Role of diradylglycerol formation in H_2O_2 and lactoferrin release in adherent human polymorphonuclear leukocytes

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Abstract: Polymorphonuclear leukocytes (PMNs) adherent to fibrinogen exhibit a delay in the release of H_2O_2 in response to fMLP. Previously, we demonstrated that H_2O_2 release in adherent PMNs coincides with the exocytosis of lactoferrin-containing specific granules and activation of phospholipase D (PLD). We also found that chelation of intracellular calcium blocked both lactoferrin and H_2O_2 release in stimulated PMNs in spite of the fact that adhesion and spreading remained normal. Since diradylglycerol (DRG) formation has been implicated in PMN secretion and oxidant release, we determined the effect of intracellular calcium chelation on PLD activation and DRG formation to ascertain whether DRG formation was coupled to lactoferrin and H_2O_2 release. We observed that chelation of intracellular calcium with bis-(O-aminophenoxy)-ethanol-N,N,N'- tetraacetic acid (BAPTA) prevented PLD activation as monitored by inhibition of phosphatidylethanol formation. Formation of DRG derived from phosphatidic acid (PA) was also inhibited in the presence of BAPTA. Following the addition of the calcium ionophore ionomycin to the BAPTA-treated PMNs, lactoferrin and H_2O_2 release was coincident with the onset of DRG formation. Also the addition of sn-1,2-didecanoylglycerol to the BAPTA-treated PMNs stimulated them to release H_2O_2. Our studies support the hypothesis that DRG derived from PLD activation is required for degranulation of specific granules and associated H_2O_2 release from adherent PMNs. J. Leukoc. Biol. 56: 105–109; 1994.

Key Words: calcium · phospholipids · neutrophil · activation

INTRODUCTION

Previously, we correlated the exocytosis of lactoferrin-containing specific granules and H_2O_2 release in polymorphonuclear leukocytes (PMNs) by taking advantage of the long lag period and protracted kinetics of oxidant generation in adherent PMNs [1]. The correlation observed between the release of LF-containing specific granules and H_2O_2 in adherent PMNs is consistent with the concept that translocation of an active NADPH oxidase from the specific granule membranes to the plasma membranes results in the delivery of H_2O_2 to the external milieu. Ultrastructural and subcellular fractionation studies have shown that cytochrome b_{558} plays a critical role in the assembly of the cytosolic components p47[phox] and p67[phox], which leads to the generation of O_2^-/H_2O_2 by PMNs in suspension [2, 3]. About 85% of the total cytochrome b_{558} is associated with LF-containing specific granule fractions in unactivated PMNs and the remainder with the plasma membrane [3]. Therefore, it is not surprising that in employing a cell-free system, an active

NADPH oxidase can be assembled on both plasma membranes and specific granules, both of which contain cytochrome b_{558} [4, 5]. Likewise, in adherent PMNs the NADPH oxidase can be assembled on specific granules and this assembled oxidase can be linked to the release of O_2^- into phagosomes [6].

We previously observed that diradylglycerol (DRG) increased following phospholipase D activation and was correlated with the exocytosis of LF-bearing specific granules and H_2O_2 release [7]. We also found that chelation of intracellular calcium blocked both LF and H_2O_2 release in stimulated PMNs, in spite of the fact that adhesion and spreading remained normal [1]. Since DRG has been implicated in PMN secretion and oxidant release [8], we determined the effect of intracellular calcium chelation on PLD activation and DRG formation in order to ascertain whether DRG formation remained coupled to LF and H_2O_2 release in activated, adherent PMNs.

MATERIALS AND METHODS

Reagents

N-Formylmethionyl-lencyl-phenylalanine (fMLP), imidazole, horseradish peroxidase, H_2O_2, phospholipase D (PLD) cabbage (type I), O-phenylenediamine hydrochloride, cardiolipin from bovine heart, β-octylglycoside, scopoletin, sodium azide, dioleoylphosphatidylcholine, and ionomycin were purchased from Sigma (St. Louis, MO). Dithiothreitol, human LF, and sn-1,2-diacylglycerol kinase from E. coli were purchased from Calbiochem Corp (San Diego, CA). Silica gel 60 thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). [32P]Adenosine-5'-triphosphate (25 Ci/mmol) was obtained from ICN Pharmaceuticals (Irvine, CA), and 1-O-[3H]octadecyl-sn-glycero-3-phosphocholine (148 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Fibrinogen was purchased from Kabi Diagnostics (Franklin OH), goat anti-human LF antibody from Nordic Immunological products (El Toro, CA), and rabbit anti-human LF and peroxidase-

conjugated goat anti-rabbit antibodies from Organon-Teknika Corp. (Malverne, PA). sn-1,2-Didecanoylglycerol was obtained from Avanti Polar-Lipids (Alabaster, AL).

Neutrophil preparation and functional assays

Human PMNs were isolated from human peripheral blood as previously described [9] using dextran sedimentation followed by hypotonic lysis to remove the majority of erythrocytes and centrifugation through Ficoll-Paque. Assays were conducted in 24-well, flat-bottom, polystyrene Falcon Primaria tissue culture plates coated with fibrinogen (50 μg/ml) as previously outlined [10]. To measure H_2O_2 release, PMNs (1×10^5/well) suspended in KRBG were added to fibrinogen-coated wells containing Krebs-Ringer phosphate buffer with glucose (KRBG), 24 μM scopoletin, 5 μg of horseradish peroxidase, 1 mM sodium azide, and the indicated agonists and/or inhibitors in a final volume of 1 ml [1]. Samples were also evaluated for LF content [1].

DRG formation in PMNs plated onto fibrinogen

PMNs were plated onto fibrinogen-coated 24-well plates under the same experimental conditions as those outlined for H_2O_2 release [7]. At the indicated time points, PMNs from eight wells were extracted according to the method of Shayman et al. [11] as combined as recently described [7]. DRG was quantified by enzymatic conversion of DRG to $[^{32}P]PA$ using E. coli diacylglycerol kinase [12, 7]. The protein concentration of each sample was determined using the Pierce BCA protein assay reagent (Pierce, Rockford, IL).

BAPTA loading of PMNs

PMNs (1.3×10^6/ml) were incubated with the intracellular Ca^{2+} chelator BAPTA at 10 μM in Ca^{2+}-free phosphate-buffered saline (PBS) for 30 min at 37°C. At the end of this incubation, 1×10^5 cells were added directly to fibrinogen-coated wells containing prewarmed KRBG with 100 nM fMLP and Ca^{2+} and assayed for H_2O_2, LF release, and DRG formation at various times [1, 7].

Labeling of PMNs with [³H]lysoPAF

For assessment of phosphatidylethanol (PEt) formation, PMNs were prelabeled with lyso-PAF. PMNs (1×10^7/ml) were incubated in [³H]lysoPAF (10^{-8} M) for 30 min at 37°C as previously described [14]. After labeling, PMNs were pelleted at 300g for 5 min, washed twice with PBS, and resuspended in PBS containing 1 mM Ca^{2+} and Mg^{2+}. PMNs were then preincubated in ethanol (200 mM) or buffer for 5 min at 37°C before plating onto fibrinogen-coated plates, with ethanol being present throughout the time course. Lipids were obtained and analyzed as previously described [7].

Statistical analysis

Data were analyzed by Student's paired t-tests.

RESULTS

Effect of BAPTA on H_2O_2 and lactoferrin release and DRG formation in PMNs plated onto fibrinogen

Previously, we found a correlation between LF exocytosis and H_1O_2 release in PMNs plated onto fibrinogen-coated plastic and activated with fMLP [1]. As noted in **Figure 1A,** the time course of LF release paralleled that of H_2O_2 release.
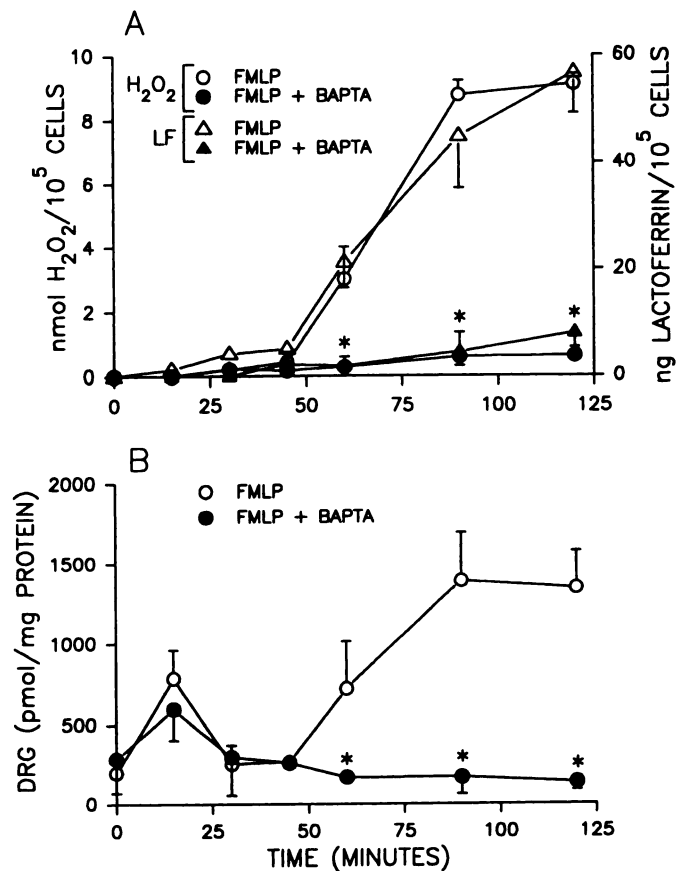

Fig. 1. Effect of BAPTA on the time course of H_2O_2 release, LF release, and DRG formation from PMNs plated onto fibrinogen and activated with fMLP. (A) PMNs (1×10^5/ml) were added to fibrinogen-coated 24-well plates containing 100 nM fMLP in the presence (●, ▲) and absence (○, △) of 10 μM BAPTA. The plates were then incubated at 37°C and samples withdrawn at varying times and monitored for LF and H_2O_2 release. Values represent the mean ± SD of three separate experiments. (B) In parallel wells, DRG formation was determined over the same time interval for fMLP-stimulated PMNs in the presence (●) or absence (○) of 10 μM BAPTA. Values represent the mean ± SD of three separate experiments. An asterisk (*) indicates values that are significantly different ($P < .01$) from controls at time 0.

Although there was a correlation between LF and H_2O_2 release in adherent PMNs, it was not clear whether the exocytosis of LF-containing granules was required for H_2O_2 generation. Therefore, we employed the intracellular calcium chelator BAPTA to block the exocytosis of LF-containing specific granules and determined the effect of this treatment on H_2O_2 release. BAPTA-treatment of the PMNs significantly inhibited ($P < .01$) fMLP-mediated LF and H_2O_2 release in adherent PMNs (Fig. 1A). When PMNs were plated onto fibrinogen and stimulated with fMLP under the conditions that promote LF and H_2O_2 release, we observed two peaks of DRG formation as noted previously [7]. The initial increase in DRG was similar in magnitude to the DRG levels reported for PMNs activated with fMLP in suspension. The second phase of DRG generation occurs through a PLD-mediated pathway and corresponds to degranulation and oxidant release in PMNs adherent to fibrinogen [7]. As shown in Figure 1B, BAPTA treatment blocked the second the phase of DRG generation without affecting the first phase.

Reversal of BAPTA/AM treatment on adherent PMN function

Following the addition of ionomycin to indo-1/AM-labeled PMNs in the presence or absence of BAPTA/AM, intracellular Ca^{2+} can be restored to very high levels in both resting and agonist-stimulated PMNs [6]. We therefore assessed the effect of ionomycin on adherent PMNs. Addition of 100 nM ionomycin to the BAPTA-treated adherent PMNs at 90 min resulted in significant release of both H_2O_2 and LF within 15 min (**Fig. 2A**). The generation of DRG following addition of ionomycin at 90 min coincided with the onset of H_2O_2 and LF release, suggesting an association between these events (Fig. 2B). These studies indicate that PLD-mediated DRG formation is a calcium-dependent process and closely associated with degranulation and oxidant release in adherent PMN.

Effect of BAPTA/AM on PLD activity in adherent PMNs plated onto fibrinogen

To confirm that the increase in DRG occurring coincidentally with LF and H_2O_2 was generated through the action of a calcium-dependent PLD, we assessed the effect of ethanol
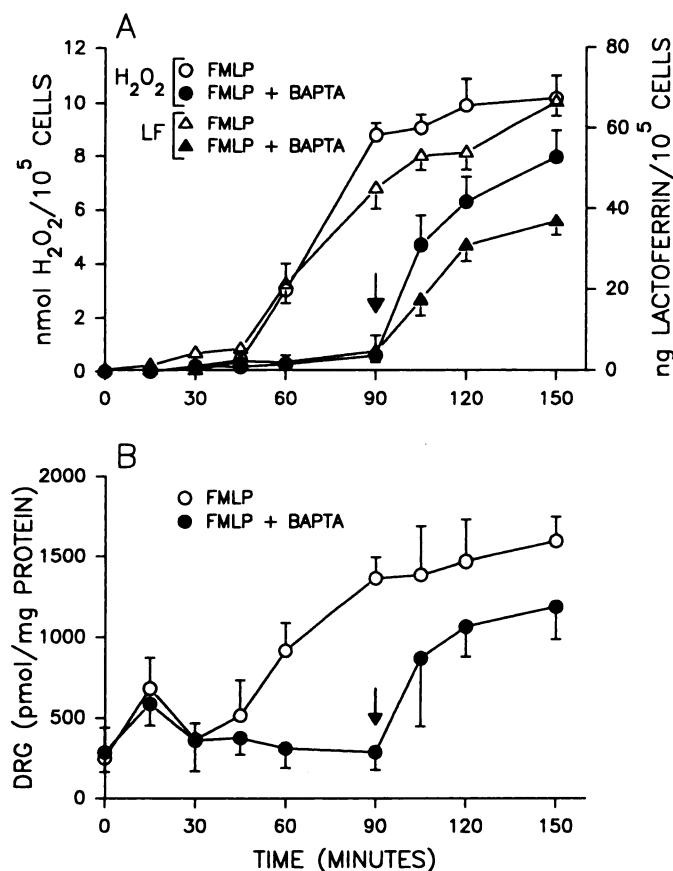
Fig. 3. Effect of BAPTA on the formation of PEt in PMNs plated onto fibrinogen. PMNs were labeled with [³H]lysoPAF. Labeled cells (1 × 10⁵/ml) were preincubated with ethanol (200 mM) or buffer for 5 min at 37°C and then plated onto fibrinogen-coated 24-well plates containing 100 nM fMLP, in the presence or absence of BAPTA and ethanol. Samples were withdrawn at the indicated times and assayed for PEt formation. Values represent the mean ± SD of three separate experiments.

Fig. 2. Effect of ionomycin on reversing BAPTA inhibition of H_2O_2 and LF release and DRG formation. (A) LF release and H_2O_2 release in PMNs in the presence and absence of 10 μM BAPTA. PMNs (1 × 10⁵/ml) were added to fibrinogen-coated 24-well plates containing either 100 nM fMLP (O, △) or fMLP and 10 μM BAPTA (●, ▲) incubated at 37°C until 90 min. At 90 min (indicated by arrow) 100 nM ionomycin was added to the BAPTA-treated adherent PMNs. Throughout the study samples were withdrawn at varying times and monitored for LF and H_2O_2 release. (B) In parallel wells, DRG formation in both fMLP-stimulated (O) and fMLP-stimulated and BAPTA-treated (●) PMNs was measured over the indicated times. At 90 min, as indicated by the arrow, 100 nM ionomycin was added to the BAPTA-treated PMNs. Values represent the mean ± SD of three separate experiments.
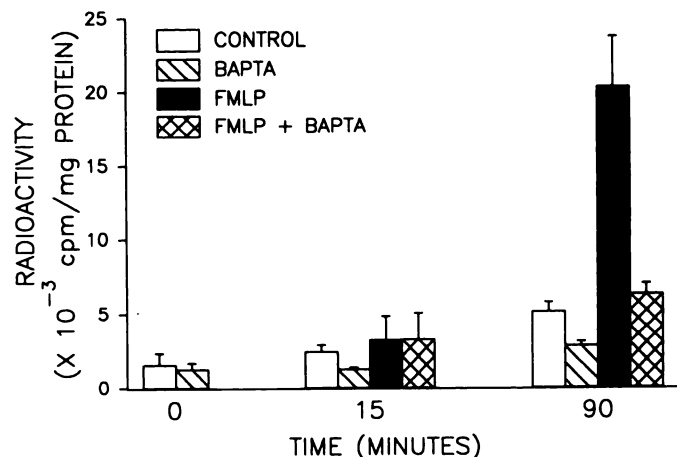
on PMN functional responses. In the presence of ethanol, PLD activation results in a transphosphatidylation reaction that generates phosphatidylethanol rather than PA. Previously, we found that fMLP-stimulated adherent PMNs failed to release significant amounts of H_2O_2 or LF, or generate the second wave of DRG, in the presence of ethanol [7]. These previous results suggested that DRG generated through the action of PLD is critical for degranulation and oxidant release in fMLP-stimulated adherent PMNs. In the current studies, we labeled cells with [³H]lysoPAF and measured PEt formation at 15 and 90 min in control and BAPTA-treated PMNs after addition to fibrinogen-coated plates. We then determined whether intracellular calcium chelation with BAPTA would prevent PLD activation. In the presence of ethanol and absence of fMLP stimulation, there was a modest increase in PEt formation at 90 min (**Fig. 3**). fMLP stimulation of PMNs in the presence of ethanol did not affect PEt formation at 15 min but resulted in about a 13-fold increase in PEt by 90 min, corresponding to the second wave of DRG formation. To determine whether the action of PLD was Ca^{2+} dependent, PMNs were incubated with 10 μM BAPTA/AM. As noted in Figure 3, BAPTA-treated PMNs were impaired in their ability to generate PEt at 90 min following activation with fMLP. These results indicate that BAPTA blocks DRG formation by chelating intracellular Ca^{2+}, thereby preventing the PLD activity in the adherent PMNs.

Effect of DiC10 on BAPTA/AM-treated PMNs

Because BAPTA treatment blocked the formation of DRG generated through the action of a calcium-dependent PLD, we assessed the effect of exogenously added DiC10 on restoring oxidant generation in the adherent PMNs (**Fig. 4**). We observed that 50 μM DiC10 by itself stimulated the cells to release H_2O_2 upon addition of DiC10 90 min after BAPTA treatment.

DISCUSSION

In the present study, we have focused on the effect of dampening changes in $[Ca^{2+}]_i$ on the activation of PLD by fMLP
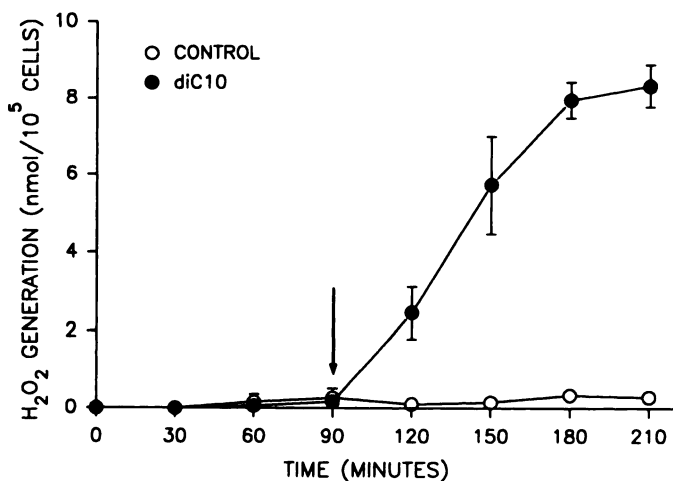
Fig. 4. Effect of DiC10 on BAPTA-loaded PMNs. PMNs (1 × 10⁵/ml) were added to fibrinogen-coated 24-well plates containing 10 μM BAPTA in the presence of 50 μM DiC10 (●), which was added at 90 min as indicated by the arrow, or the absence of DiC10 (○). The plates were incubated at 37°C, and samples were withdrawn at varying times and monitored for H_2O_2 release. Values represent the mean ± SD of three separate experiments.

in adherent human PMNs. Others have studied in more detail the effect of changes in $[Ca^{2+}]_i$ on PLD activation in both intact and electropermeabilized PMNs in suspension [13]. There appears to be an absolute requirement for a rise in $[Ca^{2+}]_i$ to induce PLD activation in intact or electropermeabilized suspended PMNs. However, in suspended, fMLP-activated PMNs, PLD activation does not appear to be required for induction of the respiratory burst [13]. In contrast, we found that chelation of intracellular calcium prevented PLD activation and the coincident release of LF-containing specific granules and H_2O_2 from adherent PMNs.

Mammalian PLD is membrane bound and uses phosphatidylcholine as its primary substrate, although under certain conditions, phosphatidylethanolamine and phosphatidylinositol may be degraded [14]. PLD acting on phosphatidylcholine exhibits two distinct enzymatic activities. In addition to the hydrolytic activity that generates phosphatidic acid, PLD catalyzes a transphosphatidylation reaction in the presence of primary alcohols in which the phosphatidyl moiety of phosphatidylcholine is transferred to a primary alcohol to produce phosphatidylalcohol [15]. In this report, we observed that chelation of intracellular calcium prevented PLD activation as monitored by inhibition of PEt formation. It is not surprising then that generation of DRG, derived from PA by activation of phosphatidic phosphohydrolase, was also prevented in the presence of BAPTA. In contrast, the initial phase of formation of DRG, likely derived from PLC activation, was unaffected by intracellular calcium chelation.

Following the addition of the calcium ionophore ionomycin to the BAPTA-treated cells, LF and H_2O_2 release resumed coincident with the onset of DRG formation. Studies using cell-permeant short-chain DRG indicate that these phospholipid products may be involved in degranulation, superoxide generation, and protein kinase C activation by PMNs in suspension [8]. Although the same studies indicated that DiC10 will activate lysosomal release, DiC10 failed to activate the respiratory burst and was a poor competitor for [³H]PDBU binding to intact PMNs compared with other short-chain DRG. These studies indicate that DiC10 stimulated PMNs in suspension by means other than protein kinase C activation [8]. In our study, however, DiC10 proved

to be a potent agonist for adherent PMNs by stimulating these cells to release H_2O_2. Our studies continue to support the notion that DRG, derived from PLD activation, is required for degranulation of specific granules and the associated release of H_2O_2 from adherent PMNs. In summary, unlike PMNs in suspension, in which fMLP will preferentially activate PLC to generate a respiratory burst, activated adherent PMNs release H_2O_2 via a PLD-mediated pathway [14, 16].

ACKNOWLEDGMENTS

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