Selective roles for α-PKC in positive signaling for $\mathbf{O}_2^-$ generation and calcium mobilization but not elastase release in differentiated HL60 cells

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Abstract

Protein kinase C (PKC) isotypes and Ca$^{2+}$ mobilization have been implicated in phagocytic cell functions such as $\mathbf{O}_2^-$ generation. Ca/DG-dependent α-PKC and β-PKC have similar substrate specificities and cofactor requirements in vitro. However it is not known if these isotypes play redundant or unique roles in the intact cell. In the present study, a role for α-PKC in positive signaling for fMet-Leu-Phe- and PMA-activated $\mathbf{O}_2^-$ generation was probed using an siRNA strategy in HL60 cells differentiated to a neutrophilic phenotype (dHL60 cells). A selective decrease in α-PKC in dHL60 cells attenuated $\mathbf{O}_2^-$ generation but not degranulation, and reduced ligand-induced phosphorylation of p47phox as previously shown for β-PKC. However α-PKC, unlike β-PKC, was a positive regulator of fMet-Leu-Phe-triggered Ca$^{2+}$ uptake via SOCC (Store Operated Calcium Channels). The ability of a selective SOCC inhibitor, MRS1845, to decrease fMet-Leu-Phe induced Ca$^{2+}$ uptake and $\mathbf{O}_2^-$ generation confirmed that Ca$^{2+}$ uptake via SOCC was required for $\mathbf{O}_2^-$ generation. These results indicate that α-PKC and β-PKC are required for optimal $\mathbf{O}_2^-$ generation, but play different roles in Ca$^{2+}$ signaling for phagocytic responses such as $\mathbf{O}_2^-$ generation.

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1. Introduction

A role for protein kinase C (PKC) isotypes has been implicated in signaling for proinflammatory responses of phagocytic cells such as generation of $\mathbf{O}_2^-$ [1–5]. PKC is a family of structurally related isotypes which have different cofactor and substrate specificities [6,7]. Neutrophils, monocytes/macrophages and HL60 cells differentiated to a neutrophilic phenotype (dHL60 cells), contain multiple isotypes of PKC, including Ca$^{2+}$/DG-dependent isotypes α-PKC, alternately spliced βI-PKC and βII-PKC, Ca$^{2+}$-independent DG-dependent isotype, δ-PKC and PS-dependent, Ca$^{2+}$/DG-independent ζ-PKC [4,5,8–12]. The phorbol ester PMA, which activates DG-dependent α-PKC, β-PKC and δ-PKC, but not ζ-PKC, triggers $\mathbf{O}_2^-$ generation but not degranulation (elastase release) in dHL60 cells [5,13]. In neutrophils and dHL60 cells, ligands such as fMet-Leu-Phe, but not PMA, elicit the twin signals of elevated cytosolic Ca$^{2+}$ and activation of protein kinase C (PKC) [14]. Elevation of cytosolic Ca$^{2+}$ is essential for optimal fMet-Leu-Phe-induced $\mathbf{O}_2^-$ generation and degranulation [15–18]. fMet-Leu-Phe triggers activation of a phospholipase Cβ which cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to generate diglyceride (DG), an activator of DG-dependent PKC isotypes, and inositol 1,4,5-trisphosphate (IP$_3$), a trigger for release of intracellular Ca$^{2+}$ stores [15–18]. IP$_3$-initiated depletion of the intracellular Ca$^{2+}$ stores can trigger uptake of extracellular Ca$^{2+}$ via store operated calcium channels (SOCC) [19–21]. Ligands such as fMet-Leu-Phe can also trigger entry of extracellular Ca$^{2+}$ through receptor-operated non-
selective cation channels which conduct Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\) [22–26].

Assembly of an active NADPH oxidase for \(\text{O}_2\) generation, requires translocation of cytosolic factors \(p47^{\text{phox}}\), \(p67^{\text{phox}}\) and rac2 to the plasma membrane where they interact with the integral membrane protein cytochrome \(b_558\) [27–32]. \(p47^{\text{phox}}\) is phosphorylated in ligand-activated phagocytic cells. \(p47^{\text{phox}}\) contains multiple phosphorylation sites, including a number of classical PKC target sites and is phosphorylated by multiple PKC isoforms in vitro [10,33]. However in vitro activity does not necessarily predict a role for a particular PKC isotype in the intact cell, where access to both substrate and cofactors is critical in controlling signaling specificity.

Previously, a role for \(\beta\)-PKC in regulating \(\text{O}_2\) generation was established in neutrophils and dHL60 cells both in vitro and in vivo [5,9,33,34]. However, \(\beta\)-PKC does not regulate \(\text{O}_2\) generation in monocytes [36], indicating selective involvement of different PKC isoforms in a manner that is cell-type dependent. Roles for \(\alpha\)-PKC and \(\delta\)-PKC have also been implicated in activation of \(\text{O}_2\) generation in monocytes, RAW246.7 cells, and in transgenic COS-phox cells [4,35–37], but it is not known whether \(\alpha\)-PKC is also involved in signaling for \(\text{O}_2\) generation in dHL60 cells and neutrophils where \(\beta\)-PKC is required for optimal \(\text{O}_2\) generation [5,9,34]. \(\alpha\)-PKC and \(\beta\)-PKC are Ca/DG-dependent PKC isoforms which have strong structural homology, identical cofactor requirements for \(\text{Ca}^{2+}\) and DG, and have similar substrate specificity in vitro. The present study asks the question whether \(\alpha\)-PKC and \(\beta\)-PKC play redundant roles in the intact cell in signaling for \(\text{O}_2\) generation, or whether in the intact cell these different PKC isoforms play unique roles.

In the present study, a role for \(\alpha\)-PKC in positive signaling for fMet-Leu-Phe- and PMA-activated \(\text{O}_2\) generation was probed using an siRNA strategy. A selective decrease in \(\alpha\)-PKC in dHL60 cells attenuated \(\text{O}_2\) generation but not degranulation, and reduced ligand-induced phosphorylation of \(p47^{\text{phox}}\) as previously shown for \(\beta\)-PKC. \(\alpha\)-PKC, unlike \(\beta\)-PKC, was a positive regulator of ligand-initiated \(\text{Ca}^{2+}\) uptake via SOCC a positive signal for \(\text{O}_2\) generation, suggesting that \(\alpha\)-PKC and \(\beta\)-PKC have different roles in signaling for phagocytic responses such as \(\text{O}_2\) generation.

2. Experimental procedures

2.1. Reagents

Cytochalasin B, cytochrome \(c\), protease inhibitors (leupeptin and aprotinin), BSA, PMA, fMet-Leu-Phe, elastase substrate MeO-Suc-AAPV-MCA, MRS1845, protease inhibitor cocktail, phosphatase inhibitor cocktail, and protease inhibitor AEBSF were purchased from Sigma. PMA was stored as a concentrated stock in DMSO, and diluted with HEPES Buffer before use. f-Met-Leu-Phe was stored as a stock solution in ethanol and diluted in buffer prior to use. Fura-2-AM was purchased from Molecular Probes and thapsigargin from Biomol. Anti-peptide polyclonal antibodies to \(\alpha\)-PKC, \(\beta\)-PKC, \(\delta\)-PKC, \(\zeta\)-PKC, and \(p47^{\text{phox}}\), and peroxidase conjugated goat anti-rabbit IgG, donkey anti-goat IgG, and A/G plus agarose were obtained from Santa Cruz Biotechnology, Inc. A polyclonal antibody to phosphoserine was purchased from Zymed. Protein A agarose was obtained from Invitrogen.

2.2. HL60 cell culture

\(\beta\)-PKC null clones of human promyelocytic HL60 leukemic cells which express \(\alpha\)-PKC, \(\beta\)-PKC, \(\delta\)-PKC and \(\zeta\)-PKC, were used as previously described [9]. The cells were grown in suspension culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% MEM vitamin solution, 0.1% gentamicin and 10% heat-inactivated fetal bovine serum (FBS). The cell cultures were maintained at 37 °C in a 5% CO2 humidified atmosphere.

2.3. siRNA synthesis and sequences

siRNA targeting the second variable V2 domain of \(\alpha\)-PKC was designed by Irie and synthesized by Dharmacon with 3' overhangs of 2'-deoxyuridymidine [38]. The siRNA sense strand was 5’ AAAGCGCUAGGUUUCCUGAATT 3’, and the antisense strand was 5’ AUCCAGCAACCUCAGCCUUUTT 3’ [38]. We also used validated stealth siRNA to \(\alpha\)-PKC, target sequences Duplex 1 5’-CCAUCCGAUUGUUCCUUCUCAUA 3’, and Duplex 2 GCCUC-CAUUUGGAGAAGAGU, for effective reduction in \(\alpha\)-PKC. The sequences duplex 1 5’-CCAUCCAUACAGAAACCUAGAAU-3’ and duplex 2 5’-CCAUCCACAAGGAUAGCUAGCAAA-3’ were used for \(\delta\)-PKC reduction (Invitrogen). siRNA with equivalent % GC nucleotide content was used as controls [39].

2.4. Treatment of cells with siRNA

Delivery of the siRNA (250 nM) was enhanced by 4 μg/ml of cationic lipid TransIT-TKO (Mirus) or by electroporation. HL60 cells (10–15 × 10^6) were differentiated for 3 days with 1.3% DMSO at a cell density of 1 × 10^6/ml. Delivery of Irie/Dharmacon siRNA to \(10–15 \times 10^6\) cells/800 μl with TransIT-TKO was followed by culture in 3 ml RPMI containing 10% heat inactivated FBS and 1.3% DMSO for 48 h. Delivery of Stealth siRNA to \(10–15 \times 10^6\) cells that had been cultured for 3 days in 1.3% DMSO, was enhanced by electroporation in 800 μl OptiMEM (10–15 × 10^6 cells/800 μl) containing 1.3% DMSO, in a BioRad Gene Pulser at 270 V and 500 μF, followed by culture in 3 ml RPMI containing 10% heat inactivated FBS plus 1.3% DMSO for 48 h [40]. Cells were harvested and suspended in a HEPES buffer (pH 7.3) having the composition Na+ 150 mM, K+ 5 mM, Ca\(^{2+}\) 1.29 mM, Mg\(^{2+}\) 1.2 mM, HEPES 10 mM, Cl\(^{-}\) 155 mM and HEPES 10 mM. Equivalent \(\alpha\)-PKC reduction and cell responses were obtained with these different protocols.

2.5. Superoxide anion generation

The generation of superoxide anion \((\text{O}_2^-)\) by dHL60 cells was measured as superoxide dismutase inhibitable cytochrome \(c\) reduction by either a continuous recording method [5] or endpoint analysis. Cells were activated by 1 μM fMet-Leu-Phe in the presence of 5 μg/ml cytochalasin B, or by 1 μg/ml PMA in the absence of cytochalasin B.

2.6. Elastase release

dHL60 cells were incubated with the fluorescent substrate peptide MeO-Suc-AAPV-MCA (50 μM) in the presence of 5 μg/ml cytochalasin B followed by addition of 1 μM fMet-Leu-Phe. Elastase release was monitored fluorometrically in a Flexstation Plate Reader (Molecular Devices) at an excitation wavelength of 380 nm, and an emission wavelength of 460 nm.

2.7. Western blots

dHL60 cell lysates (1 × 10^6 cells/sample) were prepared by heating the cells at 95 °C for 5 min in 2X SDS-PAGE Sample Buffer. The samples were briefly sonicated (12 s) to reduce viscosity. The dHL60 cell lysates were run on a 4–16% gradient SDS-PAGE, transferred to PVDF membrane and blocked for 1 h at room temperature with Tris-buffered saline pH 7.5 containing 0.1% Tween 20 and 1% BSA/3% casein. To identify the different PKC isoforms, the membrane
was incubated with a panel of PKC antibodies, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG [5]. To identify phosphorylation of serine, membranes were blocked with Zymed Blocking Buffer, and then incubated with a phosphoserine antibody (Zymed), followed by incubation with peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive bands were visualized by Pierce SuperSignal ULTRA chemiluminescence substrate. The software SigmaPlot (Jandel/SPSS) was used for densitometric analysis and results expressed in arbitrary density units (ADU).

2.8. Immunoprecipitation of α-PKC and p47phox

dHL60 cells (50 × 10⁶ cells/ml) were stimulated with either buffer alone, fMet-Leu-Phe (1 μM) for 1 min, or PMA (1 μg/ml) for 5 min. The reaction was stopped by the addition of cold immunoprecipitation (IP) buffer. Immunoprecipitation (IP) buffer consisted of 10 mM HEPES (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1 mM Na orthovanadate, 2 mM AEBSF, 0.2% NP-40, 20 μl Sigma Antiprotease Cocktail, 10 μl/mg Sigma Antiphosphatase Cocktail, 2 μg/ml leupeptin and 5 mg/ml BSA. The samples were then vortexed for 20 min at 4 °C to solubilize the membrane fraction and the supernatant collected after microfuging for 5 min. A rabbit polyclonal antibody to p47phox or α-PKC cross-linked to Protein A agarose was added and incubated overnight at 4 °C with shaking. The reaction tubes were then microfuged for 30 s and the supernatants discarded. The agarose pellets were washed four times with IP buffer, and the sample eluted by incubation for 5 min at 95 °C in 2X SDS-PAGE sample buffer.

2.9. Preparation of particulate fractions from fMet-Leu-Phe- and PMA-stimulated dHL60 cells

dHL60 cells (25 × 10⁶ cells/ml) were incubated at 37 °C in the presence of buffer, 1 μM fMet-Leu-Phe (1 min) or 1 μg/ml PMA (5 min). The suspension was centrifuged for 10 min at 300 × g and the cell pellet resuspended in buffer containing 131 mM NaCl, 1 mM EGTA, 100 mM potassium phosphate Buffer, pH 7.0, 2 mM PMSE, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 20 μg/ml pepstatin (Buffer A) [32]. The cells were disrupted in a microprobe sonicator, centrifuged at low speed (500 × g × 5 min) and the supernatant layered over a 15% calcium insensitive isosbestic point for fura-2; uptake of Mn²⁺ through a Ca²⁺ channel quenches fura-2 fluorescence [42].

2.12. Statistical analysis

Results are expressed as mean±SEM (n). Data were analyzed by Student’s one-tailed t-test for paired samples.

3. Results

3.1. Decreased expression of α-PKC by siRNA and inhibition of 0₂⁻ generation

To determine a role for α-PKC in O₂⁻ generation, we used α-PKC siRNA (αAS) transfected with TransIT-TKO to selectively decrease α-PKC expression (see Experimental procedures). Treatment with αAS-siRNA resulted in a decrease in the level of α-PKC as compared to control αC-siRNA treated dHL60 cells (Fig. 1A). Reduced α-PKC was confirmed by Western blotting and densitometry. α-PKC was selectively decreased by treatment with αAS-siRNA to 52.2 ± 7.7% of control (n=9, p<0.002). In contrast, levels of βII-PKC, δ-PKC and ζ-PKC were not significantly affected.

O₂⁻ generation triggered by fMet-Leu-Phe in cells with decreased α-PKC expression was significantly decreased to 38.9±10.1% of control (n=7, p<0.002) (Fig. 1B). Generation of O₂⁻ triggered by PMA in cells with decreased α-PKC expression was reduced to 55.9±6.5% of control (n=7, p<0.001) (Fig. 1B). A similar inhibition in O₂⁻ generation was obtained using a second siRNA system using Stealth α-PKC siRNA delivered by electroporation (data not shown) (see Experimental procedures). Therefore α-PKC is essential for optimal O₂⁻ generation in response to both the ligand fMet-Leu-Phe and the PKC activator PMA, however the effect of decreased α-PKC was greater for O₂⁻ generation triggered by fMet-Leu-Phe as compared to PMA.

Our previous studies demonstrated that decreased β-PKC also significantly inhibited O₂⁻ generation triggered by either fMet-Leu-Phe or PMA in a manner similar to decreased α-PKC [5,9].

Reduction of β-PKC to 62.7±10.0% control (n=4), significantly inhibited O₂⁻ generation triggered by fMet-Leu-Phe to 41.7% of control, and by PMA to 48.7% of control [9]. However, selective reduction of δ-PKC by stealth siRNA to 50.4±6.2% of control (n=9, p<0.001) had no significant effect on O₂⁻ generation triggered by fMet-Leu-Phe or PMA. In cells treated with δ-PKC siRNA O₂⁻ generation was 10.7±1.9 nmol O₂⁻/10⁶ cells/10 min in cells treated with control siRNA, and 12.1±1.9 nmol O₂⁻/10⁶ cells/10 min in cells with decreased δ-PKC, i.e. 104.7±7.1% control (n=9, p=ns). Similarly in cells activated by PMA, O₂⁻ generation was 50.7±6.1 nmol O₂⁻/10⁶ cells/10 min in control cells and 46.5±7.3 nmol O₂⁻/10⁶ cells/10 min in cells with decreased δ-PKC expression, i.e. 104.1±4.4% of control (n=9, p=ns). Therefore, both α-PKC and β-PKC, but not δ-PKC, play essential roles in activation of O₂⁻ generation triggered by fMet-Leu-Phe or PMA in dHL60 cells in suspension.
In contrast to $O_2^-$ generation, which is triggered by both $f$Met-Leu-Phe and PMA, elastase release is triggered by $f$Met-Leu-Phe, but not by PMA. The release of elastase triggered by $f$Met-Leu-Phe was not affected by decreased $\alpha$-PKC. Elastase release was 105.5 ±5.0% of control levels in cells with decreased $\alpha$-PKC as compared to control cells (Fig 1C).

Therefore, $\alpha$-PKC plays a selective role in positive signaling for $f$Met-Leu-Phe induced $O_2^-$ generation but not the simultaneous release of elastase.

3.2. Translocation of $p47^{phox}$ and $\alpha$-PKC to the membrane in response to $f$Met-Leu-Phe and PMA

Translocation of $p47^{phox}$ to the membrane, and association of phosphorylated $p47^{phox}$ with cytochrome $b_558$, are essential steps in the assembly of an active NADPH oxidase complex. Our previous studies demonstrated that activation of dHL60 cells triggered translocation of $p47^{phox}$ and $\beta$II-PKC from the cytosol to the membrane fraction [5]. We now demonstrate that activation of dHL60 cells by $f$Met-Leu-Phe or PMA elicited translocation of $\alpha$-PKC as well as $p47^{phox}$ from the cytosol to the membrane fraction (Fig. 2). Activation by $f$Met-Leu-Phe or PMA triggered a significant reduction in the levels of $\alpha$-PKC and $p47^{phox}$ in the cytosolic fraction, and a simultaneous increase in the levels of $\alpha$-PKC and $p47^{phox}$ in the membrane fraction of dHL60 cells (Fig. 2). This finding is concordant with a role for $\alpha$-PKC as well as $\beta$-PKC in assembly of an active NADPH oxidase.

Fig. 2. Translocation of cytosolic $p47^{phox}$ and $\alpha$-PKC isotypes to cell membranes in resting, $f$-Met-Leu-Phe- and PMA-activated dHL60 cells. dHL60 cells were treated with buffer, 1 $\mu$M $f$Met-Leu-Phe (1 min) or 1 $\mu$g/ml PMA (5 min). Membrane (10 x 10^6 cell equivalents/lane) and cytosol (3 x 10^6 cell equivalents/lane) fractions were prepared, run on 4–12% gradient SDS-PAGE, blotted to PVDF membrane and probed with antibodies to $p47^{phox}$ and $\alpha$-PKC. (A) Western blot to $\alpha$-PKC, $\beta$II-PKC, $\delta$-PKC and $\zeta$-PKC in cells treated with $\alpha$-AS-siRNA and $\alpha$-C-siRNA. (B) $O_2^-$ generation was triggered by 1 $\mu$M $f$Met-Leu-Phe plus 5 $\mu$g/ml cytochalasin B, or 1 $\mu$g/ml PMA. $O_2^-$ generation was measured as the superoxide dismutase inhibitable reduction of cytochrome C (see Experimental procedures) in control siRNA ($\alpha$-C-siRNA) and antisense siRNA ($\alpha$-AS-siRNA) pretreated dHL60 cells. Data shown are expressed as nmol $O_2^-$/10^6 cells/10 min (n=7, **p<0.001 and *p<0.002 $\alpha$-AS-siRNA vs. $\alpha$-C-siRNA). (C) Elastase release triggered by 1 $\mu$M $f$Met-Leu-Phe plus 5 $\mu$g/ml cytochalasin B, was measured as cleavage of the fluorescent peptide MeO-Suc-AAPV-MCA. Data are shown as AFU/10^6 cells/10 min. (n=4, p=ns $\alpha$AS-siRNA vs. $\alpha$C-siRNA).
To determine if α-PKC associates with p47phox in activated dHL60 cells, p47phox was immunoprecipitated from cells treated with buffer, fMet-Leu-Phe or PMA. Activation of dHL60 cells by fMet-Leu-Phe and PMA triggered an increase in the level of α-PKC associated with immunoprecipitated p47phox (Fig. 3A). Conversely, when α-PKC was immunoprecipitated from cells treated with buffer, fMet-Leu-Phe or PMA, there was an increase in p47phox associated with immunoprecipitated α-PKC in cells activated by fMet-Leu-Phe or PMA (Fig. 3B). Therefore, fMet-Leu-Phe and PMA triggered association of p47phox with α-PKC.

3.3. Decreased α-PKC expression reduced ligand-initiated phosphorylation of p47phox

fMet-Leu-Phe and PMA trigger phosphorylation of p47phox on multiple serines, an essential step for assembly of the NADPH oxidase. To determine whether α-PKC was essential for phosphorylation of p47phox in response to activation by fMet-Leu-Phe and PMA, dHL60 cells were treated with αAS-siRNA to decrease α-PKC expression. Control cells and cells with decreased α-PKC expression contained equivalent amounts of p47phox immunoreactivity (Fig. 4A and B). However, serine phosphorylation of the p47phox band was significantly reduced in the cells with decreased αPKC expression, as compared to the control cells as shown by Western Blotting and densitometry (Fig. 4A and B). Thus, serine phosphorylation of p47phox in response to fMet-Leu-Phe and PMA was significantly reduced in the cells with diminished α-PKC, concordant with a role for α-PKC in phosphorylation of p47phox in ligand-initiated signaling and in the assembly of an active NADPH oxidase.

3.4. Decreased α-PKC inhibited fMet-Leu-Phe-triggered mobilization of Ca^{2+}

A key difference in signaling between fMet-Leu-Phe and PMA is the ability of fMet-Leu-Phe, but not PMA, to elicit Ca^{2+} mobilization. In order to test a role for α-PKC in phosphorylation of p47phox in ligand-initiated signaling and in the assembly of an active NADPH oxidase.
cytosolic Ca\(^{2+}\) which peaked at a level of 308.0±29.7 nM in control cells (Fig. 5A). However, a decrease in \(\alpha\)-PKC inhibited the fMet-Leu-Phe-triggered increase in cytosolic Ca\(^{2+}\). In cells with decreased \(\alpha\)-PKC, the peak cytosolic Ca\(^{2+}\) level triggered by 1 \(\mu\)M fMet-Leu-Phe decreased to 242.9±24.9 nM (n=7, p<0.002) (Fig. 5A).

In the presence of 1.25 mM EGTA, where cytosolic Ca\(^{2+}\) changes are derived only from intracellular stores, resting cytosolic Ca\(^{2+}\) was not significantly different in control cells as compared to cells having decreased \(\alpha\)-PKC (Fig. 5B). When the cells were activated by addition of 1 \(\mu\)M fMet-Leu-Phe (Fig. 5B), cytosolic Ca\(^{2+}\) rose to peak values of 109.7±12.4 (n=7) nM in control cells, and to a level of 104.4±5.7 (n=7) nM in \(\alpha\)AS siRNA pretreated cells respectively, a decrease that was not statistically significant. Thus, no alteration in ligand-initiated mobilization of intracellular Ca\(^{2+}\) stores was observed following a reduction in \(\alpha\)-PKC. The decrease in peak cytosolic Ca\(^{2+}\) observed in the presence of extracellular Ca\(^{2+}\) in cells with decreased \(\alpha\)-PKC, reflects a decrease in Ca\(^{2+}\) uptake from extracellular sources. These findings suggest a role for \(\alpha\)-PKC in positive signaling for fMet-Leu-Phe induced Ca\(^{2+}\) uptake.

### 3.5. Store operated calcium channel (SOCC) opening is regulated by \(\alpha\)-PKC

Ca\(^{2+}\) uptake pathways were assessed as fMet-Leu-Phe-induced uptake of Mn\(^{2+}\) into Fura-2 loaded cells, and quenching of Fura-2 fluorescence [42,43]. Addition of 1 \(\mu\)M fMet-Leu-Phe triggered a prompt increase in Mn\(^{2+}\) influx, and thus a decrease in fluorescence of Fura-2, which continued over the 7 min monitored after addition of the stimulus (Fig. 6A). fMet-Leu-Phe triggered a decrease in fluorescence of 11066±399 AFU/min (n=4) in control siRNA pretreated cells, while in cells with decreased \(\alpha\)-PKC (Stealth \(\alpha\)AS siRNA treated cells) the
decrease in fluorescence was significantly decreased to 6091 ± 954 AFU/min (n=4, p<0.05) (Fig. 6A). Therefore, fMet-Leu-Phe-induced Ca2+ uptake was inhibited in dHL60 cells with decreased α-PKC, further implicating α-PKC in positive regulation of ligand-induced Ca2+ uptake.

The SERCA inhibitor thapsigargin, inhibits the Ca2+-ATPase responsible for pumping Ca2+ into the endoplasmic reticulum, and induces Ca2+ uptake through SOCC. Thapsigargin triggered Ca2+ uptake in dHL60 cells, concordant with activation of SOCC [21]. The role of α-PKC in regulation of thapsigargin-induced Ca2+ uptake was investigated in dHL60 cells pretreated with αAS siRNA or control siRNA (Fig. 6B). Thapsigargin (100 nM) triggered a loss of fluorescence of 10397± 444 AFU/min in control siRNA-treated cells, while in cells with decreased α-PKC expression, fluorescence quenching was significantly decreased to 4568±345 AFU/min (44.4±4.0% control, n=4, p<0.002) (Fig. 6B). Therefore, α-PKC is a positive regulator of ligand-initiated Ca2+ uptake via a thapsigargin sensitive channel, concordant with a role for α-PKC in positive regulation of Ca2+ uptake via SOCC.

3.6. Correlation of Ca2+ uptake via SOCC with ligand-initiated activation of O2 generation

In order to further probe a role for α-PKC in positive regulation of Ca2+ uptake via SOCC and therefore in the positive regulation of O2 generation by α-PKC, we used MRS1845, a selective inhibitor of Ca2+ uptake through SOCC [44]. MRS1845 inhibited Ca2+ uptake triggered by both fMet-Leu-Phe and thapsigargin (Fig. 7A); 10 μM MRS1845 on O2 generation triggered both fMet-Leu-Phe and Thapsigargin by 53.1% and 62.8% respectively. MRS1845 (10 μM) also inhibited O2 generation induced by fMet-Leu-Phe to 51.4±12.7 (n=5, p<0.02) % of control (Fig. 7B) concordant with a role for Ca2+ uptake via the SOCC in activation of O2 generation. In contrast, elastase release triggered by fMet-Leu-Phe was 105.5±5.0 (n=4) % control in the presence of 10 μM MRS1845, a non-significant difference. Therefore, the ability of diminished α-PKC to inhibit Ca2+ uptake via SOCC may contribute to the observed decrease in O2 generation triggered by fMet-Leu-Phe in α-PKC deficient dHL60 cells.

4. Discussion

A selective role for α-PKC in ligand-initiated responses in neutrophilic dHL60 cells was probed using an antisense approach. A decrease in α-PKC inhibited O2 generation triggered by fMet-Leu-Phe and PMA. In contrast, elastase release triggered by fMet-Leu-Phe, was not affected by a decrease in α-PKC. Therefore α-PKC plays a selective role in positive signaling for O2 generation. These findings are in agreement with other workers who determined that α-PKC is required for an optimal respiratory burst measured as O2 or H2O2 generation triggered by opsonized zymosan in monocytes and RAW264.7 cells [35,36].

We and other workers previously demonstrated that β-PKC is required for O2 generation; a role for β-PKC in O2 generation was demonstrated in dHL60 cells by an antisense strategy, and in neutrophils derived from a β-PKC knockout mouse [5,9,34]. Moreover βII-PKC, but not βI-PKC, was required for O2 generation triggered by fMet-Leu-Phe and PMA in dHL60 cells [9]. In addition, a role for ζ-PKC in activation of O2 generation triggered by fMet-Leu-Phe, but not by PMA, has been demonstrated [45]. Therefore the ability of a decrease in α-PKC expression to inhibit O2 generation triggered by fMet-Leu-Phe and PMA in dHL60 cells was not unique to α-PKC. In contrast, δ-PKC was not required for O2 generation in dHL60 cells in suspension, as compared to findings of other workers where δ-PKC was required for O2 generation in monocytes or in adherent transgenic COS-phox cells [4,37]. In addition, β-PKC was not required by monocytes for optimal O2 generation in response to zymosan [4]. Usage of discrete PKC isotypes is context sensitive and depends on cell type, the ligand and the specific response [6,7]. These differences may reflect...
involvement of different PKC isotypes for activation of the NADPH oxidase in monocytes as compared to dHL60 cells and neutrophils, or may reflect different requirements for a phagocytic stimulus as compared to a chemotactic peptide such as fMet-Leu-Phe or the PKC activator PMA.

Roles for both α-PKC and β-PKC in O2− generation by dHL60 cells may reflect unique or redundant roles for these isotypes in positive signaling for assembly of the NADPH oxidase in dHL60 cells. Identifying key substrate(s) is important in defining a role for α-PKC in positive signal transduction for O2− generation. The mechanism of assembly and activation of the NADPH oxidase for O2− generation, and targets for PKC-based phosphorylation have been defined. Phosphorylation of a cytosolic component of the NADPH oxidase, p47phox, and translocation and binding of p47phox to cytochrome b558 is an essential step in ligand-initiated activation of the NADPH oxidase. Previous studies demonstrated translocation of β-PKC from cytosol to the membrane or cytoskeleton in fMet-Leu-Phe and PMA-activated neutrophils and dHL60 cells [5,27,46]. The present study demonstrates that α-PKC as well as p47phox is also translocated from the cytosol to the membrane in dHL60 cells activated by fMet-Leu-Phe and by PMA. Co-immuno-precipitation of α-PKC with p47phox, or p47phox with α-PKC, demonstrated that fMet-Leu-Phe and PMA triggered a more specific association of α-PKC with p47phox. Such translocation of PKC would allow α-PKC or β-PKC to access the activating lipid cofactors PS and DG which are located in the membrane, as well as membrane-associated substrates.

Phosphorylation is required for a conformational change in p47phox which releases binding of p47phox to itself and to p40phox [31], and allows translocation and binding of p47phox to membrane-associated cytochrome b558 [1,47]. The importance of a role for phosphorylation of p47phox by PKC for assembly of an active NADPH oxidase was demonstrated in a neutrophil cell free system [33]. PKC is promiscuous in vitro, and multiple PKC isotypes phosphorylate p47phox in vitro indicating that in vivo studies are essential to define roles for discrete PKC isotypes [33,48]. In the present study, phosphorylation of p47phox activated by fMet-Leu-Phe or PMA was inhibited in dHL60 cells having diminished α-PKC, concordant with a role for α-PKC in phosphorylation of p47phox and activation of the NADPH oxidase in intact cells. In contrast, decreased α-PKC in monocytes had no effect on phosphorylation of p47phox triggered by opsonized zymosan possibly reflecting cell type or ligand specificity [4]. Previous work demonstrated that depleted levels of β-PKC in dHL60 cells also inhibited phosphorylation of p47phox and activation of the NADPH oxidase. Therefore in dHL60 cells, both α-PKC and β-PKC are active in phosphorylation of p47phox [5]. Work by other investigators has shown that several serines in the C-terminus of p47phox, are consensus sequences for phosphorylation by PKC and are phosphorylated during activation of the NADPH oxidase [33,49]. Separate roles for α-PKC and β-PKC might occur by a direct phosphorylation of different serine residues on p47phox, perhaps phosphorylated sequentially by these PKC isotypes [50]. Alternatively, α-PKC or β-PKC might act to trigger a signaling pathway (s) upstream from p47phox, possibly involving different kinases such as Akt, and the MAP kinases ERK and P38 [5,33,51].

Decreased α-PKC was a more potent inhibitor of O2− generation in cells activated by fMet-Leu-Phe than in cells activated by PMA. fMet-Leu-Phe, but not PMA, triggers Ca2+-mobilization which is essential for optimal O2− generation. Thus a role(s) for α-PKC in regulation of Ca2+-mobilization in response to fMet-Leu-Phe was examined. An alteration in intracellular Ca2+-homeostasis has profound effects on many cell functions including O2− generation and elastase release. In this study we demonstrated a role for α-PKC in positive regulation of fMet-Leu-Phe-initiated uptake of extracellular Ca2+, but not of intracellular Ca2+ release. A decrease in α-PKC inhibited the fMet-Leu-Phe-induced uptake of Mn2+, which acts as a surrogate for Ca2+ during opening of the ligand-operated Ca2+ channels in HL60 cells [42], and of store operated Ca2+ channels in numerous cell types [14, 52]. Two distinct channels for Ca2+ uptake are potential targets for PKC. fMet-Leu-Phe triggers Ca2+ uptake via a non-selective cation channel(s) which is activated by elevation of cytosolic Ca2+ [22]. In addition, Ca2+ mobilizing ligands such as fMet-Leu-Phe activate SOCC, initiated by IP3-triggered emptying of ER Ca2+ stores. The SERCA inhibitor thapsigargin can bypass ligand-initiated depletion of Ca2+ stores and directly activate the store operated Ca2+ entry pathway [21]. Ca2+ uptake through SOCC activated by thapsigargin, and by fMet-Leu-Phe, was inhibited by decreased α-PKC. Therefore fMet-Leu-Phe-initiated opening of the SOCC is positively regulated by α-PKC. We previously demonstrated that reduction of β-PKC by an antisense approach, enhanced Ca2+ uptake via a non-selective cation channel, but not mobilization of intracellular Ca2+ [24]. Therefore α-PKC and β-PKC play distinct roles in ligand initiated Ca2+ mobilization and in O2− generation. β-PKC is a negative regulator of a non-selective cation channel, while the current study demonstrated that α-PKC acts as a positive regulator of Ca2+ uptake via SOCC.

Mobilization of Ca2+ is a key element in signaling for O2− generation and degranulation triggered by fMet-Leu-Phe. Chelation of intracellular and extracellular Ca2+, inhibits both O2− generation and degranulation [16,17]. Nevertheless, O2− generation and degranulation have different requirements for calcium signaling. We previously demonstrated that prolonged fMet-Leu-Phe receptor occupancy is required to generate optimal Ca2+ uptake as well as O2− generation, but not degranulation [52]. The present study demonstrated that α-PKC is a positive regulator of Ca2+ uptake through SOCC, an event that would serve to prolong cell responses, in particular O2− generation. Indeed, an inhibitor of SOCC, MRS1845, inhibited Ca2+ uptake and O2− generation triggered by fMet-Leu-Phe in dHL60 cells, in agreement with findings by other workers in neutrophils [53]. In contrast, degranulation, measured as elastase release, was not affected by inhibition of Ca2+ uptake via SOCC. Thus a role for α-PKC in positive regulation of O2− generation may be mediated by Ca2+ uptake via SOCC, but does not preclude an action of α-PKC on...
other signaling pathways as demonstrated in PMA activated cells where Ca\(^{2+}\) mobilization is not triggered.

In summary, using siRNA to decrease \(\alpha\)-PKC we have demonstrated a selective role for \(\alpha\)-PKC in signaling for fMet-Leu-Phe, and PMA-induced O\(_2\)\(^{-}\) generation but not fMet-Leu-Phe induced degranulation. Reduction of \(\alpha\)-PKC inhibited ligand-induced phosphorylation of p47\(^{phox}\), and inhibited O\(_2\)\(^{-}\) generation, as shown previously for decreased \(\beta\)-PKC. However, \(\alpha\)-PKC, unlike \(\beta\)-PKC, was also involved in positive signaling for ligand initiated Ca\(^{2+}\) uptake via SOCC, an event that can enhance O\(_2\)\(^{-}\) generation, suggesting that \(\alpha\)-PKC and \(\beta\)-PKC have different roles in signaling for phagocyte responses such as O\(_2\)\(^{-}\) generation.

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