Activation of protein kinase C-α is essential for stimulation of cell proliferation by ceramide 1-phosphate

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We previously demonstrated that ceramide-1-phosphate (C1P) stimulates fibroblast and macrophage proliferation, but the mechanisms involved in this action have only been partially described. Here we demonstrate that C1P induces translocation of protein kinase C-alpha (PKC-α) from the soluble to the membrane fraction of bone marrow-derived macrophages. Translocation of this enzyme was accompanied by its phosphorylation on Ser 657 residue. Activation of PKC-α was independent of prior stimulation of phosphatidylinositol-dependent or phosphatidylcholine-dependent phospholipase C activities, but required activation of sphingomyelin synthesis. Inhibition of PKC-α activation also blocked C1P-stimulated macrophage proliferation indicating that this enzyme is essential for the mitogenic effect of C1P.

1. Introduction

Ceramide 1-phosphate (C1P) is a bioactive sphingolipid that regulates critical biological functions including, apoptosis [1–3], phagocytosis [4,5], inflammation [6,7], and cell proliferation [8–10]. A major mechanism by which C1P stimulates cell division in macrophages involves activation of the phosphoinositol 3-kinase (PI3-K)/protein kinase B (PKB, also known as Akt), mitogen-activated protein kinase kinase (MAPKK)/extracellularly regulated (ERK) 1-2, and c-Jun N-terminal kinase (JNK) pathways [8]. Concerning inhibition of apoptosis, we found two major mechanisms whereby C1P exerts this action: (i) direct inhibition of acid sphingomyelinase, a signal-activated enzyme that generates ceramide from sphingomyelin [2], and (ii) inhibition of serine palmitoyltransferase, which is the key regulatory enzyme of the de novo ceramide synthesis pathway [11]. Inhibition of either of these enzymes causes potent reduction in the levels of proapoptotic ceramides thereby blocking apoptosis. In addition, C1P can contribute to the inhibition of apoptosis through stimulation of the prosurvival PI3-K/PKB pathway [1], and stimulation of nitric oxide production through stimulation of the inducible form of nitric oxide synthase [3]. Other actions of C1P that might be important for controlling cell proliferation, or apoptosis, are stimulation of intracellular calcium mobilization, or calcium entry from the extracellular milieu [12–14]. However, it should be pointed out that these actions are likely to be cell type specific, as C1P failed to alter calcium levels in macrophages, neutrophils, or A549 lung carcinoma cells [9,10]. More recently, C1P was found to potently stimulate cell migration [15]. The latter work was performed using macrophages as the experimental system, a fact that is particularly important because these cells are associated to the inflammatory response, which is partly controlled by C1P [16,17].

In the present work, we demonstrate that C1P stimulates translocation of protein kinase C-alpha (PKC-α) from the cytosol to the cell membranes, and that this action is essential for the stimulation of cell proliferation by C1P.

2. Materials and methods

2.1. Materials

Ceramide 1-phosphate (from bovine brain, containing predominantly stearic and nervous acids), culture medium RPMI 1640, tricyclodecan-9-yl xanthogenate (D609), L-α-phosphatidylcholine and dequalinium were from Sigma–Aldrich. Fetal bovine serum
(FBS) was supplied by Gibco. C-12 NBD Ceramide was from Cayman. [Methyl-\(^{3}\)H] thymidine and [Methyl-\(^{3}\)H] choline were from Perkin–Elmer. The U73122 inhibitor was supplied by Tocris. Antibodies to phosphoprotein kinase B (p-PKB; Ser 473), phospho-ERK 1-2 (Thr 202/Tyr 204), PKB, ERK 1-2, and goat anti-rabbit IgG horseradish peroxidase secondary antibody were purchased from Cell Signalling, and those to phosphoprotein kinase C-\(\alpha\) (p-PKC-\(\alpha\); Ser 657), and \(\beta\)-actin (H-196) were from Santa Cruz Biotechnology. 4\(\beta\)-Phorbol 12-myristate 13-acetate (PMA), and the Protein Kinase C kit were all from Calbiochem.

2.2. Cell culture

BMDM were isolated from femurs of 6–8-week old female CD-1 mice as described [18]. Cells were plated for 24 h in RPMI 1640 medium containing 10% FBS and 10% L-cell conditioned medium as the source of macrophage-colony stimulating factor (M-CSF) [19]. The non-adherent cells were removed and cultured for 4–6 days in the same medium until about 80% confluence was reached, at which point they were used in experiments.

2.3. Delivery of C1P to cells in culture

An aqueous dispersion of natural C1P (in the form of liposomes) was added to cultured macrophages as previously described [1,2]. Stock solutions were prepared by sonicating C1P (1 mg) in sterile nanopure water (1 ml) on ice using a probe sonicator until a clear dispersion was obtained. Final concentration of the stock solution was approximately 1.47 mM. We believe this procedure is preferable to dispersions created by adding C1P in organic solvents because droplet formation is minimized and there are no solvent effects on cells.

2.4. Determination of DNA synthesis

BMDM were seeded at 500 000 cells/well in 12-well plates and they were grown in the medium indicated above. Before experiments, macrophages were pre-incubated for 24 h in RPMI 1640 with 10% FBS and 1.5% L-cell conditioned medium (as the source of M-CSF) and then incubated in the presence or absence of agonists for 24 h. [\(^{3}\)H]Thymidine (0.5 \(\mu\)Ci/ml) was added for the first 2 h of incubation. The medium was then removed and cells were washed twice with phosphate-buffered saline (PBS). [\(^{3}\)H]Thymidine incorporation into DNA was measured as described previously [8–10].

2.5. Western blotting

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [18]. Twenty to 40 \(\mu\)g of protein from each sample were loaded and separated by SDS–PAGE, using 12% separating gels. Proteins were transferred onto nitrocellulose paper and blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.01% NaN\(_3\) and 0.1% Tween 20, and then incubated

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**Fig. 1.** C1P stimulates PKC-\(\alpha\) translocation. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. (A) Levels of PKC-\(\alpha\) in the microsomal and soluble fractions of BMDM. Macrophages were treated with C1P (50 \(\mu\)M) for the indicated times. Results are representative of one experiment and were confirmed in an additional experiment. (B) Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to control value and are the means ± S.D. of two replicate experiments. (C and D) BMDMs were treated with C1P (50 \(\mu\)M) or PMA (100 nM) for 15 min. PKC activity was determined using a commercial kit from Calbiochem according to the manufacturer’s instructions. Results are expressed as fold stimulation relative to control value and are the means ± S.E.M. of five independent experiments performed in duplicate for panel C (*\(P<0.05\); **\(P<0.01\)), and means ± S.E.M. of three independent experiments performed in duplicate for panel D.
overnight with the primary antibody in TBS/0.1% Tween at 4 °C. After three washes with TBS/0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1 h. Bands were visualized by enhanced chemiluminescence.

**2.6. Determination of PKC-α translocation by Western blotting**

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [18]. Nuclei were then removed by centrifugation at 500\(\times\)g for 5 min, and then the membrane and soluble fractions were separated by centrifugation at 100 000\(\times\)g at 4 °C for 30 min. Ten to 30 μg of protein from each sample were loaded and separated by SDS–PAGE, using 12% separating gels, and analyzed as indicated above.

**2.7. Determination of protein kinase C activity**

Protein kinase C activity was analyzed using a Protein Kinase Non-Radioactive kit from Calbiochem, according to the manufacturer’s instructions.

**2.8. Measurement of inositol phosphate accumulation**

These experiments were performed as previously described [20]. Briefly, the macrophages were labeled with myo-[\(^{3}\)H]inositol (1.5 μCi/ml) for 24 h in RPMI 1640 with 10% FBS and 1.5% L-cell conditioned medium (as the source of M-CSF). The cells were then washed and treated for 2.5 h with 10 mM LiCl, and after this time they were incubated in the presence or absence of agonists for 15 min. Incubations were ended by removing the medium and

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**Fig. 2.** C1P stimulates PKC-α phosphorylation. Involvement of PKC-α in C1P-induced DNA synthesis. (A) BMDMs were pre-incubated as in Fig. 1 and treated with C1P (50 μM) for the indicated times. Phosphorylation of PKC-α was examined by immunoblotting as described in Section 2. Equal loading of protein was monitored using a specific antibody to β-actin. Similar results were obtained in each of three replicate experiments. (B) Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to control value and are the means ± S.E.M. of three replicate experiments. (C) BMDMs were pre-incubated as previously indicated, and then treated with vehicle (Ctrl) or dequalinium (1 μM) in the presence or absence of C1P (50 μM) for 10 min. Phosphorylation of PKC-α was examined by immunoblotting as described in Section 2. Equal loading of protein was monitored using a specific antibody to β-actin. Similar results were obtained in each of three replicate experiments. (D) Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to control value and are the means ± S.E.M. of three replicate experiments (*\(P < 0.05\)). (E) BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium, and were then treated with vehicle or dequalinium (1 μM) in the presence or absence of C1P (50 μM) for 24 h, as indicated. The incorporation of [\(^{3}\)H]thymidine into DNA was determined as indicated in Section 2. Results are expressed as fold stimulation relative to the control value without inhibitor, and are the means ± S.E.M. of four independent experiments performed in triplicate (***\(P < 0.01\)).
adding 0.5 ml of 5% HClO₄, and 100 µl BSA (20 mg/ml). Inositol phosphates were separated by ion exchange chromatography on a Dowex AG1 X 8 column or eluted together, as previously described [20]. Radioactivity in the inositol phosphate fractions was measured by liquid scintillation counting.

2.9. Measurement of [³H] choline-labeled sphingomyelin levels

Levels of [³H] sphingomyelin were determined as described [21]. Briefly, the macrophages were labeled with [methyl-³H] choline chloride (0.5 µCi/ml) for 48 h in RPMI 1640 with 10% FBS and 1.5% L-cell conditioned medium (as the source of M-CSF). After this time, BMDM were washed with PBS and incubated for another 2 h. The cells were then incubated in the presence or absence of agonists for the indicated times, and harvested in distilled water. Lipids were extracted with 2.5 ml of chloroform/methanol (2:1, v/v). Total lipid extracts were subject to mild alkaline hydrolysis as described [22] and [³H] sphingomyelin was determined by thin-layer chromatography analysis in chloroform/methanol/acetic acid/H₂O (50:30:8.5, v/v/v/v), followed by scraping and counting the radioactivity by liquid scintillation counting.

2.10. Determination of sphingomyelin synthase (SMS) activity

SMS activity was performed as previously described [21]. Briefly, the macrophages were harvested and lysed in ice-cold homogenization buffer containing 250 mM sucrose, 5 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin, by 15 passages through a 25-gauge × 0.5-in needle. The cell lysates were centrifuged at 1000 g for 10 min at 4 °C to remove the nuclei. Protein in the supernatants was quantified using a commercial kit of BioRad. SMS activity was determined using 30–40 µg protein per assay. Samples were pre-incubated for 10 min at 30 °C in a final volume of 50 µl of a solution containing 50 mM Tris–HCl, pH 7.4, 25 mM KCl, and 0.5 mM EDTA. The reaction was started by adding 2 nmol of NBD C₁₂-ceramide and 12 nmol of phosphatidylcholine, and was allowed to proceed for 30 min. The reaction was stopped by the addition of 200 µl of chloroform/methanol (1:1, v/v). Lipids were extracted and NBD-sphingomyelin was separated by thin-layer chromatography analysis in chloroform/methanol/15 mM anhydrous CaCl₂ (90:52.5:12, v/v/v) and eluted with three washes of 3 ml of chloroform/methanol/acetic acid/water (50:39:1:1, v/v/v/v). The silica gel was removed by centrifugation, and all supernatants were pooled. One milliliter of water was added to separate the phases. The organic phase was washed once with 1 ml of methanol/water (1:1, v/v) and NBD-sphingomyelin was quantified by fluorometric analysis.

2.11. Statistical analyses

Results are expressed as means ± S.E.M. of three independent experiments performed in triplicate, unless indicated otherwise. Statistical analyses were performed using Student’s t-test as appropriate, with the level of significance set at P < 0.05.

3. Results and discussion

We previously found that C1P-stimulated cell division in macrophages involved activation of protein kinase B (PKB or Akt), ERK1-2 and JNK [8]. We now demonstrate that a major factor leading to stimulation of cell proliferation is activation of conventional PKC-α. It is known that conventional PKC isoforms require their recruitment to cell membranes for activation. Fig. 1A shows that C1P causes a significant increase in the level of PKC-α in the membrane fraction after 15 min of incubation with C1P, at a concentration (50 µM) that is optimal for stimulation of cell proliferation [8]. However, a corresponding decrease in the levels of PKC-α in the soluble fraction could not be detected by Western blotting. This was probably due to the high concentration of this enzyme in the cytosol. Therefore, to ensure that PKC-α decreased in the soluble fraction, the activity of PKC was measured after 15 min of treatment with C1P. Fig. 1C shows that C1P significantly decreased PKC activity in the cytosolic fraction, and this was in agreement with the increased activity of PKC-α observed in the membrane fraction (Fig. 1D). This effect of C1P was reproduced by the phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA), a well-known activator of PKC (Fig. 1C and D).

It is well established that optimal catalytic output of PKC isoforms requires phosphorylation on their activation loops. In particular, Ser 657 is a critical site for phosphorylation of PKC-α because it controls the accumulation of phosphate at other sites on PKC-α [23]. Therefore, we tested whether C1P was able to induce phosphorylation of this important site of PKC-α. Fig. 2A and B shows that C1P promoted phosphorylation of PKC-α at Ser 657 in a time
dependent manner, with maximal effect attained at 10–15 min of incubation. As expected, phosphorylation of PKC-α was sensitive to inhibition by the selective inhibitor dequalinium (Fig. 2C and D). Since C1P stimulates cell division, and activation of PKC-α is involved in mitogenesis, experiments were aimed at elucidating whether phosphorylation of PKC-α was relevant for the stimulation of cell growth by C1P. We and others have previously suggested that primary cultures are the best model to study the mechanisms involved in the regulation of macrophage proliferation. However, transfection of BMDM is highly inefficient ([24] and our unpublished work). Therefore, chemical inhibitors are usually used to study the contribution of specific effectors or pathways to macrophage growth. The implication of PKC-α in the mitogenic effect of C1P was evaluated by preincubating the cells in the presence or absence of dequalinium. This inhibitor completely blocked the stimulation of DNA synthesis by C1P, as determined by the incorporation of [3H]thymidine into DNA, thereby demonstrating that phosphorylation of PKC-α at Ser 657 is essential for the mitogenic effect of C1P (Fig. 2E).

It is well established that diacylglycerol (DAG) is the physiological activator of conventional and novel PKCs, including PKC-α. DAG can be produced after stimulation of a variety of signal-activated enzymes, including different phospholipases. A major phospholipase involved in PKC activation is phosphatidylinositol specific phospholipase C (PI-PLC), which generates DAG and inositol phosphates directly by degrading phosphatidylinositol (PI). However, inhibition of this enzyme with the selective inhibitor U73122 did not significantly alter C1P-stimulated DNA synthesis (Fig. 3A). In addition, C1P did not change the levels of inositol phosphates (Fig. 3B), and intracellular Ca²⁺ levels remained unchanged after treatment with C1P, as previously reported [8]. Activation of PKC-α could also occur through generation of DAG by a phosphatidylycholine-specific phospholipase C (PC-PLC), which would produce phosphocholine in addition to DAG. However, C1P did not cause significant accumulation of phosphocholine in the macrophages, suggesting that PC-PLC activity was unaltered (data not shown). Another enzyme capable of generating DAG is sphingomyelin synthase (SMS), which catalyzes the transfer of...
phosphocholine from phosphatidylcholine (PC) to ceramide forming sphingomyelin (SM). Fig. 4A shows that C1P increased SM levels after challenging the macrophages with C1P for 10 min. As expected, the activity of SMS was significantly increased [enzyme activity was enhanced by about 18.6 ± 2.0%, mean ± S.E.M. of three independent experiments performed in triplicate (**P < 0.01) over control values after treatment with C1P for 10 min]. To evaluate whether this enzyme activity was involved in the stimulation of cell proliferation by C1P we used tricyclodecan-9-yl-xanthogenate (D609), a compound initially shown to inhibit PC-PLC but that is a potent inhibitor of SMS [21,25]. Fig. 4B and C shows that D609 completely blocked the incorporation of [3H]thymidine into DNA and the phosphorylation of PKC-α that were stimulated by C1P, respectively. These data, together with the lack of phosphocholine accumulation after treatment with C1P suggest that SMS is important for PKC-α activation and the subsequent stimulation of DNA synthesis by C1P.

We also found that activation of ERK1-2 and PKB by C1P depend upon stimulation of PKC-α, as inhibition of this kinase by prolong treatment (16–18 h) with PMA (Fig. 5A), or with dequalinium (Fig. 2C and D), blocked both PKB and ERK1-2 phosphorylation (Fig. 5B–E). These results are consistent with previous reports showing that PKC-α is upstream of PKB and ERK1-2 activation in other cell types [26–28]. In addition, the SMS inhibitor D609,
immunoblotting as described in Section 2. Equal loading of protein was monitored using specific antibodies against total ERK 1-2 and PKB. Similar results were obtained in each of two replicate experiments.

Fig. 6. Involvement of SMS activity in CIP-stimulated ERK 1-2 and PKB phosphorylation. BMDMs were pre-incubated for 24 h in 1.5% L-cell conditioned medium. (A) BMDMs were treated with or without D609 (20 μM) for 10 min. Phosphorylation of ERK 1-2 and PKB was examined by immunoblotting as described in Section 2. Equal loading of protein was monitored using specific antibodies against total ERK 1-2 and PKB. Similar results were obtained in each of two replicate experiments. (B) Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to control value and are the means ± S.D. of two replicate experiments.

blocked phosphorylation of PKB and ERK1-2 (Fig. 6), thereby supporting the notion that SMS and PKC-α stimulation preceded the activation of PKB and ERK1-2 in macrophages. In a previous report, we demonstrated the existence of a plasma membrane G protein-coupled receptor that is specific for C1P [15]. Also, we demonstrated that C1P-stimulated cell proliferation requires activation of PKB and ERK1-2 [8]. However, these actions were not affected by pertussis toxin (PTX) [15], a potent and well-established inhibitor of G proteins, suggesting that these effects of C1P were receptor-independent. Likewise, C1P-stimulated macrophage proliferation was unaltered upon treatment with PTX [29] and unpublished work.

Taken together these data demonstrate that PKC-α is a key enzyme for regulation of cell proliferation by C1P, and that SMS is required for activation of this PKC isoform by C1P in macrophages.

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