Ceramide-induced translocation of protein kinase C ζ in primary cultures of astrocytes

Ismael Galve-Roperh[a], Amador Haro[a], Inés Díaz-Laviada[b,*]

[a]*Department of Biochemistry and Molecular Biology I, Faculty of Biology, Complutense University, 28040 Madrid, Spain*
[b]*Department of Biochemistry and Molecular Biology, Faculty of Medicine, Alcalá de Henares University, 28871 Alcalá de Henares, Madrid, Spain*

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Abstract The present research was undertaken to study the possible involvement of the atypical protein kinase C (PKC) ζ in ceramide signal transduction in primary cultures of rat astrocytes. As shown by Western blot analysis, translocation of immunoreactive PKCζ to the particulate fraction occurred upon exposure of astrocytes to cell-permeable ceramide analogs or to exogenous sphingomyelinase. The particulate fraction may correspond to a perinuclear area, as indicated by immunocytochemical techniques. Furthermore, treatment of cells with *N*-octanoylsphingosine led to an increased phosphorylation of PKCζ. Results thus show that stimulation of PKCζ may be one of the intracellular events triggered by activation of the sphingomyelin pathway.

Key words: Protein kinase C ζ; Ceramide; Sphingomyelin; Astrocyte

1. Introduction

Sphingolipids are currently recognized as active participants in the regulation of a wide range of cellular responses such as regulation of cell growth, differentiation, transformation and death [1]. Sphingomyelin (SM) breakdown occurs in multiple cell types in response to a variety of extracellular mediators including cytokines e.g. tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), interferon-γ and other agents such as nerve growth factor (NGF), 1α,25-dihydroxyvitamin D_3, Fas ligand and ionizing radiation (reviewed in [1–3]). Receptor-mediated hydrolysis of SM generates ceramide, which may function as a second messenger activating several serine/threonine protein kinases and phosphatases [4]. Among the former, one of the atypical isoforms of protein kinase C (PKC), PKCζ, has been proposed as an intracellular target of ceramide action [5,6]. These atypical PKC members (ζ and λ/ι) are dependent on phosphatidylserine but are not affected by diacylglycerol, phorbol esters or Ca^{2+} [7,8]. Although PKCζ has been shown to be involved in the control of a number of cellular functions including mitogenic signal transduction [9], neuronal differentiation [10] and long-term potentiation [11], the signal mechanisms downstream PKCζ activation are not well known. PKCζ has been suggested to play a role in the activation of the mitogen-activated protein kinase (MAPK) pathway [12]. PKCζ has also been shown to be involved in the activation of nuclear factor κB (NF-κB), which is a landmark of the TNFα mechanism of action. Little is known about the specific signal transduction pathways linking TNF receptors to NF-κB. A connection has been proposed between TNFα receptor and phosphatidylcholine phospholipase C (PC-PLC), which is coupled to sphingomyelinase (SMase), resulting in the generation of ceramide [6,13,14]. We have recently demonstrated that PC-PLC induces cellular redistribution and phosphorylation of PKCζ in glial cells [15]. The present work was thus undertaken to test whether PKCζ may also be a target for ceramide action in primary cultures of astrocytes.

2. Materials and methods

2.1. Materials

Anti-PKCζ antibody, raised against amino acids 577–592 of rat PKCζ, was from Life Technologies Inc. (Gaithesburg, MD, USA). Fluorescein-conjugated donkey anti-rabbit IgG was from Amersham (Little Chaltfont, UK). D-erythro-*N*-acetylsphingosine (C_2-ceramide), D-erythro-*N*-octanoylsphingosine (C_8-ceramide) and D-erythro-dihydro-*N*-acetylsphingosine (DHC) were from Calbiochem (La Jolla, CA, USA). Protein A-agarose was from Transduction Laboratories (Lexington, KY, USA). Neutral SMase (*S. aureus*) and all other reagents were from Sigma Chemicals (St Louis, MO, USA).

2.2. Primary cultures of astrocytes

Cortical astrocytes were derived from 1–2 day old rats and cultured as previously described [16]. Cells were seeded at a density of 3×10^4 cells/cm^2 on plastic plates previously coated with 5 μg/ml dl-polyornithine in water. The primary cultures consisted of 95% astrocytes as judged by immunocytochemical staining of glial fibrillary acidic protein.

Three days before the experiment, the serum-containing medium was removed and cells were transferred to a chemically defined medium consisting of serum-free DMEM:Ham's F12 (1:1, v:v) medium supplemented with 25 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, and 30 nM sodium selenite.

2.3. Western blot analysis of PKCζ

After stimulation, cells were washed with ice-cold PBS and subsequently homogenized in 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml benzamidine. Soluble and particulate fractions were obtained after centrifugation for 60 min at $40\,000\times g$. Proteins were then resolved by SDS-PAGE and transferred onto nitrocellulose. Immunodetection of PKCζ was carried out by incubating membranes with anti-PKCζ polyclonal antibody and developing with an enhanced chemiluminescence reaction kit (Amersham).

2.4. Immunocytochemical procedures

Astrocytes were grown in glass coverslips in serum-containing medium and at three weeks made quiescent by serum starvation. Cells were stimulated with different agents, washed with phosphate buffer saline (PBS) and coverslips immediately immersed in 3.7% formaldehyde/PBS for 5 min. After washing in PBS, cells were treated with

*Corresponding author. Fax: +34 (1) 885 45 85.
E-mail: bqidm@bioqui.alcala.es

Abbreviations: C_2-ceramide, *N*-acetylsphingosine; C_8-ceramide, *N*-octanoylsphingosine; DHC, dihydro-*N*-acetylsphingosine; IL, interleukin; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PC-PLC, phosphatidylcholine phospholipase C; PKC, protein kinase C; SM, sphingomyelin; TNF, tumor necrosis factor

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0.05% Triton X-100 and subsequently incubated with 40 μg/ml polyclonal anti-PKCζ antibody for 60 min and fluorescein-conjugated anti-rabbit IgG for 60 additional min. Coverslips were then mounted with glycerol containing 0.1% *p*-phenylenediamine and subjected to fluorescence microscopy.

2.5. Phosphorylation of PKCζ

Phosphorylation of PKCζ was performed after loading of cells with ^{32}Pi and immunoprecipitation as previously described [17]. Immunoprecipitation was carried out by incubation with 7.5 μg/ml of anti-PKCζ polyclonal antibody and precipitation with agarose-linked protein A. Phosphorylation was determined in the immunoprecipitates by SDS-PAGE and autoradiography. Gels were previously stained with Coomasie blue in order to verify the appropriate loading of the gels. Autoradiography Fuji films were subjected to densitometric analysis using the Sigma-Gel program.

3. Results and discussion

3.1. Translocation of PKCζ and identification by immunoblotting

To study whether SMase and ceramides may have an effect on the subcellular distribution of PKCζ, primary cultures of astrocytes were treated with neutral SMase or cell-permeable ceramide analogs, C_2-ceramide and C_8-ceramide. As shown in Fig. 1, PKCζ appears to be expressed in primary cultures of astrocytes as a single 75 kDa isoform in both the particulate and the soluble fraction, with approximately equal amounts of total enzyme in each fraction in non-stimulated cells (55% and 45% respectively) [18]. C_8- and C_2-ceramide, as well as exogenous SMase, induced a significant increase in immunoreactive PKCζ in the particulate fraction with a concomitant decrease in the soluble fraction (Fig. 1). The time course of C_8-ceramide-induced PKCζ translocation to the particulate fraction is shown in Fig. 2. PKCζ redistribution was already evident after 5 min treatment and was maximal after 15 min of ceramide stimulation. These findings indicate that activation of the SM pathway induces PKCζ translocation to the particulate fraction of rat astrocytes.

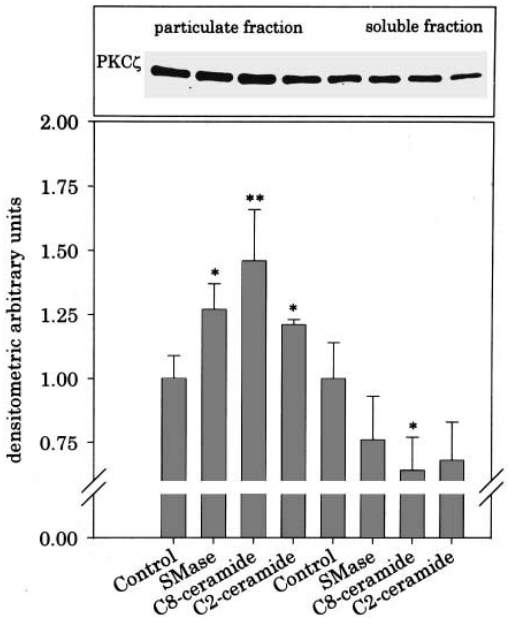

Fig. 1. Localization of PKCζ by immunoblotting. Astrocytes were stimulated with vehicle, 1 U/ml SMase (*S. aureus*), 25 μM C_8-ceramide or 25 μM C_2-ceramide for 15 min. The upper panel shows a representative luminogram of the immunoblots. The lower panel shows the means ± S.D. of densitometric analyses from three independent experiments expressed in arbitrary units referred to densitometric units from control cells. Statistical analysis was performed by Student's *t*-test. Significantly different vs. the corresponding subcellular fraction of control cells: *: $P < 0.025$; **: $P < 0.01$.

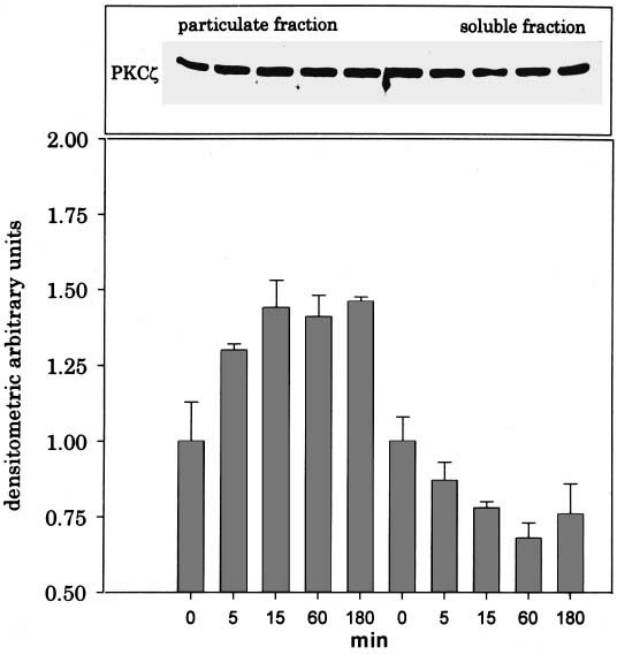

Fig. 2. Time course of C_8-ceramide-induced translocation of PKCζ. Astrocytes were stimulated with 25 μM C_8-ceramide for 0, 5, 15, 60 and 180 min. The upper panel shows a representative luminogram of the immunoblots. The lower panel shows the means ± S.E.M. of densitometric analyses from two independent experiments expressed in arbitrary units referred to densitometric units from control cells.

3.2. Redistribution of PKCζ in primary astrocytes

To further study the intracellular localization of PKCζ, immunocytochemical analyses were performed. As shown in Fig. 3, exposure of cells to either SMase or ceramide analogs induced PKCζ translocation to a perinuclear area. The effect of added SMase indicates that endogenously produced ceramides are also able to induce PKCζ cellular redistribution, therefore corroborating the specificity of ceramide analog action. Perinuclear signal was stronger in SMase-treated and C_8-ceramide-treated cells (Fig. 3C and D) than in C_2-ceramide (Fig. 3E). This may be probably due to the longer alkyl chain of the former, which makes it more similar to natural occurring ceramides. Like in Western blot analysis (see above), C_8-ceramide and SMase-generated ceramides produced a greater effect than the short chain C_2-ceramide. Therefore alkyl chain length may play an important role in ceramide action. This effect has also been observed in raf-1 activation by ceramides [19]. The emerging hypothesis from this observations is that such structural requirements may explain divergent effects of different ceramides as a consequence of its subcellular origin, and the specific SM pool involved [20]. In this context, a different action of intracellularly generated ceramides and exogenously added ceramides in the induction of apoptosis has been recently reported [21].

The specificity of the effect of ceramides is supported by the fact that inactive ceramide analog DHC did not induce any change in the subcellular localization of PKCζ (Fig. 3B).

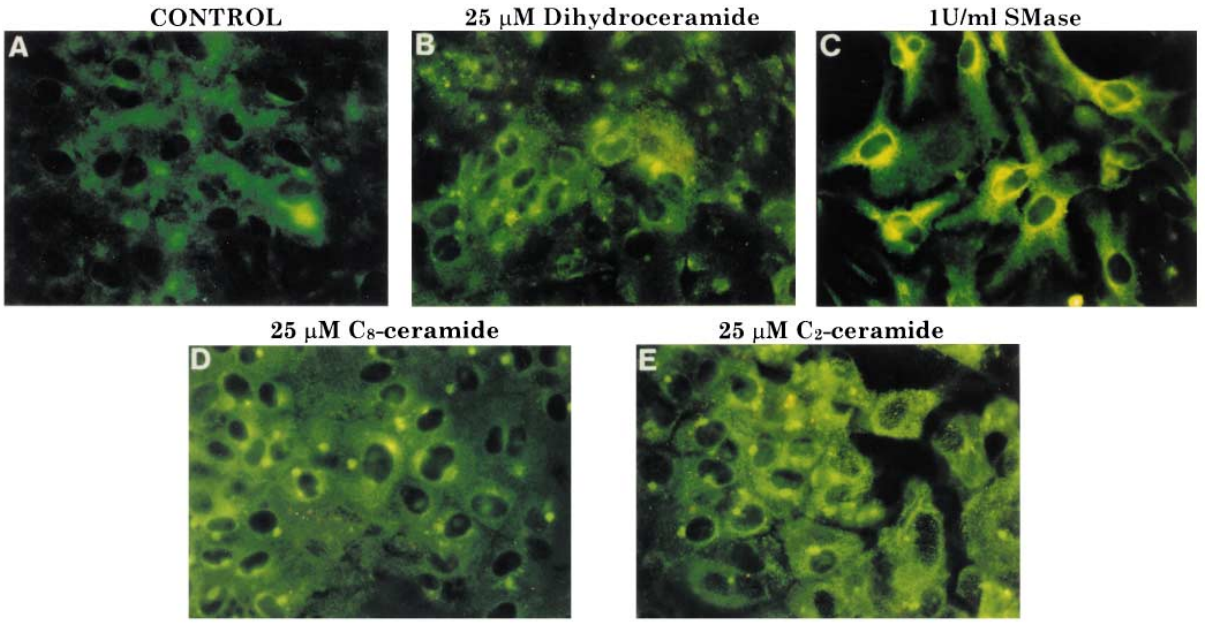

Fig. 3. Immunofluorescence localization of PKCζ in primary astrocytes. Cells were stimulated for 15 min with vehicle (A); 25 μM DHC (B); 1 U/ml SMase (*S. aureus*) (C); 25 μM C_8-ceramide (D) and 25 μM C_2-ceramide (E).

These data are in line with previous observations by other authors who have also found PKCζ located in a perinuclear area in intimate association with the β-tubulin fraction of the cytoskeleton [22,23]. PKCζ has also been found associated to the Golgi apparatus and probably involved in the formation of transport vesicles [24].

3.3. *Phosphorylation state of PKCζ*

PKC translocation has been related to its functional state [8]. One of the molecular events observed during PKC activation is an increase in its phosphorylation state [25]. To investigate the effect of ceramides in the phosphorylation level of PKCζ, astrocyte cultures were incubated with $^{32}P_i$, and then immunoprecipitation studies were carried out. Fig. 4 shows that short-term treatment with ceramide analogs induced an increase in the phosphorylation state of PKCζ, that was specific for biologically active ceramide analogs, as shown by the lack of effect of DHC. Subcellular redistribution of PKCζ to the particulate cell fraction was in the same interval of time than ceramide-induced increase of PKCζ phosphorylation, indicating that both events may be related.

The precise mechanism of the regulation of PKCζ activity is still controversial. Contradictory results have been reported regarding the subcellular redistribution of PKCζ upon activation. Numerous reports indicate that active PKCζ is found concentrated in membrane fractions or cytoskeleton structures [15,22,24,26]. Translocation of PKCζ from the cytosol to nuclear myofibrilar fraction occurs in the ischemic fetal brain [27]. However, recent data have shown a translocation of activated PKCζ to the cytoplasm, with a concomitant increase in the phosphorylation state and activity of the enzyme in response to changes in intracellular cAMP levels [28]. This indicates that the phosphorylated enzyme would display a diminished membrane affinity [8]. Association of PKCζ with both cytosol and membranes may be mediated through a recently identified protein kinase C-binding protein, i.e. zeta-interacting protein which has been shown to interact with the regulatory domain of the kinase [29].

The importance of ceramide signaling in rat astrocytes has been evidenced in our laboratory by the fact that ceramide analogs, SMase and PC-PLC induce NGF production in these glial cells [16,17]. We report here that both SMase and ceramide analogs induce the translocation of PKCζ to a perinuclear membrane region and increase the PKCζ phosphorylation level, suggesting the possible involvement of PKCζ in ceramide signaling in primary cultures of rat astrocytes.

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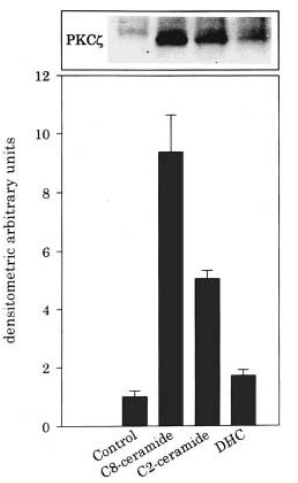

Fig. 4. Phosphorylation of PKCζ. ^{32}P-labelled astrocytes were stimulated with vehicle, 25 μM C_8-ceramide, 25 μM C_2-ceramide, and 25 μM dihydroceramide for 15 min. The upper panel shows a representative autoradiogram. The lower panel shows the means ± S.E.M. of densitometric analyses from two independent experiments expressed in arbitrary units referred to densitometric units from control cells.

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References

[1] Spiegel, S., Foster, D. and Kolesnick, R. (1997) Curr. Opin. Cell Biol. 8, 159–167.
[2] Hannun, Y. (1996) Science 274, 1855–1859.
[3] Testi, R. (1996) Trends Biochem. Sci. 21, 468–471.
[4] Ballou, L.R., Lauderkind, S.J.F., Rosloniec, E. and Raghow, R. (1996) Biochim. Biophys. Acta 1301, 273–287.
[5] 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.
[6] Müller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D. and Pfizenmaier, K. (1995) EMBO J. 14, 1961–1969.
[7] Jaken, S. (1996) Curr. Opin. Biol. 8, 168–173.
[8] Newton, A.C. (1996) Curr. Biol. 6, 806–809.
[9] Berra, E., Diaz-Meco, M.T., Dominguez, I., Municio, M.M., Sanz, L., Lozano, J., Chapkin, R.S. and Moscat, J. (1993) Cell 74, 555–563.
[10] Coleman, E.S. and Wooten, M.W. (1994) J. Mol. Neurosci. 5, 39–57.
[11] Hrabetova, S. and Sacktor, T. (1996) J. Neurosci. 16, 5324–5333.
[12] Berra, E., Díaz-Meco, M.T., Lozano, J., Frutos, S., Municio, M.M., Sánchez, P., Sanz, L. and Moscat, J. (1995) EMBO J. 14, 6157–6163.
[13] Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Krönke, M. (1992) Cell 71, 765–776.
[14] Machleidt, T., Krämer, B., Adam, D., Neumann, B., Schütze, S., Wiegmann, K. and Krönke, M. (1996) J. Exp. Med. 184, 725–733.
[15] Galve-Roperh, I., Malpartida, J.M., Haro, A. and Díaz-Laviada, I. (1996) Neurosci. Lett. 219, 68–70.
[16] Laviada, I.D., Baudet, C., Galve-Roperh, I., Naveilhan, P. and Brachet, P. (1995) FEBS Lett. 364, 301–304.
[17] Galve-Roperh, I., Haro, A. and Díaz-Laviada, I. (1997) Mol. Brain Res. (in press).
[18] Chen, C.C., Chang, J. and Chen, W.C. (1995) Mol. Pharmacol. 48, 39–47.
[19] Huwiler, A., Brunner, J., Hummel, R., Vervoordeldonk, M., Stabel, S., Van Den Bosch, H. and Pfeilschifter, J. (1996) Proc. Natl. Acad. Sci. USA 93, 6959–6963.
[20] Andrieu, N., Salvayre, R. and Levade, T. (1996) Eur. J. Biochem. 236, 738–745.
[21] Zhang, P., Liu, B., Jenkins, G.M., Hannun, Y. and Obeid, L.M. (1997) J. Biol. Chem. 272, 9609–9612.
[22] Lehrich, R.W. and Forrest, J.N. (1994) J. Biol. Chem. 269, 32446–32450.
[23] García-Rocha, M., Avila, J. and Lozano, J. (1997) Exp. Cell. Res. 230, 1–8.
[24] Westermann, P., Knoblich, M., Maier, O., Lindschau, C. and Haller, H. (1996) Biochem. J. 320, 651–658.
[25] Keranen, L.M., Dutil, E.M. and Newton, A.C. (1995) Curr. Biol. 5, 1394–1403.
[26] Gomez, J., Martinez de Aragon, A., Bonay, P., Pitton, C., García, A., Silva, A., Fresno, M., Alvarez, F. and Rebollo, A. (1995) Eur. J. Immunol. 25, 2673–2678.
[27] Yavin, E., Inserte-Igual, J. and Gil, S. (1995) J. Neurochem. 65, 2594–2602.
[28] Wooten, M.R., Seibenhener, L.M., Matthews, L.H., Zhou, G. and Coleman, E.S. (1996) J. Neurochem. 67, 1023–1031.
[29] Puls, A., Schmidt, S., Grawe, F. and Stabel, S. (1997) Proc. Natl. Acad. Sci. USA 94, 6191–6196.