Interleukin-1-induced Ether-linked Diglycerides Inhibit Calcium-insensitive Protein Kinase C Isotypes

IMPLICATIONS FOR GROWTH SENESCENCE*

(Received for publication, April 11, 1997)

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It is hypothesized that inflammatory cytokines and vasoactive peptides stimulate distinct species of diglycerides that differentially regulate protein kinase C isotypes. In published data, we demonstrated that interleukin-1, in contrast to endothelin, selectively generates ether-linked diglyceride species (alkyl, acyl- and alkenyl, acylglycerols) in rat mesangial cells, a smooth muscle-like pericyte in the glomerulus. We now demonstrate both in intact cell and in cell-free preparations that these interleukin-1 receptor-generated ether-linked diglycerides inhibit immunoprecipitated protein kinase C ε and δ but not ζ activity. Neither interleukin-1 nor endothelin affect de novo protein expression of these protein kinase C isotypes. As down-regulation of calcium-insensitive protein kinase C isotypes has been linked to antimitogenic activity, we investigated growth arrest as a functional correlate for IL-1-generated ether-linked diglycerides. Cell-permeable ether-linked diglycerides mimic the effects of interleukin-1 to induce a growth-arrested state in both G-protein-linked receptor- and tyrosine kinase receptor-stimulated mesangial cells. This signaling mechanism implicates cytokine receptor-induced ether-linked diglycerides as second messengers that inhibit the bioactivity of calcium-insensitive protein kinase C isotypes resulting in growth arrest.

Interleukin-1 (IL-1)1-induced activation of rat glomerular mesangial cells (MC) culminates in an inflammatory phenotype often observed in vivo in models of glomerulosclerosis. Our laboratory has been investigating the early, lipid-mediated signal transduction pathways for inflammatory cytokines in MC with particular emphasis on the distinct molecular species of diglycerides (DG) generated and their regulation of protein kinase C (PKC) activity. We have demonstrated previously that the inflammatory cytokine interleukin-1α and the vasoconstrictor peptide, endothelin-1 (ET-1) generate distinct species of DG from different membrane-associated phospholipids in MC (1–5). IL-1 receptor activation selectively generates ether-linked species of DG, namely alkyl, acyl- and alkenyl, acylglycerols, whereas ET-1 receptor activation produces predominantly ester-linked diacylglycerols (1). Our laboratory has also demonstrated previously that these IL-1 generated ether-linked DG, in contrast to the PKC-activating diacylglycerols, inhibit total PKC activity as well as inhibit diacylglycerol-stimulated PKC α activation (1). In fact, ether-linked DG may competitively inhibit PKC activation induced by diacylglycerol species (4). Additional studies support this signaling mechanism, as ether-linked DG fail to activate total PKC activity in vitro or only activate PKC in the presence of pharmacological concentrations of calcium (5–8).

In light of these findings, our interest has now turned to regulation by ether-linked DG of specific PKC isotypes. The PKC family now includes 12 distinct isotypes subdivided into three categories (9). The calcium-sensitive conventional PKCs consist of alpha (α), beta (β1 and β2), and gamma (γ) isotypes, while the calcium-insensitive novel isotypes include delta (δ), epsilon (ε), nu (ν), mu (μ), and theta (θ). PKCs zeta (ζ), iota (ι), and lambda (λ) are known as atypical PKCs as they have only one cysteine-rich zinc finger domain and are not regulated by DG or phorbol esters. We have chosen to investigate the regulation of the calcium-insensitive isotypes δ and ε by DG, as IL-1 receptor-mediated production of ether-linked DG is not accompanied by an increase in intracellular free calcium concentration (3, 10).

It is not surprising that in addition to varying in primary structure, these PKC isotypes are differentially regulated in terms of expression, subcellular localization, cofactor, or substrate specificities and ligand activation (9, 11). However, the mechanisms by which distinct receptors target specific PKC isotypes have not been elucidated. Mechanisms have focused on ligand-induced PKC translocation and/or down-regulation and catabolism as well as PKC accessory or binding proteins. We now hypothesize a novel acute mechanism by which inflammatory cytokine receptors form ether-linked DG species that competitively inhibit the bioactivity of calcium-insensitive PKC isotypes. Moreover, it is hypothesized that inhibition of calcium-insensitive PKC isotypes correlates with a growth-arrested, inflamed MC phenotype.

MATERIALS AND METHODS

ET-1 was obtained from the Peptide Institute (Osaka, Japan), while IL-1 and platelet-derived growth factor (PDGF) were purchased from Life Technologies, Inc. (Gaithersburg, MD). [3H]NADP was obtained from ICN and [14C]P32ATP from AMERPEX (Louisville, KY). All other materials were purchased from Sigma Chemical Co., Poole, UK.

1 The abbreviations used are: IL-1, interleukin-1; PKC, protein kinase C; DG, diglyceride(s); diacylglycerol, 1,2-diacyl-sn-glycerol; alkyl, acylglycerol, 1-O-alkyl-2-acyl-sn-glycerol; alkenyl, acylglycerol, 1-O-alk-1-enyl-2-acyl-sn-glycerol; PAG, palmitoyl, acetylglycerol (1-palmitoyl-2-acetylglycerol); OAG, oleoyl, acetylglycerol (1-oleoyl-2-acetylglycerol); AAG, alkenyl, acylglycerol; MC, rat glomerular mesangial cell(s); PDGF, platelet-derived growth factor.

* This work was supported by National Institutes of Health Grant AR40225. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Calbiochem. All cell culture media and reagents were purchased from Life Technologies, Inc.

**Glomerular Mesangial Cell Isolation and Culture**—MC were isolated from collagenase-digested glomeruli obtained from 100-g male Harlan Sprague Dawley rats by a sequential sieving technique. MC were grown in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 12% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium at 37 °C in 5% CO2. Cells were used in their 3rd to 15th passage. We have verified previously that the MC cultures are devoid of epithelial, endothelial, macrophage, and fibroblast contamination (12).

**Western Blot Analysis**—Western immunoblotting using polyclonal antibodies to PKC α, βγ, δ, ε, and ζ was performed primarily as described previously (1, 13) except that the lysis buffer now consisted of 20 mM Hepes, pH 7.5, 40 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM leupeptin, 1 µg/ml pepstatin, and 1 mM benzamidine hydrochloride. The cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose blots were visualized by autoradiography, and quantified by scanning densitometry. n = 3 separate experiments for each PKC isotype. Mean ± S.E.; p < 0.05 for ET versus IL/ET as well as PDGF versus IL/PDGF for immunoprecipitated PKC δ, ε, but not ζ.

**In Vitro Reconstitution Assay for Immunoprecipitated PKC**—The immunoprecipitation of PKC isotypes and the subsequent reconstitution assay were adapted from previous methods (1). The bioactivity of the immunoprecipitate has been demonstrated with our polyclonal antibody, upon which no positive bands were observed when anti-PKC δ, or ζ was immunoprecipitated isotype were subsequently stripped and reprobed with another anti-PKC antibody, upon which no positive bands were revealed. For example, Western blots of immunoprecipitated PKC δ revealed a 78-kDa band when anti-PKC δ, but not when anti-PKC ε, was used as a probe (data not shown). Also, the position of the band for each isotype corresponded to the correct molecular weight as determined by molecular weight markers. Furthermore, it has been shown that PKC type-specific peptide immunogens can abolish subsequent visualization of the PKC isotype by Western analysis.2 We determined that this procedure quantified equal masses of each isotype by visualization of the protein bands on the membranes with Ponceau S (Sigma). Specificity of anti-PKC antibodies was determined on MC immunoprecipitates as described later. Human foreskin fibroblasts served as positive controls in all experiments.

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isotypes in the presence of exogenous DAG or phorbol 12-myristate 13-acetate (15, 16). In contrast to PKC δ or ε, exogenous diacylglycerol had no effect upon either control or ET-stimulated PKC ζ bioactivity. This confirms numerous reports that activation of PKC ζ is DAG-independent. These data suggest, but do not prove, that ET, and not IL-1, treatment may induce a conformational or post-translational alteration of PKC δ, ε, and ζ, which can be maintained throughout the immunoprecipitation procedure.

MC Proliferation—Proliferation of quiescent MC was evaluated by modification of our procedure to assess [3H]thymidine uptake into acid-insoluble DNA (17). Cells were subcultured into 12-well dishes and incubated with RPMI 1640 medium supplemented with 12% bovine serum albumin overnight. Twenty-four hours subsequent to the time of subculture, cells were down-regulated by incubation with serum-free medium for an additional 24 h. Cells were then stimulated with the appropriate agonist and further incubated for an additional 18 h, after which cells were pulsed with 1 µCi of [3H]thymidine/ml of medium. Thymidine incorporation was stopped after 6 h by aspiration of medium and washing the cells twice with ice-cold Dulbecco's phosphate-buffered saline. Cells were fixed for 1 h at 4 °C with 1 ml/well of fixing solution (40:50:10, v/v/v, water:methanol:acetic acid). A solution of 1% SDS (w/v) was applied to each well (0.5 ml/well) for 5 min at 4 °C. The SDS solution was then removed from each well and counted in a liquid scintillation counter.

Statistical Analysis—Independent t tests were used to establish significant differences between groups. The p value of the individual components was adjusted for multiple comparisons by the Bonferroni method. All data points on the concentration-response curves are expressed as arithmetic means ± S.E. and analyzed by nonlinear curve fitting to logistical equations using the PRISM program (18). The goodness-of-fit for competitive and noncompetitive models were compared by an F test on the ratio of residual variance.

RESULTS AND DISCUSSION

To extend our previous studies, which demonstrate that IL-1-induced ether-linked DG inhibit total PKC activity (1), we now investigate if individual PKC isotypes are regulated via DG cofactor specificity. Utilizing intact cells, we asked if there were any effects of IL-1 pretreatment on either G-protein-coupled receptor (ET) or tyrosine kinase receptor-linked (PDGF) activation of novel and atypical calcium-insensitive immunoprecipitated PKC isotypes. IL-1- pretreatment diminished both ET- and PDGF-stimulated PKC δ and ε, but not ζ bioactivity (Fig. 1). In these experiments, IL-1 was added 5 min before a subsequent 5 min stimulation with either ET, PDGF, or vehicle. IL-1 had no significant effect, by itself, on any of these PKC isotypes. IL-1 pretreatment abrogated approximately 50% of either the ET- or PDGF-induced activation of PKC δ and ε. In additional data not shown, coinucibution of IL-1 and ET for 10 min or IL-1 pretreatment for 5 min followed by a 30-min ET stimulation also significantly reduced PKC δ and ε activity. However, a 5- or 30-min incubation with IL-1 subsequent to a 5-min pretreatment with ET failed to significantly diminish PKC δ or ε activity (data not shown). At minimum, these studies suggest that a physiological function of IL-1 is, in part, to inhibit specific PKC isotypes and that IL-1, by itself, is incapable of stimulating MC PKC isotypes. At maximum, this inhibition may reflect the generation of second messengers that inhibit, but do not activate, selected PKC isotypes.

We next asked if an IL-1-induced second messenger, ether-linked DG, can mimic the effect of IL-1 to inhibit PKC isotypes in a cell-free system. Immunoprecipitated PKC isotypes from control MC were treated with exogenous alkenyl, acylglycerol (AAG, consisting primarily of 1-O-palmit-1'-ene-yl-2-oleyl-1-glycerol) and/or DAG (1-palmitoyl-2-oleoyl-sn-glycerol) in the kinase assay buffer (Fig. 2). A dose-response relationship was determined with concentrations of AAG ranging from 10⁻⁸ M to 10⁻¹⁰ M, which were added to the kinase assay buffer already containing a fixed concentration of DAG (10⁻⁷ M or 10⁻⁶ M). Increasing concentrations of AAG dose-dependently inhibited PKC δ and ε activated with 0.1 M DAG. Curve fitting analysis showed that inhibition of PKC δ by AAG was both highly potent (IC₅₀ = 13 ± 2 nM) and effective (E₅₀ = 87 ± 3% reduction in activity). AAG was even more potent against PKC ε (IC₅₀ = 19 ± 0.6 nM, E₅₀ = 55 ± 1%). Neither AAG nor DAG stimulated PKC ζ activity (data not shown). Also in data not shown, alkenyl, acylglycerol (1-O-palmitoyl-2-oleoyl-glycerol) inhibited activated PKC δ and ε with a similar dose-response profile. This suggests that it is most likely the sn-1 ether or vinyl ether linkage and not the chain length or degree of saturation that leads to inhibition of PKC isotypes.

To determine whether AAG inhibition was competitive with respect to DAG, we tested the effect of AAG on PKC activity stimulated with a 10-fold higher concentration of DAG (Fig. 2). The inhibitory effect of AAG was markedly blunted in the presence of 1.0 M DAG. These data were analyzed according to either a competitive model (increase in IC₅₀, with no concomitant change in E₅₀) or a noncompetitive model (no change in IC₅₀ with a decrease in E₅₀). The competitive model gave the best fit for both PKC δ and ε (F(3,3) = 40 and 39, respectively; p < 0.01). Only the curves obtained in the competitive fit are shown in Fig. 2. These results show that DAG and AAG inte-
PKC represents experimental evidence using specific polyclonal anti-PKC isotype antibodies. The molecular masses of PKC types in MC can be differentially regulated by inflammatory cytokines or vasoactive peptides. Western blots depicting a time course of PKC expression were evident.

PKC activity competitively and probably act at the same site on PKC. It may be inferred from these experiments that IL-1-induced ether-linked DG may function as second messengers to diminish the activity of calcium-insensitive PKC isotypes by binding to the DAG cofactor domain without leading to activation of PKC.

We next investigated if the protein expression of PKC isoforms in MC can be differentially regulated by inflammatory cytokines or vasoactive peptides. Western blots depicting a time course of PKC δ, ε, and ζ expression after ET (10−8 M) and IL-1 (10 ng/ml) treatment are shown in Fig. 3. There were no significant differences observed in the level of protein expression at any of the time points for either of the treatments. PDGF-ββ (10 ng/ml) treatment also did not significantly alter PKC δ, ε, or ζ expression over time (data not shown). In addition, IL-1 cotreatment with either ET or PDGF did not affect PKC isotype expression (data not shown). The constitutive expression of these PKC isotypes suggests that regulation of bioactivity by IL-1 is most likely a post-translational event and not a consequence of translational regulation or down-regulation by catabolism.

We next investigated growth arrest as a physiological correlate for IL-1-induced ether-linked DG inhibition of selected PKC isoforms. Since physiological DG cannot penetrate the plasma membrane of intact cells, we utilized cell-permeable, sn-2 short chain, DG analogues. We initially asked if an ether-linked, cell-permeable, DG analogue could mimic the effect of DAG to reduce PKC activation. In cell-free systems, 1-palmitoyl-2-oleoylglycerol (PAG) preincubation (5 min) reduced DAG (1-palmitoyl-2-oleoylglycerol)-stimulated immunoprecipitated PKC δ and ε activity. Specifically, DAG (10−8 M) activated PKC δ and ε 58 and 56%, respectively. PAG pretreatment reduced DAG stimulated activity to 6 and 4% of vehicle control for PKC δ and ε, respectively. Thus, we are confident in using PAG as an ether-linked DG analogue to potentially demonstrate inhibition of ET- or PDGF-induced proliferation.

We next asked if either IL-1 or the cell-permeable, ether-linked, DG analogue can induce growth arrest. Quiescent MC, in the absence of serum, proliferated in response to both ET and PDGF, but not IL-1, stimulation (Fig. 4A). However, IL-1 pretreatment reduced both ET (56%) and PDGF (42%)-induced MC proliferation to a level consistent with the reduction of PKC δ and ε bioactivity by IL-1 pretreatment. We next asked if the ether-linked DG analogue, PAG, could mimic the effect of IL-1 to induce growth-arrest. PAG reduced ET-induced proliferation to basal levels (Fig. 4B). In contrast, the ester-linked DG analogue, OAG, elevated ET-induced proliferation. Both PAG and OAG did not by themselves significantly demonstrate any proliferative effect. As PAG can be metabolized into platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), we utilized a platelet-activating factor receptor blocker (BNS2021) to show that this autacoid does not mediate the inhibitory actions of PAG upon proliferation (data not shown). These data suggest that IL-1-generated ether-linked DG can induce a growth-arrested state in both G-protein-linked receptor and tyrosine kinase receptor-stimulated MC. We conclude that the cell-permeable ether-linked DG analogue, PAG, not only mimics the actions of physiological (IL-1 receptor-generated) ether-linked DG to inhibit the activation of PKC isoforms, but also reduces ET- or PDGF-induced proliferation.

We have demonstrated a signaling mechanism by which receptor generation of unique molecular species of diglycerides differentially regulate the bioactivity of individual PKC iso-
types. Recent reports have also begun to establish a role for DG cofactor specificity for individual PKC isotypes. Supplementation of 1-O-hexadecylglycerol into fibroblasts of Zellweger patients leads to accumulation of ether-linked DG and a concomitant inhibition of bradykinin-induced translocation of PKC α, but not ε or ζ (19). Phosphatidylinositol-derived DG preferentially containing arachidonate at the sn-2 position are a better cofactor for PKC α compared with PKC β or γ, in contrast to, phosphatidylethanolamine-derived DG, translocates PKC ε but not α in L929 cells (21). Our studies may begin to clarify apparent paradoxes by demonstrating an acute receptor-mediated mechanism to diminish activation of selected PKC isotypes.

Even though physiological ether-linked DG species have not been studied as growth-arresting lipids, ether-linked phospholipids and alkyl phosphocholine have recently been identified as antineoplastic, antiproliferative, and apoptosis-inducing agents (22, 23). 1-O-Octadecyl-2-O-methyl-rac-glycerophosphocholine, an alkyllysophospholipid analogue, reduced cell proliferation by decreasing serum- or PDGF-activated MAP kinases (24). Both alkyllysophospholipid and hexadecylphosphocholine reduced membrane-associated and cytosolic total PKC activity (25). 1-O-Dodecyl-sn-glycerol, a monoaoylglycerol, reduced membrane-associated total PKC activity, which correlated with cell culture contact inhibition and resulting growth arrest (26). Also, in agreement with our studies, a cell-permeable ether-linked DG analogue (1-O-hexadecyl-2-acytylglycerol), but not an ester-linked DG analogue (1-oleyl-2-acytyl-rac-glycerol), inhibited cell growth and induced the differentiation of HL60 human promyelocytic leukemia cells to mononuclear phagocytes (27). Thus, ether-linked DG inhibition of selected PKC isoforms may, in part, be the signaling mechanism by which inflammatory cytokine receptor-generated ether-linked DG induce a growth-arrested cellular phenotype.

Our studies suggest that one of the major physiological outcomes of IL-1-mediated inhibition of calcium-insensitive PKC activity may be the induction of a growth-arrested phenotype. Previous studies have also suggested that inhibition and/or down-regulation of calcium-insensitive isoforms leads to changes in the cellular phenotype and/or growth arrest. Down-regulation of PKC ε or α inhibits G1/S transition (growth arrest) in vascular smooth muscle cells, an event consistent with cytokine-induced inflammation (28, 29). Furthermore, overexpression of PKC ε induces tumorigenicity in fibroblasts (30, 31).

Our data indicate that one component of IL-1 signaling is ether-linked DG inhibition of PKC δ and ε activity. However, other signaling pathways have been suggested, including the nuclear targeting of a 16-kDa N-terminal IL-1 cleavage product that may function as a trans-activating factor (32). Alternatively, the carboxyl end of IL-1 bound to the IL-1 receptor also localizes to the nucleus (33). IL-1 has also been shown to activate upstream kinases for heat shock proteins, β-casein, NF-κB, and cap-binding protein (34–36). Our laboratory has demonstrated recently that IL-1 activates the stress-activated protein kinase cascade (also known as jun kinase) via ceramide formation (17). As IL-1 does not activate ceramidase to form the putative PKC inhibitor sphingosine (37), we can rule out a role for IL-1-induced sphingosine to augment ether-linked DG-induced inhibition of PKC. On the other hand, ceramide inhibits PKC α translocation (38) and autophosphorylation (39) while inducing PKC ζ autophosphorylation (40).

In contrast to PKC δ and ε, PKC ζ was unaffected by DG, perhaps, reflecting the lack of a integral cysteine-rich zinc finger domain (9). Regardless of mechanism, our data suggest that inhibition of activated PKC ζ is probably not essential for growth arrest. Even though IL-1 may not affect PKC ζ bioactivity directly, this inflammatory cytokine has been shown to induce translocation of PKC ζ from cytosol to a presumed membrane compartment in MC in the presence of serum (41) or after long term pretreatment (42).

In summary, we have demonstrated that IL-1-induced ether-linked DG competitively inhibit activated calcium-insensitive PKC isotypes, an event consistent with a proinflammatory, growth-arrested phenotype. DG cofactor specificity for individual PKC isotypes may offer a more sensitive and specific mechanism for overall PKC regulation. Down-regulation of PKC δ and ε by IL-1-generated ether-linked DG may illustrate one mechanism by which a growth-arrested phenotype in MC can be induced and/or maintained in models of nonproliferative immunological renal disease (43).

Acknowledgment—We thank Michael Simonson for critically reviewing this manuscript.

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