2\text{O}_2 \) production (Fig. 6). As PKCs α and β did not translocate to the membrane in Ca\textsuperscript{2+}-depleted cells (Fig. 3), these results are consistent with the hypothesis that cPKC activation is required for IgG-mediated respiratory burst in RAW 264.7 cells.

This hypothesis predicts that the cPKC selective inhibitors CGP 41251 and Gö 6976 will block the IgG-stimulated burst, but not affect phagocytosis. Fig. 7 (A and B) demonstrates that CGP 41251 and Gö 6976 caused a dose-dependent decrease in \( \text{H}_2\text{O}_2 \) production, but not phagocytosis. Interestingly, the nonselective PKC inhibitor GF109203X also blocked respiratory burst but not phagocytosis (Fig. 7C). These results are further evidence for a role for cPKC α and/or β in the respiratory burst in RAW 264.7 cells. Phagocytosis was not affected, implying that cPKCs are not involved in FcγR-mediated ingestion and that these drugs are not otherwise interfering with FcγR signaling.

nPKCs translocate to the membrane fraction during phagosome formation

cPKC and nPKC isoforms associate with membranes by binding DAG and PS (7). We reasoned that those PKC isoforms involved in FcγR-initiated signaling would translocate to the membrane fraction during IgG-mediated phagocytosis, so we measured membrane-bound cPKC α, cPKC β, nPKC δ, and nPKC ε during synchronized phagocytosis of IgG-opsonized glass beads. Time course studies showed that phagocytosis took at least 7.5 min (Fig. 8A). Few completely internalized targets were observed before 7.5 min. Internalized targets were present in most cells at 7.5 min, and
cytosis or respiratory burst assayed as described in Materials and Methods were present in the membrane at all time points. In contrast, PKCs did not increase in the membranes during phagocytosis. PKC_a depleted of intracellular Ca^{2+} always increased in the membrane in both +Ca and −Ca cells (Fig. 8B). ANOVA indicated that phagocytosis increased PKCs δ and ε in the membrane in both +Ca and −Ca cells (p < 0.05). To examine the pattern of nPKC translocation during phagosome formation, the levels of membrane PKCs δ and ε in Ca^{2+}-depleted cells were quantified (Fig. 8C). PKC δ increased gradually and was maximally elevated after 7.5 min, corresponding to the appearance of closed phagosomes. PKC ε was significantly increased in the membrane fraction by 5 min, suggesting that this isoform translocates during the formation of phagosomes.

Although the amount of membrane-bound PKC increased during phagocytosis, the cytosolic levels did not change (data not shown). Unlike PKC activation during PMA stimulation, the amount of PKC associated with the membranes during phagocytosis was small compared with that in the cytosol. This is consistent with phagocytosis being a localized membrane event, with a relatively small proportion of the PKC mass targeted to membrane sites involved in phagocytosis.

**GFP PKCs α and ε translocate to the phagosome membrane**

The detection of PKCs α and ε in membrane fractions during phagocytosis is consistent with their localization to phagosomes. To identify the membranes to which these isoforms move, we transfected GFP-conjugated PKCs α and ε into RAW 264.7 cells and examined their location during phagocytosis. In the presence of Ca^{2+}, GFP PKC α concentrated in membrane regions associated with targets at 2.5 and 7.5 min, but at 15 min, when phagosome closure was complete, little localization remains (Fig. 9, a–f). In contrast, GFP PKC α was cytosolic at all times in Ca^{2+}-depleted cells; no concentration of the GFP signal was detected (Fig. 9, g–l). Analysis of topographic images confirmed the lack of GFP PKC α concentration in Ca^{2+}-depleted cells (data not shown).

PKC ε also translocated to forming phagosomes (Fig. 9, m–r). The localization of GFP PKC ε was similar in the presence (data not shown) and absence (Fig. 9, m–r) of Ca^{2+}. That is, GFP PKC ε localized to targets at early time points (2.5 min, 7.5 min; Fig. 9, m, n, p, and q) but this localization was largely lost by 15 min. These images demonstrate that PKC ε is associated with the phagocytic cup. The results confirm the biochemical data (Fig. 8).
demonstrating translocation of PKC ε to cell membranes during phagocytosis in Ca\(^{2+}\)-depleted cells. The confocal data extend these findings by localizing the PKC ε to the target-associated membranes, consistent with our hypothesis that nPKC is involved in IgG-mediated phagocytosis.

**Discussion**

IgG-mediated phagocytosis is a key function of the immune system for identifying and destroying pathogens. Ligation and cross-linking of phagocyte IgG receptors (FcγR) results in particle internalization, but unlike the phagocytosis of complement-opsonized particles or apoptotic cells, FcγR ligation induces macrophages to produce a respiratory burst (1, 21, 24). In monocyte/macrophages, phagocytosis and respiratory burst are increased by PMA and reduced by PKC inhibitors, suggesting a requirement for PKC (5, 6, 8, 15, 18, 25).

Different PKC isoforms are likely to be involved in the multiple signaling pathways from FcγR, and research has focused on identifying required isoforms to study their functions in these pathways. Increased Ca\(^{2+}\)-dependent PKC activity has been reported during IgG-mediated phagocytosis in monocytes (8). When monocytes were stimulated by cross-linking FcγR, Ca\(^{2+}\)-dependent PKC activity increased and both cPKC β and nPKC ε translocated to the membrane fraction (9). Importantly, PKC α has been observed in nascent and fully formed phagosomes by immunostaining (10). Although these reports provide evidence that cPKCs are activated upon FcγR ligation, they are not consistent with repeated observations that phagocytosis proceeds at exceedingly low [Ca\(^{2+}\)]\(_i\) in monocyte/macrophages and related cell lines (6, 11, 12). The [Ca\(^{2+}\)]\(_i\), measured in these studies (<2 mM) is well below that required for cPKC isoforms to bind to membranes (0.8–1 μM) or to be fully activated (1.5–38 μM) (26). Thus, although cPKCs may be activated during phagocytosis, they are apparently not required for particle ingestion.

Phagocytosis in RAW 264.7 cells was Ca\(^{2+}\) independent (Fig. 2), increased by PMA and DAG (Fig. 4), and decreased by staurosporine and calphostin C (Fig. 5), indicating that PKC is critical for phagocytosis in these cells. As calphostin C acts as a competitive inhibitor for the DAG binding site on cPKC and nPKC isoforms (27, 28), inhibition of phagocytosis by calphostin C is further evidence that a DAG-requiring PKC isoform is involved. Thus, phagocytosis responded to conditions consistent with activation of a DAG-dependent, Ca\(^{2+}\)-independent nPKC isoform.

We hypothesized that PKC activated during phagocytosis would translocate to, and be detected in, the membrane fraction. In this study, PKC α increased in the membrane fraction during phagocytosis, consistent with its activation and previous reports documenting PKC α and Ca\(^{2+}\)-dependent PKC activity in phagosomes (8–10). However, Ca\(^{2+}\) depletion, which did not inhibit phagocytosis, completely prevented membrane association of PKC α and βI (Figs. 3, 8B, and 9). Both PKCs δ and ε increased in the membrane fraction with a time course consistent with their participation in phagocytosis. PKC δ was present at time points corresponding to the presence of fully formed phagosomes. Importantly, membrane-associated PKC ε increased before and during particle internalization. This agrees with previous reports that PKC activity increases after FcγR ligation but before phagosome closure (8, 9) and that membrane-associated PKC ε is increased by 2–5 min after FcγR cross-linking in monocytes (9). Thus participation of nPKC δ and/or ε in phagocytic signaling is supported by their rapid translocation to membranes upon initiation of IgG-mediated phagocytosis as well as by their activation characteristics.

**FIGURE 7.** Macrophage functions of phagocytosis and respiratory burst can be separated by selective inhibition of cPKC. Cells were incubated (30 min, 37°C) with the indicated doses of CGP 41251 (A), Go 6976 (B), or GF109203X (C), and phagocytosis or respiratory burst was assayed. Control (100%) is defined as the phagocytosis or H\(_2\)O\(_2\) release by cells treated with carrier only. Data are expressed as mean ± SEM, n = 3. *, p < 0.05.
FIGURE 8.  

A, Time-dependent internalization of IgG-opsonized glass beads. Cells were grown on glass coverslips and phagocytosis was determined as described in Materials and Methods. Data shown are average ± range, n = 2. 

B, Translocation of PKC isoforms during BlgG phagocytosis. Western blots (described in Materials and Methods) show PKC isoforms α, βI, δ, and ε in the membrane fractions of RAW 264.7 cells undergoing phagocytosis of IgG-opsonized glass beads for the indicated times. Cells were Ca replete (+Ca) or Ca depleted (−Ca) as described in Materials and Methods. Mouse brain (+) was included as a positive control. Blots are representative of a minimum of three experiments. 

C, Quantitation of membrane-associated nPKC isoforms during phagocytosis in Ca-depleted cells. Densities were normalized for cell DNA before comparison by ANOVA. *, Significantly greater than 0 time point, p < 0.05. PKC δ, n = 3; PKC ε, n = 4.
PKC activity has been implicated in regulation of respiratory burst (14, 25, 34, 35), with cPKC isoforms identified as the primary mediators (13, 14, 17, 23). In our studies, CGP 41251 and Gö 6976 inhibited respiratory burst and not phagocytosis, thus separating these pathways at the level of PKC activation (Fig. 7, A and B). Because PKC α was the only cPKC that translocated to membranes during phagocytosis, this suggests that respiratory burst is mediated by PKC α in RAW 264.7 cells, consistent with results in primary human monocytes (17). A recent report that overexpression of a dominant-negative PKC α in RAW 264.7 cells decreased their ability to kill the intracellular pathogen *Leishmania donovani*, but had no effect on their phagocytosis (36), provides additional evidence that PKC α is activated, but not required, during phagocytosis.

Our results that the cPKC inhibitor Gö 6976 reduced, but did not eliminate, respiratory burst in neutrophils are in agreement with those reported by Pongracz and Lord (13). In their studies, the magnitude of the reduction (50–60%) was similar to that seen in our experiments (Fig. 7) using the same cPKC inhibitors. Similarly, the down-regulation of PKC β in differentiated HL-60 cells using antisense oligonucleotides produced a partial inhibition of superoxide release (14). In contrast, nonselective PKC inhibitors (Fig. 5 and 7C) gave a much more complete inhibition of respiratory burst. Although the absence of a respiratory burst in the Ca²⁺-depleted cells may seem to contradict the partial inhibition of respiratory burst achieved with pharmacological inhibition of cPKC, the lack of respiratory burst in the Ca²⁺-depleted cells may also be due to the inhibition of other Ca²⁺-dependent enzymes, notably cPLA₂, also required for superoxide production (37). Taken together, these data suggest that the respiratory burst is regulated by multiple signaling pathways, with part of the burst mediated by cPKC and part by other PKC isoforms.

GF109203X is used as a nonisoform-selective inhibitor of PKC (38, 39). Unexpectedly, GF109203X inhibited respiratory burst but not phagocytosis in the same manner as the cPKC-selective drugs (Fig. 7). There are several possible explanations for this discrepancy. One is that the concentrations of GF109203X used in our study were sufficient to inhibit the cPKC but not the nPKC isoforms. The IC₅₀ values given for GF109203X vary with the different isoforms and are lower for PKC α than for PKC δ or ε (39). However, our dose curve (1–10 μM) is at and above the levels previously shown to effect PKC activity and function in vivo (38–41), so this interpretation seems unlikely.

Alternatively, phagocytosis may require PKC translocation but not kinase activity. GF109203X is a competitive inhibitor of the PKC ATP-binding site (38, 39), so this interpretation seems unlikely. Nonetheless, our results that the cPKC inhibitor Gö 6976 reduced, but did not eliminate, respiratory burst in neutrophils are in agreement with those reported by Pongracz and Lord (13). In their studies, the magnitude of the reduction (50–60%) was similar to that seen in our experiments (Fig. 7) using the same cPKC inhibitors. Similarly, the down-regulation of PKC β in differentiated HL-60 cells using antisense oligonucleotides produced a partial inhibition of superoxide release (14). In contrast, nonselective PKC inhibitors (Fig. 5 and 7C) gave a much more complete inhibition of respiratory burst. Although the absence of a respiratory burst in the Ca²⁺-depleted cells may seem to contradict the partial inhibition of respiratory burst achieved with pharmacological inhibition of cPKC, the lack of respiratory burst in the Ca²⁺-depleted cells may also be due to the inhibition of other Ca²⁺-dependent enzymes, notably cPLA₂, also required for superoxide production (37). Taken together, these data suggest that the respiratory burst is regulated by multiple signaling pathways, with part of the burst mediated by cPKC and part by other PKC isoforms.

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Alternatively, phagocytosis may require PKC translocation but not kinase activity. GF109203X is a competitive inhibitor of the PKC ATP-binding site (38, 39), and as such should not inhibit the translocation of the PKC isoforms. Calphostin C inhibits the DAG binding site and translocation of PKC ε (27) and presumably other PKC isoforms. This suggests that the translocation and presence of the PKC enzyme, but not its kinase activity, may be required during phagocytic signaling. Precedence for such a mechanism comes from studies on the regulation of neurite outgrowth by PKC ε. Zeidman and coworkers have shown that the catalytic domain of PKC ε is not required to induce neurite outgrowth, and the effect cannot be inhibited by GF109203X, indicating that kinase activity is not required (42). In a similar manner, PKC α has been shown to activate phospholipase D even in the absence of its kinase activity (43). Our results are consistent with the hypothesis that the role of PKC ε in phagocytosis is independent of its kinase activity.

In conclusion, we have separated the signaling pathways for IgG-mediated phagocytosis and respiratory burst at the level of PKC activation. We have demonstrated that cPKC inhibition or Ca²⁺ depletion inhibited respiratory burst but not phagocytosis. IgG-mediated phagocytosis was Ca²⁺-dependent and DAG sensitive, implying activation of the nPKC isoforms. Both nPKCs δ and ε increased in cell membranes during phagocytosis with a time course consistent with particle ingestion. Our results suggest that PKCs δ and ε translocate and are likely candidates for signal transduction during IgG-mediated phagocytosis in RAW 264.7 cells.
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