Interleukin 1- β -induced protein kinase C- ζ activation is mimicked by exogenous phospholipase D

Cristina LIMATOLA*†§, Benedetta BARABINO*, Anna NISTA‡, and Angela SANTONI†‡

*Biophysics and ‡Physiopathology Laboratories, Centro Ricerca Sperimentale IRE, Via delle Messi d'Oro 156, 00158 Rome, Italy, and †Department of Experimental Medicine and Pathology, University of Rome 'La Sapienza', 00161 Rome, Italy

Interleukin 1- β (IL1- β) is a pleiotropic cytokine that stimulates a number of signal transduction pathways in cells, leading to different cellular responses. In this study we investigated the signal transduction pathways activated by IL1- β in two different human cell lines: RD/TE671, a rhabdomyosarcoma, and EJ, a bladder-derived carcinoma. We showed that this cytokine induced the activation of protein kinase C- ζ (PKC- ζ) and the accumulation of a putative physiological PKC- ζ activator, phosphatidic acid [Limatola, Schaap, Moolenaar and van Blitterswijk (1994) *Biochem. J.* **304**, 1001–1008]. Exogenously

supplied phospholipase D, which generated cellular phosphatidic acid, was able to mimic the cytokine effect, supporting the hypothesis that this lipid second messenger might contribute to cytokine-induced PKC- ζ activation. In addition, we show that IL1- β stimulation of BOSC23 cells, transiently overexpressing PKC- ζ , induced an increase in PKC- ζ autophosphorylation. These results give the first direct evidence that IL1- β can activate this atypical PKC isoform and suggest that this enzyme might be involved in mediating some of the biological effects induced by IL1- β .

INTRODUCTION

Interleukin 1- β (IL 1- β) is a pleiotropic cytokine that exerts a wide range of biological activities in inflammatory processes [1]. The downstream effectors linking receptor activation with the different cellular responses are still largely to be identified, although several signalling pathways have been described (reviewed in [2]). The involvement of protein kinase C (PKC) in IL1 signal transduction has often been proposed [3–6]. The variety of isoforms that comprise the PKC family differ, among other characteristics, in their activation requirement [7]. Lipid second messengers known to be produced during IL1- β stimulation could theoretically activate only members of the 'novel' and 'atypical' subgroups of the PKC family, as this cytokine does not induce phosphatidylinositol 4,5-bisphosphate hydrolysis and consequent Ca^{2+} mobilization [8,9]. Recent data indicate that tumour necrosis factor- α (TNF- α), a cytokine that shares many biological activities with IL1- β and induces an almost identical pattern of intracellular signalling [10], activates PKC- ζ [11,12], one of the atypical isoforms of this enzyme family. PKC- ζ , the activity of which is Ca^{2+} - and diacylglycerol (DAG)-independent [13], can be activated *in vitro* by phosphatidylinositol 3,4,5-trisphosphate [14], phosphatidic acid (PA) [15] and ceramide [12,16], lipid compounds that can all be produced rapidly in activated cells. PA exerts a wide range of biological activities in different cell types [17–21] and, in recent years, a role for it as a second messenger has often been proposed. Evidence has been presented that this lipid can activate a number of cellular enzymes [22–24], including PKC- ζ [15]. Ceramide has been suggested to be an important mediator of some of the effects triggered by cytokines, such as cell growth inhibition, nuclear factor κB activation and apoptosis [25]. The functions of ceramide could be mediated by some of its proposed intracellular targets: a ceramide-activated protein phosphatase [26], a 97 kDa proline-directed Ser-Thr kinase [27], and PKC- ζ itself [12,16], but a direct demonstration of their role is still lacking.

In the present study we report that IL1- β stimulation of RD/TE671 and EJ cells induces the activation of PKC- ζ , together with PA production, whereas ceramide accumulation is clearly detectable only in RD/TE671 cells. We also report that both exogenously supplied phospholipase D (PLD), which generates cellular PA, and short-chain ceramide (*N*-hexanoylsphingosine; C_6 -Cer) can mimic the cytokine effect. These data confirm that both lipid second messengers can be involved in PKC- ζ activation, but suggest that IL1- β -mediated enzyme activation is more likely to be controlled by the PA produced from phosphatidylcholine (PC)-derived DAG. In addition, we report that BOSC23 cells, a human embryonic kidney cell line transiently overexpressing PKC- ζ , showed enhanced PKC- ζ autophosphorylation after IL1- β stimulation. Overall, these results suggest that this atypical isoform of the PKC family is a likely candidate as one of the enzymes involved in the transduction of the biological activities attributed to this pleiotropic cytokine.

EXPERIMENTAL

Materials

All radioactive materials were from Amersham, except that [γ - ^{32}P]ATP was from NEN–du Pont. Polyclonal antiserum against PKC- ζ was from Gibco BRL; IL1- β (human recombinant; 10000 units/ml) and TNF- α (human recombinant; 10 $\mu\text{g}/\text{ml}$) were from Boehringer Mannheim; ϵ -peptide was from Peninsula Laboratories; C_6 -Cer and *N*-acetylsphingosine (C_2 -Cer) were from Calbiochem; Protein A–agarose was from Oncogene Science; BSA (fraction V, fatty-acid free), PLD type VII from *Streptomyces* species and all other materials were from Sigma.

Cells

TE671 is a human rhabdomyosarcoma cell line from American Type Culture Collection. EJ is a human epithelial bladder-

Abbreviations used: IL1- β , interleukin 1- β ; TNF- α , tumour necrosis factor- α ; PC, phosphatidylcholine; PC-PLC, PC-specific phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; PEt, phosphatidylethanol; DAG, diacylglycerol; C_2 -Cer, *N*-acetylsphingosine; C_6 -Cer, *N*-hexanoylsphingosine; PKC, protein kinase C; HPTLC, high-performance thin-layer chromatography.

§ To whom correspondence should be addressed, at the Biophysics Laboratory, Centro Ricerca Sperimentale IRE.

derived cell line. BOSC23 is a human cell line derived from HEK 293 [28].

Sphingomyelinase activation

Sphingomyelin hydrolysis was measured using the procedure described by Schutze et al. [29]. Serum-starved cells were labelled for 2 h with 1-[1-¹⁴C]palmitoyl L-lyso-3-PC at 0.1 μ Ci/ml (specific radioactivity 56 mCi/mmol) in RPMI medium containing 2% (w/v) BSA, washed and stimulated with IL1- β (1 ng/ml). Stimulation was stopped with ice-cold methanol and lipids were extracted according to the procedure of Bligh and Dyer [30], with minor modifications. For analysis of sphingomyelin hydrolysis, lipids were subjected to mild alkaline hydrolysis with 0.1 M methanolic KOH for 1 h at 37 °C. Lipids were then analysed by high-performance thin-layer chromatography (HPTLC) on Silica Gel 60 (Merck AG, Darmstadt, Germany) in a solvent system of chloroform/methanol/acetic acid/water (100:60:20:5, by vol.) for sphingomyelin and chloroform/methanol/7 M ammonia/water (85:15:0.5:0.5, by vol.) for ceramide. Sample spots co-migrating with authentic lipid standards were analysed for radioactivity by liquid scintillation counting. As a further control for analysis of sphingomyelin hydrolysis, in separate experiments, cells were also labelled with [methyl-³H]choline (1 μ Ci/ml; sp. radioactivity 60 Ci/mmol) or L-[³H]serine (1 μ Ci/ml; sp. radioactivity 20 Ci/mmol) for 48 h, serum-starved for 1 h in medium supplemented with 2% BSA, and then stimulated and processed as described above.

PC-specific phospholipase C (PC-PLC) and PLD activation

Serum-starved cells were labelled for 16 h with 100 nCi/ml [9,10(n)-³H]myristic acid (50 Ci/mmol) and/or for 4 h with 25 nCi/ml [1-¹⁴C]arachidonic acid (46 Ci/mmol), washed and stimulated with agonists. In experiments performed to detect phosphatidylethanol (PEt) accumulation, 1% ethanol (v/v) was present in the medium. PEt standard was prepared as described [31]. The reaction was stopped with ice-cold methanol and lipids were extracted as described above. Lipids were analysed by HPTLC with a solvent system with an upper phase of ethyl acetate/iso-octane/acetic acid/water (13:2:3:10, by vol.) for PtdOH and PtdEt, and an upper phase of ethyl acetate/iso-octane/acetic acid/water (10:5:2:10, by vol.) for half-plate height and, after drying, of hexane/diethyl ether/methanol/acetic acid (90:20:3:2, by vol.) for DAG. Lyso-PA production and release was analysed as described by Jalink et al. [32]. Sample spots co-migrating with authentic lipid standards were analysed for radioactivity content by liquid scintillation.

PKC- ζ activity in immunoprecipitates

The kinase reaction was performed essentially as described by Diaz-Meco et al. [11]. After stimulation, the reaction was blocked with ice-cold PBS and cells were extracted with lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μ g/ml aprotinin, 1 mM benzamidine) and clarified for 20 min at 15000 *g*. Lysates were precleared by incubation for 30 min at 4 °C with Protein A-agarose precoated with normal rabbit serum, followed by two incubations for 10 min at 4 °C with Protein A. PKC- ζ was immunoprecipitated with a polyclonal antibody (Gibco-BRL) at 1 μ g/ml for 1–2 h at 4 °C. Immunocomplexes were then precipitated with Protein A-agarose and washed twice with lysis buffer and twice with kinase buffer (35 mM Tris/HCl pH 7.5, 15 mM MgCl₂, 1 mM MnCl₂, 0.5 mM EGTA, 0.1 mM CaCl₂, 1 mM dithiothreitol, 10 mM p-nitrophenyl phosphate, 1 mM

PMSF, 20 μ g/ml aprotinin and 20 μ g/ml leupeptin). The kinase reaction was performed by resuspending the immunocomplexes in 50 μ l of this same buffer containing 10 μ M ATP, 3 μ Ci of [γ -³²P]ATP (6000 Ci/mmol) and 5 μ g of ϵ -peptide for 30 min at 30 °C. The reaction was blocked by adding 2-fold concentrated SDS buffer. Samples were boiled for 5 min and separated on 15% polyacrylamide gels. Dried gels were exposed at –80 °C for 3–16 h to Hyperfilm MP (Amersham) with one intensifying screen. Alternatively, half of the sample was spotted on to phosphocellulose filters (Pierce Phosphocellulose Unit), washed twice with 75 mM phosphoric acid and counted for radioactivity.

PKC- ζ autophosphorylation in transfected cells

BOSC23 cells were seeded 24 h prior to transfection at 2×10^5 per 30 mm plate in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. The medium was replaced 3 h before transfection, which was performed using the calcium phosphate method, as described in [15]. Briefly, 1.8 μ g of plasmid DNA per plate was used for transfections. The plasmid used was pmtSM-PKC- ζ , which is a pMT2-derived COS cell expression vector containing the cDNA for PKC- ζ [15]. At 48 h after transfection, cells were labelled for 4 h with 50 μ Ci of [³²P]P_i per ml in minimum essential medium (Eagle modified) without phosphate (ICN Biomedicals), washed twice and stimulated as described above. Reactions were blocked with ice-cold PBS and cells were lysed in RIPA buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 2 mM EDTA) containing 20 μ M leupeptin, 20 μ M aprotinin and 1 mM PMSF. Samples of 2 μ l of cell lysate were counted for radioactivity and sample normalization. Immunoprecipitations were performed as described above, and immunocomplexes were run on 10% polyacrylamide gels. Dried gels were exposed at –80 °C with one intensifying screen for 1–7 days to Hyperfilm MP (Amersham).

Western blotting

The PKC- ζ expression level in transfected BOSC23 cells was measured both in total cell lysates and after immunoprecipitation. Proteins were separated on a 10% polyacrylamide gel and blotted to nitrocellulose (Amersham; Hybond C) for 2 h at 250 mA. Blots were incubated for 2 h with anti-PKC- ζ serum, washed extensively with PBS containing 0.1% Triton X-100 and incubated with a goat anti-rabbit peroxidase-conjugated secondary antibody. ECL (Amersham) was performed according to the manufacturer's instructions.

RESULTS

Sphingomyelinase activation

Activation of a neutral sphingomyelinase has been described previously on cell stimulation with IL1- β [33]. We examined the effect of IL1- β on sphingomyelin hydrolysis in RD/TE671 and EJ cells labelled with lyso-[¹⁴C]PC as described by Schutze et al. [29]. As shown in Figure 1, treatment of RD/TE671 cells with IL1- β induced significant sphingomyelin hydrolysis, to give sphingomyelin levels of $75 \pm 5\%$ of control values after 30 s, followed by rapid recovery in the next 2 min. Ceramide accumulation peaked after 1 min of IL1- β treatment, achieving $137 \pm 4\%$ of control values (Figure 1). When EJ cells were stimulated with IL1- β , no significant effects on sphingomyelin hydrolysis were evident and, in addition, we could not detect increased ceramide accumulation at any time points analysed (Figure 1). Similar degrees of sphingomyelin hydrolysis upon

Figure 1 IL1- β induces sphingomyelin hydrolysis in RD/TE671 cells but not in EJ cells

RD/TE671 and EJ cells were labelled for 2 h with 0.1 μ Ci/ml 1-[1- 14 C]palmitoyl L-lyso-3-PC and stimulated with IL1- β , as described in the Experimental section. At the indicated time points, the reactions were rapidly stopped with ice-cold methanol, and lipids were extracted and separated on HPTLC for analysis of radioactivity content in sphingomyelin (●) and ceramide (○). Results are means $\pm$ S.D. of at least three different experiments, and are expressed as percentages of control values. Radioactivity incorporated into sphingomyelin and ceramide in unstimulated cells was, respectively, approx. 6080 and 665 d.p.m./mg of protein for RD/TE671 cells (A) and 4800 and 1400 d.p.m./mg of protein for EJ cells (B).

Figure 2 Stimulation by IL1- β induces PA accumulation in RD/TE671 and EJ cells

Serum-starved cells were labelled for 4 h with 25 nCi/ml [1- 14 C]arachidonic acid or for 24 h with 100 nCi/ml [9,10(n)- 3 H]myristic acid, washed and stimulated with IL1- β as described in the Experimental section. At the indicated times, the reactions were blocked with ice-cold methanol and lipids were extracted and separated on HPTLC for analysis of radioactivity content in PA. Results are means $\pm$ S.D. of at least three independent experiments, and are expressed as percentages of control values. Radioactivity incorporated into [3 H]myristoyl-PA (○) and [14 C]arachidonoyl-PA (●) in unstimulated cells was approx. 14250 and 380 d.p.m./mg of protein respectively for TE671 cells and 18380 and 440 d.p.m./mg of protein respectively for EJ cells.

IL1- β stimulation were obtained when cells were labelled for 48 h with either [methyl- 3 H]choline or L-[3- 3 H]serine (results not shown).

Table 1 Cytokine-induced PKC- ζ activation

Serum-starved RD/TE671 or EJ cells were stimulated for 10 min with vehicle (BSA) alone (control), TNF- α (5 ng/ml), IL1- β (1 ng/ml) or C $_6$ -Cer (30 μ M), or for 30 min with PLD (3 units/ml), and PKC- ζ was immunoprecipitated as described in the Experimental section. Immunocomplexes were incubated with ϵ -peptide in the presence of [γ - 32 P]ATP, in appropriate kinase buffer (containing 15 mM MgCl $_2$, 0.5 mM EGTA and 0.1 mM CaCl $_2$; see the Experimental section) for 30 min. Phosphorylated peptides were applied to P81 phosphocellulose paper, washed twice with 75 mM phosphoric acid and counted for radioactivity. Results are expressed as fmol of 32 P incorporated per μ g of ϵ -peptide, and represent half of the samples shown in Figure 3. Values shown are representative of three independent experiments, where the variation did not exceed 15% of control values taken as 100%. Numbers in parentheses are the fold stimulation relative to unstimulated cells. * Statistically significant increases over basal ($P < 0.05$).

	32 P transferred (fmol)	
	RD/TE671	EJ
Control	0.89 (1)	0.54 (1)
TNF- α	1.38 (1.6)*	2.8 (5.2)*
IL1- β	1.12 (1.3)*	2.19 (4)*
C $_6$ -Cer	0.96 (1.1)	1.97 (3.6)*
PLD	1.03 (1.2)	2.72 (5)*

PA accumulation

IL1- β -responsive PC-PLC has been described in different cellular systems [8,9]. To study whether IL1- β stimulation of RD/TE671 and EJ cells induces PC-PLC activation, cellular phospholipids were labelled with [1- 14 C]arachidonic acid or [9,10(n)- 3 H]myristic acid, and levels of radiolabelled DAG were analysed at various times of cell stimulation from 30 s to 15 min. Increased DAG accumulation over basal levels was barely detectable, at least up to 15 min of IL1- β cell stimulation (results not shown), possibly because of its rapid metabolism. Changes in monoacylglycerols, triacylglycerols and non-esterified fatty acids were also not significant (results not shown). Interestingly, however, after IL1- β stimulation, significant accumulation of PA was observed, which was probably derived from the activity of a DAG kinase acting on PC-PLC derived DAG. Figure 2 shows that, within the first few minutes of cell stimulation, IL1- β had induced PA production; this accumulation was sustained for up to 15 min, at which time it had reached $159 \pm 24\%$ and $130 \pm 20\%$ of control values in EJ and RD/TE671 cells respectively when cells were labelled with [14 C]arachidonic acid, and $147 \pm 6\%$ and $113 \pm 3\%$ respectively of control values when cells were labelled with [3 H]myristic acid. To investigate whether this PA was at least partially produced by the direct action of a PLD, cell stimulation was performed in the presence of 1% (v/v) ethanol, to monitor the accumulation of PEt produced by the transphosphatidyl reaction catalysed by PLD [34]. IL1- β was unable to induce significant formation of PEt in comparison with unstimulated samples, indicating that IL1- β is not coupled to PLD stimulation (results not shown).

PKC- ζ activation

It has been reported that, in U937 [12] and NIH-3T3 [11] cells, PKC- ζ , one of the atypical isoforms of the PKC family, can be activated by TNF- α . Because TNF- α and IL1- β induce similar pathways of biological and biochemical activities [10], we investigated whether in our cellular systems these cytokines were both able to induce PKC- ζ activation. Immunoprecipitated PKC- ζ from stimulated cells was assayed for kinase activity using ϵ -peptide as exogenous substrate. Table 1 shows that cytokine

Figure 3 Immunoprecipitated PKC- ζ from stimulated cells induces ϵ -peptide phosphorylation

Serum-starved RD/TE671 (**A**) and EJ (**B**) cells were stimulated with the indicated agonists, as described in the Experimental section. Stimulation was performed for 10 min with vehicle (BSA) alone (C), TNF- α (5 ng/ml), IL1- β (1 ng/ml) or C₆-Cer (C6; 30 μ M), and for 30 min with PLD (3 units/ml). Immunoprecipitated PKC- ζ was incubated in kinase buffer with ϵ -peptide (5 μ g), in the presence of [γ -³²P]ATP, for 30 min. Reactions were blocked by adding 2-fold concentrated hot SDS Laemmli buffer. Samples were boiled for 5 min and run on an SDS/15%-polyacrylamide gel, and autoradiographs were exposed for 5 h. Only regions containing the peptide are shown. Similar results were obtained in three separate experiments.

stimulation induced a large increase in ϵ -peptide phosphorylation in EJ cells, while RD/TE671 cells showed only a minor increase. TNF- α and IL1- β stimulation of EJ cells induced 5.2- and 4.0-fold increases respectively in ϵ -peptide phosphorylation, compared with 1.6- and 1.3-fold increases in RD/TE671 cells. C₆-Cer and PLD stimulation induced comparable increases in enzyme activity in EJ cells only, of 3.6 and 5.0 times the basal values, compared with 1.1- and 1.2-fold increases in RD/TE671 cells. Unexpectedly, C₂-Cer, another synthetic ceramide analogue, at the same concentration used for C₆-Cer, was completely ineffective in both cell lines (results not shown). Similar results are shown in Figure 3, for which samples from the same experiment as shown in Table 1 were separated on an SDS/15% polyacrylamide gel. EJ cells (Figure 3B) always had a lower basal PKC- ζ activity than RD/TE671 cells (Figure 3A); this could explain the different degrees of enzyme activation observed upon cytokine stimulation of the two cell lines.

Control experiments confirmed that stimulation with bacterial PLD of EJ and RD/TE671 cells prelabelled with [³H] myristic acid induced PA production to $180 \pm 15\%$ and $490 \pm 20\%$ respectively of control values after 5 min of cell stimulation, and to $207 \pm 15\%$ and $408 \pm 18\%$ respectively after 30 min. Part of this [³H]PA was converted into [³H]DAG, which peaked at $135 \pm 15\%$ and $138 \pm 22\%$ of control values respectively in EJ and RD/TE671 cells after 5 min of stimulation, while [³H]monoacylglycerol and [³H]lyso-PA levels were unchanged up to at least 30 min of cell stimulation (results not shown). DAG generation does not induce sphingomyelinase activation, as shown for other tumour cells [29]; PLD stimulation of EJ and RD/TE671 cells prelabelled with [¹⁴C]lyso-PC in fact did not induce sphingomyelin hydrolysis at any time point analysed (from 2 to 30 min; results not shown). This indicates that PKC- ζ activation is mediated by PA because, during PLD stimulation, ceramide is not generated and DAG cannot activate this atypical isoform [13]. In addition, C₆-Cer stimulation of both cell lines did not induce any significant PA, lyso-PA or DAG accumulation over basal values at any time point analysed (results not shown).

In another set of experiments, human BOSC23 cells transiently overexpressing PKC- ζ were labelled with [³²P]P_i, stimulated and immunoprecipitated with anti-PKC- ζ serum. Figure 4(A) shows

Figure 4 Cytokine-induced PKC- ζ autophosphorylation in PKC- ζ -over-expressing BOSC23 cells

BOSC23 cells were transfected with pmtSM-PKC- ζ , as described in the Experimental section. (**A**) At 48 h after transfection, cells were labelled for 4 h with [³²P]P_i and stimulated with vehicle (BSA) alone (C), TNF- α (5 ng/ml), IL1- β (1 ng/ml), C₆-Cer (30 μ M) or PLD (3 units/ml) for 10 min. Phosphorylated PKC- ζ was immunoprecipitated as described in the Experimental section, and analysed on an SDS/10%-polyacrylamide gel. The dried gel was exposed for 1 day. Similar results were obtained in three separated experiments. Only the region containing the immunoprecipitated PKC- ζ is shown. (**B**) Western blot with anti PKC- ζ serum of same samples shown in (**A**).

that both IL1- β and TNF- α induced an increase in enzyme autophosphorylation in comparison with unstimulated samples. Cell stimulation with exogenous PLD could also induce PKC- ζ autophosphorylation, whereas C₆-Cer was ineffective (Figure 4A). Control experiments showed that stimulation of PKC- ζ -transfected BOSC23 cells by exogenous PLD induced [³H]myristoyl-PA accumulation ($180 \pm 10\%$ of control values); at the time points analysed (from 10 to 20 min), no DAG, lyso-PA or ceramide was generated. Figure 4(B) is the Western blot with anti-PKC- ζ serum of the same samples shown in Figure 4(A), and indicates that comparable amounts of transfected protein were present in the different samples. Because PKC autophosphorylation reflects its activation [35], these results further confirm that IL1- β activates PKC- ζ .

DISCUSSION

In this study we have investigated the signal transduction pathways activated by IL1- β in RD/TE671 and EJ cells. The main results obtained are as follows. First, IL1- β stimulation of RD/TE671 and EJ cells induces an increase in PKC- ζ activity, measured as exogenous substrate (ϵ -peptide) phosphorylation, that, at least in EJ cells, can be mimicked by both exogenous PLD and C₆-Cer. Secondly, cytokine stimulation of both cell lines induces a rapid and sustained accumulation of PA, which is probably derived from DAG produced by PC-PLC activation. Thirdly, ceramide production, derived from sphingomyelin hydrolysis, is clearly evident upon IL1- β stimulation of RD/TE671 cells only. Fourthly, IL1- β stimulation of BOSC23 cells, which overexpress PKC- ζ , results in PKC- ζ autophosphorylation, which is a further indication of its activation.

Our result that PKC- ζ becomes activated upon stimulation by IL1- β is in line with the finding that most of the signal transduction pathways employed by IL1- β are common to TNF- α [10]. It has been hypothesized that TNF- α -induced activation of PKC- ζ could be mediated by sphingomyelin-derived ceramide, which has been proposed to function as a bifunctional mediator of PKC- ζ activation, in a concentration-dependent manner [12]. However, our observation that IL1- β stimulation induces ceramide accumulation only in TE671 cells, while cytokine

stimulation induces PKC- ζ activation in both cell lines, suggests that other lipid activator(s) must be responsible for IL1- β -induced enzyme activation. We propose that PA is the second messenger responsible for PKC- ζ activation during cell stimulation with IL1- β , because it rapidly accumulates in both cell lines and, in addition, cell treatment with PLD can mimic the cytokine effect in both EJ (Figure 3) and BOSC23 (Figure 4) cells. Alternatively, IL1- β -mediated PKC- ζ activation could be mediated in different cell types by different sets of lipid second messengers.

IL1- β stimulates PC-PLC in different cell types [8,9], generating DAG in the absence of intracellular Ca^{2+} . In our cellular systems we could not detect significant DAG accumulation upon cell stimulation with this cytokine, but we measured PA accumulation (Figure 2); this was not derived from a PLD activity because, when cell stimulation was performed in the presence of ethanol, no PEt was produced. This PA either could be a product of a DAG kinase acting on DAG derived from IL1- β -induced PC-PLC activation [10,11] or, as demonstrated for human mesangial cells stimulated with IL1, could also originate from the activity of lysophosphatidate acyltransferase [36]. This last hypothesis seems unlikely in our system, because in IL1-stimulated human mesangial cells the authors described a rapid (15–30 s) conversion of the newly generated PA into DAG.

It has been shown that, depending on the activating lipid, PKC can induce different patterns of substrate phosphorylation [15,37]. Our observation that both PA and ceramide can be involved in mediating PKC- ζ activation leads us to hypothesize that, upon cell stimulation with IL1- β , the differential production of the two lipids in different cell types could induce the phosphorylation of distinct substrates, activating different cellular responses.

Several studies have described the involvement of PKC- ζ in a number of signalling pathways and cellular functions [11,12,16,38–40], but the role of this PKC isoform in cell growth control has been questioned [41,42]. An understanding of the role of PKC- ζ in IL1- β -mediated signalling could be important for defining the biochemical link between receptor stimulation and the plethora of physiological effects induced by this cytokine.

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REFERENCES

- Dinarelli, C. A. (1991) *Blood* **77**, 162–165
- Dinarelli, C. A. (1994) *FASEB J.* **8**, 1314–1325
- Munoz, E., Beutner, U., Zubiaga, A. and Huber, B. T. (1990) *J. Immunol.* **144**, 964–969
- Kracht, M., Heiner, A., Resch, K. and Szamel, M. (1993) *J. Biol. Chem.* **268**, 21066–21072
- Ostrowski, J., Meier, K. E., Stanton, T. H., Smith, L. L. and Bromshtyk, K. (1988) *J. Biol. Chem.* **263**, 13786–13790
- Lee, W. Y., Butler, A. P., Locniskar, M. F. and Fisher, S. M. (1994) *J. Biol. Chem.* **269**, 17971–17980
- Nishizuka, Y. (1992) *Science* **258**, 607–614
- Rosoff, P. M., Savage, N. and Dinarello, C. A. (1988) *Cell* **54**, 73–81
- Kester, M., Siomomson, M. S., Mene, P. and Sedor, J. R. (1989) *J. Clin. Invest.* **83**, 718–723
- Wright, S. D. and Kolesnick, R. N. (1995) *Immunol. Today* **16**, 297–302
- Diaz-Meco, M., Dominguez, I., Sanz, L., Dent, P., Lozano, J., Municio, M. M., Berra, E., Hay, R. T., Sturgill, T. W. and Moscat, J. (1994) *EMBO J.* **13**, 2842–2848
- Muller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D. and Pfizenmaier, K. (1995) *EMBO J.* **14**, 1961–1969
- Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3099–3103
- Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993) *J. Biol. Chem.* **268**, 13–16
- Limatola, C., Schaap, D., Moolenaar, W. H. and van Blitterswijk, W. J. (1994) *Biochem. J.* **304**, 1001–1008
- Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L. and Moscat, J. (1994) *J. Biol. Chem.* **269**, 19200–19202
- Liscovitch, M. and Amsterdam, A. (1989) *J. Biol. Chem.* **264**, 11762–11767
- Metz, S. A. and Dunlop, M. (1990) *Biochem. J.* **270**, 427–435
- Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O. and Hsuan, J. J. (1994) *Science* **263**, 523–526
- Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. and de Laat, S. W. (1986) *Nature (London)* **323**, 171–173
- van Corven, E. J., van Rijswijk, A., Jalink, K., van der Bend, R. L., van Blitterswijk, W. J. and Moolenaar, W. H. (1992) *Biochem. J.* **281**, 163–169
- Moritz, A., De Graan, P. N. E., Gispens, W. H. and Wirtz, K. W. A. (1992) *J. Biol. Chem.* **267**, 7207–7210
- Nakanishi, H. and Exton, J. H. (1992) *J. Biol. Chem.* **267**, 16347–16354
- Zhao, Z., Shen, S. H. and Fisher, E. H. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4251–4255
- Kolesnick, R. N. (1992) *Trends Cell Biol.* **2**, 231–236
- Dobrowsky, R. T. and Hannun, Y. A. (1992) *J. Biol. Chem.* **267**, 5048–5051
- Mathias, S., Dressler, K. A. and Kolesnick, R. N. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10009–10015
- Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8392–8396
- Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Kronke, M. (1992) *Cell* **71**, 765–776
- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Pacini, L., Limatola, C., Frati, L., Luly, P. and Spinedi, A. (1993) *Biochem. J.* **289**, 269–275
- Jalink, K., Eichholtz, T., Postma, F. R., van Corven, E. J. and Moolenaar, W. H. (1993) *Cell Growth Differ.* **4**, 247–255
- Mathias, S., Younes, A., Kan, C., Orlow, I., Joseph, C. and Kolesnick, R. N. (1993) *Science* **259**, 519–522
- Liscovitch, M. (1989) *J. Biol. Chem.* **264**, 1450–1456
- Mitchell, F. E., Marais, R. M. and Parker, P. J. (1989) *Biochem. J.* **261**, 131–136
- Bursten, S. L., Harris, W. E., Bomshtyk, K. and Lovett, D. (1991) *J. Biol. Chem.* **266**, 20732–20743
- Newton, A. C. and Koshland, D. E. (1990) *Biochemistry* **29**, 6656–6661
- Berra, E., Diaz-Meco, M. T., Lozano, J., Frutos, S., Municio, M. M., Sanchez, P., Sanz, L. and Moscat, J. (1995) *EMBO J.* **14**, 6157–6163
- Dominguez, I., Sanz, L., Arenzana-Seisdedos, F., Diaz-Meco, M. T., Virelizier, J. L. and Moscat, J. (1993) *Mol. Cell. Biol.* **13**, 1290–1295
- Dominguez, I., Diaz-Meco, M. T., Municio, M. M., Berra, E., Frutos, S., Sanz, L. and Moscat, J. (1992) *Mol. Cell. Biol.* **12**, 3776–3783
- Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J., Chapkin, R. and Moscat, J. (1993) *Cell* **74**, 555–563
- Montaner, S., Ramos, A., Perona, R., Esteve, P., Carnero, A. and Lacal, J. C. (1995) *Oncogene* **10**, 2213–2220