Involvement of phosphatidylinositol 3-kinase in nuclear translocation of protein kinase C ζ induced by C2-ceramide in rat hepatocytes

M.C. Calcerrada[a], B.G. Miguel[a], L. Martín[a], R.E. Catalán[b,*], A.M. Martínez[a]

[a] *Departamento de Bioquímica y Biología Molecular I, Facultad de Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain*
[b] *Departamento de Biología Molecular, Centro de Biología Molecular 'Severo Ochoa', Universidad Autónoma de Madrid, E-28049 Madrid, Spain*

Received 11 December 2001; revised 30 January 2002; accepted 4 February 2002

First published online 15 February 2002

Edited by Guido Tettamanti

Abstract In this study we report that protein kinase C ζ (PKC ζ), one of the atypical isoforms of the PKC family located predominantly in cytosol, is redistributed by C2-ceramide treatment in isolated hepatocytes. PKC ζ increased in membrane and nuclear fractions after 30 min of treatment with C2-ceramide in a dose- and time-dependent manner. The action of C2-ceramide was inhibited by wortmannin and LY 294002, indicating that C2-ceramide-induced PKC ζ increase in both nucleus and membrane fractions is mediated by phosphatidylinositol 3-kinase (PI3-kinase) activation. In addition, a significant translocation of PI3-kinase to the nucleus was observed after C2-ceramide treatment.

Key words: Protein kinase C ζ; Phosphatidylinositol 3-kinase; C2-ceramide; Hepatocyte

1. Introduction

Sphingolipids are found in all eukaryotic cells, where they are primarily components of the plasma membrane [1]. Evidence has emerged during the last few years that sphingolipid metabolites such as ceramide, sphingosine and sphingosine-1-phosphate play an important role as intracellular signaling molecules for a variety of different targets [2]. In rat hepatocytes, sphingosylphosphorylcholine activates phospholipase C (PLC) and inhibits adenylyl cyclase [3], and a role for the sphingomyelin pathway in liver regeneration has been proposed [4].

Although the intracellular distribution of protein kinase C (PKC) isoforms varies among tissues and cells, and no general pattern can be established, they are located in their inactive state in cytosol and they translocate, upon stimulation to the plasma membrane and/or nucleus [5]. The sphingolipid derivatives, originally proposed as negative regulators of PKC [6], may modulate the activity of different PKC isoforms. Thus, PKC ζ may be a direct target for ceramide stimulation [7,8]. Besides, it has been shown that ceramides selectively inhibit the PKC α [9] and stimulate autophosphorylation of PKC β leading to its proteolysis [10]. On the other hand, the presence and function of sphingolipid derivatives in nuclei have been described in a number of papers, e.g. the involvement of sphingolipid concentration in DNA stabilization [11] and the presence of sphingomyelinase activity in rat liver nuclei [12]. More recently, we have described an increase in calcium concentration mediated by sphingolipids in isolated rat liver nuclei [13]. All these results suggest a potential role of this class of lipids in nuclear functions.

Some evidence has revealed that the activity of phosphatidylinositol 3-kinase (PI3-kinase) was increased by sphingosine-1-phosphate [14] and C2-ceramide [15]. The enzyme, composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit, phosphorylates the 3′ position in the inositol ring of phosphoinositides, which in turn act as second messenger [16]. Recent work has revealed the existence of cross-talk between PI3-kinase and PLC–PKC signaling. Thus, the activity of PLC is enhanced by lipid products of PI3-kinase [17–19]. Receptors coupled to the inhibitory G protein Gi, such as that for lysophosphatidic acid, have been shown to activate the PKC ζ isoform through PI3-kinase activation [20,21]. Moreover, the involvement of PI3-kinase in nuclear translocation of PKC ζ has been suggested [22]. The fact that the nuclear PI3-kinase exists in both membranes and intranuclear locations suggests that it plays a diverse role in the regulation of nuclear functions [23], and that nuclear PI3-kinase and its lipid products may provide a link for the communication between cytosolic and nuclear signaling pathways.

Here we study the action of C2-ceramide on the translocation of PKC ζ isoform to nucleus and the involvement of PI3-kinase activation in isolated hepatocytes.

2. Materials and methods

2.1. Materials

C2-ceramide was obtained from ICN Biomedicals Inc. LY 294002, wortmannin, RNase A, DNase I, PI and protease inhibitors were obtained from Sigma. Collagenase was obtained from Boehringer Mannheim. [^{32}P]ATP (185 TBq/mmol) was from Amersham Iberica S.A. Nitrocellulose filters were purchased from Schleicher&Schuell. Protein-A Sepharose and ECL kit were obtained from Amersham Pharmacia Biotech. Anti-phosphotyrosine antibody (PY20), anti-mouse and anti-rabbit IgGs were purchased from Transduction Laboratories. Anti-PKC ζ antibody was obtained from Santa Cruz Biotechnology.

2.2. Isolation of rat hepatocytes

All procedures followed the guidelines of the Institutional Animal Care and Use Committee. The hepatocytes were isolated from male Wistar rats as described by Berry et al. [24]. Briefly, rats were anaesthetized with sodium pentobarbital (10 mg/100 g body weight). The liver was perfused first through the portal vein with Ca^{2+}-free Krebs Henseleit (11.7 mM NaCl, 4.6 mM KCl, 1.17 mM $MgSO_4$, 1.15 mM KH_2PO_4, 15 mM glucose, 0.7 mM EGTA, 25 mM $NaHCO_3$) that had been equilibrated with 95% O_2/5% CO_2. Then the tissue was perfused with collagenase type I, 90 UI/ml in Krebs Henseleit con-

*Corresponding author. Fax: (34)-91-3974870.
E-mail address: anamart@solea.quim.ucm.es (R.E. Catalán).

PII: S0014-5793(02)02401-8

taining 5 mM Ca^{2+} at 37°C until the liver was soft (usually 8–10 min). The liver capsule was removed and the isolated hepatocytes were shaken into fresh Krebs Henseleit containing 2.5 mM Ca^{2+}. Parenchymal cells were washed by two centrifugations at $600\times g$ for 5 min. Cell viability was always higher than 95% as assessed by trypan blue exclusion. Hepatocytes (1×10^7) were incubated at 37°C with C2-ceramide and the different inhibitors for the time indicated in each case.

2.3. Subcellular fractionation

Hepatocytes treated with C2-ceramide and the different inhibitors were resuspended in an ice-cold homogenization buffer (20 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin) and lysed with ~50 strokes with a Potter–Elvehjem homogenizer on an ice bath. Homogenates were centrifuged at $800\times g$ for 5 min at 4°C. The pellet was used to isolate nuclei and the supernatant was used to isolate cytosolic and particulate fractions.

The $800\times g$ supernatant was centrifuged at $15\,000\times g$ for 15 min at 4°C. The supernatant obtained was used as the non-nuclear fraction. In some cases this fraction was centrifuged at $100\,000\times g$ for 60 min at 4°C, and the supernatant obtained was used as the cytosolic fraction. The pellet was resuspended in a buffer containing: 50 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1% Nonidet P-40 and 0.1% 2-mercaptoethanol and was shaken at 4°C for 60 min and centrifuged at $100\,000\times g$ for 60 min at 4°C. The supernatant obtained was used as the membrane fraction as described previously [25].

The nuclear fraction was obtained by the method of Fields et al. [26]. Briefly, the $800\times g$ pellet was resuspended in STM buffer (50 mM Tris–HCl, pH 7.4, 250 mM sucrose and 5 mM $MgSO_4$) and capped over a buffer containing 2.1 mM sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$ and 1% 2-mercaptoethanol. Nuclei were successively pelleted at $70\,000\times g$ for 60 min in an SW 28 rotor. When the nuclear fraction was used for immunoblot analysis, the isolated nuclei were resuspended in STM buffer and incubated for 60 min at 4°C with DNase I and RNase A (100 μg/ml each). Nuclease-treated nuclei were sedimented at $800\times g$ for 10 min and resuspended in 50 mM Tris–HCl, pH 7.4, at 5×10^8 nuclei/ml. Marker enzymes were used in conjunction with electron microscopy to ensure that the preparations contained intact nuclei and were free of contaminating organelles, and determination of DNA content indicated a mean reactivity of approximately 20%, as described previously [27].

2.4. PKC ζ determination by Western blotting

Immunoblot analysis was carried out on cytosol, membrane and nuclear fractions basically as described previously [25]. Equal amounts of protein (100 μg) from each fraction were separated and analyzed by SDS–PAGE and then transferred to nitrocellulose membranes. After blocking non-specific sites with 0.1% (v/v) Tween-20 in 20 mM Tris–base, 137 mM NaCl adjusted to pH 7.6 with 1 M HCl, containing 1% (w/v) bovine serum albumin for 3 h at room temperature, the membranes were incubated with anti-PKC ζ antibody overnight at 4°C (1/1000 dilution). After washing, the membranes were incubated for 2 h at room temperature with horseradish peroxidase-linked secondary antibody (1/2000 dilution in a solution containing 4% skimmed milk). Bound antibody was detected by the enhanced chemiluminescence method according to the manufacturer's (Amersham) instructions. The scanned images of autoradiograms were quantified by the Sigmagel image program. Protein concentration was determined by the method of Lowry et al. [28].

2.5. PI3-kinase assay

PI3-kinase was immunoprecipitated essentially as described previously [29]. Both nuclear and non-nuclear fractions were incubated with anti-phosphotyrosine PY20 antibody (10 μg/mg protein) overnight at 4°C and then with protein-A Sepharose beads for 2 h at 4°C. Immunoprecipitates were collected by centrifugation at $10\,000\times g$ for 5 min at 4°C, then washed three times with Nonidet P-40 buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium vanadate and 1 mM PMSF), once with LiCl solution (0.5 M LiCl, 0.1 M Tris–HCl, pH 7.5), once with distilled water and once with washing buffer (0.1 M NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 7.5) and, finally, subjected to PI3-kinase assay.

PI3-kinase activity was measured by following the PIP formation from phosphorylation of PI with [γ-^{32}P]ATP basically as described by Yano et al. [29]. Briefly, immunoprecipitates were suspended in 50 μl of a buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM NaCl and 0.5 mM EGTA. Then 1 μl of 10 mg/ml phosphatidylinositol dissolved in dimethyl sulfoxide was added to the reaction mixture. The mixture was preincubated at 25°C for 10 min. [γ-^{32}P]ATP (2.5 μCi/assay, final concentration 10 μM) and $MgCl_2$ (final concentration 20 mM) were added to start the reaction, and the mixture was incubated at 25°C for 30 min. The reaction was stopped by the addition of 100 μl of chloroform, methanol, 11.6 N HCl (100:200:2). The lipid-containing organic phase was resolved on oxalate-coated thin-layer chromatography plates (Silica Gel 60) developed in chloroform/methanol/acetic acid/acetone/water (40:13:12:15:8 by volume) as described previously [30]. ^{32}P-labeled PI_3P was visualized by autoradiography and the spots were quantified by scintillation counting.

3. Results and discussion

Sphingolipid derivatives have been reported to regulate PKC action in different cell types [6–10]. To study the possible action of C2-ceramide on redistribution of PKC ζ in rat hepatocytes, in this paper we tested the effect of 35 μM C2-ceramide on PKC ζ in cytosol, membrane and nuclei for different times. In untreated hepatocytes, PKC ζ was distributed between the cytosolic (about 55%), membrane (about 35%) and nuclear (about 10%) fractions (data not shown), and after C2-ceramide treatment a notable increase of PKC ζ in nucleus and membrane was found (Fig. 1). A maximal effect was observed after 90 min of C2-ceramide treatment, about 250% of the control value (100%). Although a translocation from cytosol to both nucleus and membrane occurred, no decrease of the enzyme in cytosol was observed. The difficulty in detecting variations in the amount of PKC ζ in the cytosolic fraction may be explained by the high immunoreactivity of the enzyme observed in cytosol. After C2-ceramide treatment, there is likely to be an undetectable percentage of PKC ζ that is translocated to nucleus and membrane as described previously [31–33]. A discrepancy exists in the literature regarding whether the activation of PKC requires the translocation from the cytosol to membrane. However, this phenomenon appears to be dependent on the type of cell and the stimulus. A number of growth factors have been reported to stimulate and increase the cytosolic form of PKC and, moreover, several studies have documented that activation of PKC may result in an increase in total cellular PKC content [34]. From our results, we cannot exclude the possibility that a generalized increase of PKC ζ occurs.

Results from experiments performed at different concentrations of C2-ceramide, in a range from 5 to 35 μM, indicate that a maximal increase of PKC ζ is observed in nucleus at the highest concentration tested (Fig. 1B). It must be pointed out that higher concentrations of C2-ceramide (i.e. 50 μM) resulted in similar changes to those observed with 35 μM (data not shown). It has been reported that PKC ζ may reside in or translocate to the nucleus, in response to mitogenic signals, where it directly phosphorylates nuclear regulatory proteins or can activate downstream protein kinase cascades that modulate the gene expression [34].

PKC ζ activity can be stimulated by PI3-kinase with a negligible effect on other PKC isoforms [20,21]. To assess the contribution of PI3-kinase in C2-ceramide-induced redistribution of PKC ζ in hepatocytes, we first examined the effect of different concentrations of wortmannin, a fungal PI3-kinase inhibitor, on the effect evoked by C2-ceramide. As can be seen in Fig. 2A, wortmannin, at concentrations as low as 5 nM, partially reverses the effect evoked by 35 μM C2-ceram-

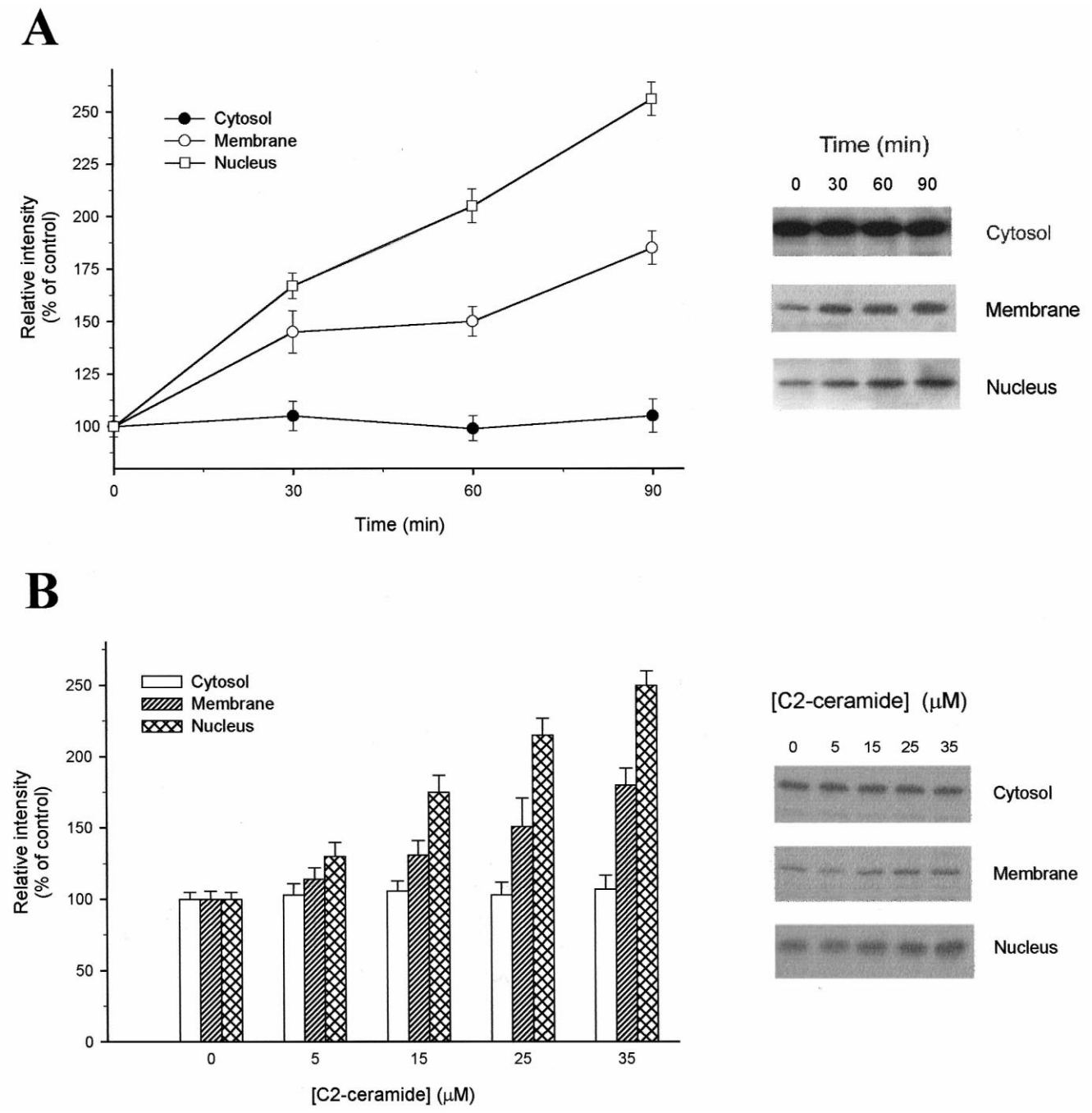

Fig. 1. Effect of C2-ceramide on subcellular localization of PKC ζ in rat hepatocytes. Isolated hepatocytes were incubated with 35 μM C2-ceramide for the indicated times (A) or with different C2-ceramide concentrations for 90 min (B), and cytosolic, membrane and nuclear fractions were separated and subjected to immunoblotting with anti-PKC ζ antibody. The results for each experiment were normalized to the density of PKC ζ of the nuclear fraction, which was arbitrary adjusted to 1.0. Basal values in arbitrary units of intensity were: 5.5, 3.5 and 1.0 for cytosol, membrane and nucleus respectively. Results are expressed as percentage of control (100) and are means ± S.E.M. of three experiments.

ide after 90 min of treatment, in both membrane and nuclear fractions. Higher concentrations of wortmannin totally inhibit C2-ceramide-induced PKC ζ increase. Bearing in mind that the effect of wortmannin may be due to PI3-kinase inhibition, we investigated whether inhibition of PI3-kinase by an alternative, unrelated compound such as LY 294002 was also able to modify the effect of C2-ceramide. As shown in Fig. 2B, LY 294002 at a concentration of 1 μM, which completely inhibits PI3-kinase in hepatocytes [35], has also an inhibitory effect on C2-ceramide-induced PKC ζ increase, similar to that of wortmannin.

To study the involvement of PI3-kinase in C2-ceramide actions further, we measured the PI3-kinase activity in both nuclear and non-nuclear fractions in isolated hepatocytes. As shown in Fig. 3A, the PI3-kinase activity, immunoprecipitated by using anti-phosphotyrosine antibody (PY20), increased in a time-dependent manner in the nuclear fraction, reaching a maximum of 2.5 times that of controls after 35 μM C2-cer-

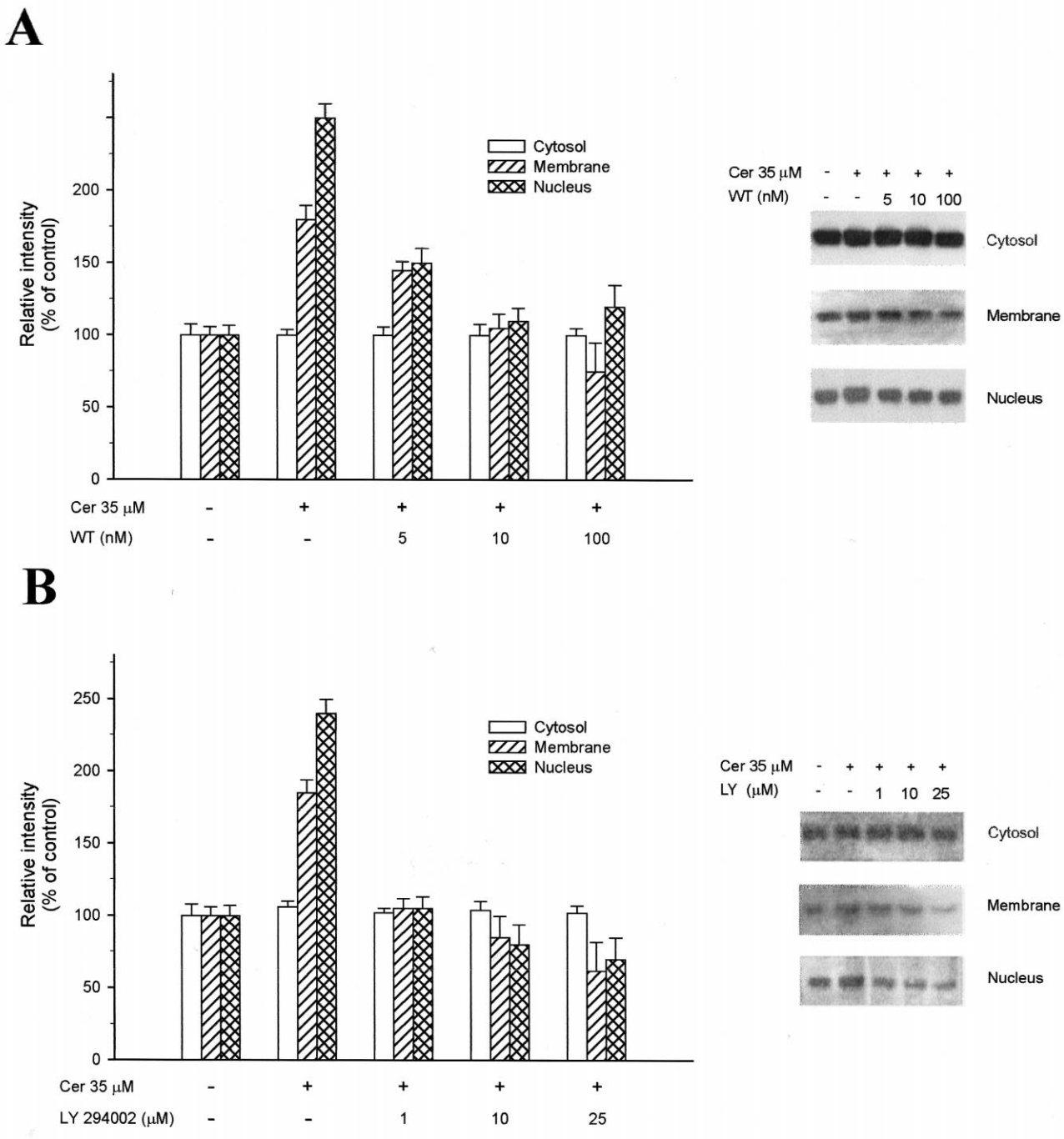

Fig. 2. Effect of PI3-kinase inhibition on C2-ceramide-induced PKC ζ translocation in rat hepatocytes. Isolated hepatocytes were incubated with 35 μM C2-ceramide (Cer) for 90 min in the presence or absence of wortmannin (WT) (A) or LY 294002 (LY) (B), and cytosolic, membrane and nuclear fractions were separated and subjected to immunoblotting with anti-PKC ζ antibody. Results are expressed as percentage of control and are means ± S.E.M. of three experiments.

amide treatment for 30 min. After longer times, a smaller increase was observed. Concomitantly, PI3-kinase activity decreased in the non-nuclear fraction, prepared as described above. As can be seen in Fig. 3B, the C2-ceramide-induced effect on PI3-kinase activity was also concentration-dependent. Thus, a concentration as low as 5 μM causes the increase of PI3-kinase activity in the nuclear fraction (about 1.2 times that of controls). These findings indicate that the activation of PI3-kinase by C2-ceramide involves the translocation of the enzyme to the nucleus. Previous studies have indicated that PI3-kinase is translocated into the nucleus after agonist stimulation, while no nuclear association of the enzyme could be detected prior to the activation [36,37]. Our finding supports the hypothesis that nuclear $PI(3,4,5)P_3$, generated by PI3-kinase activity, is the driving force for PKC ζ translocation to the nucleus. Nevertheless, in order to assess whether the reported observations are specific for C2-ceramide, studies with other forms of ceramide are required. The

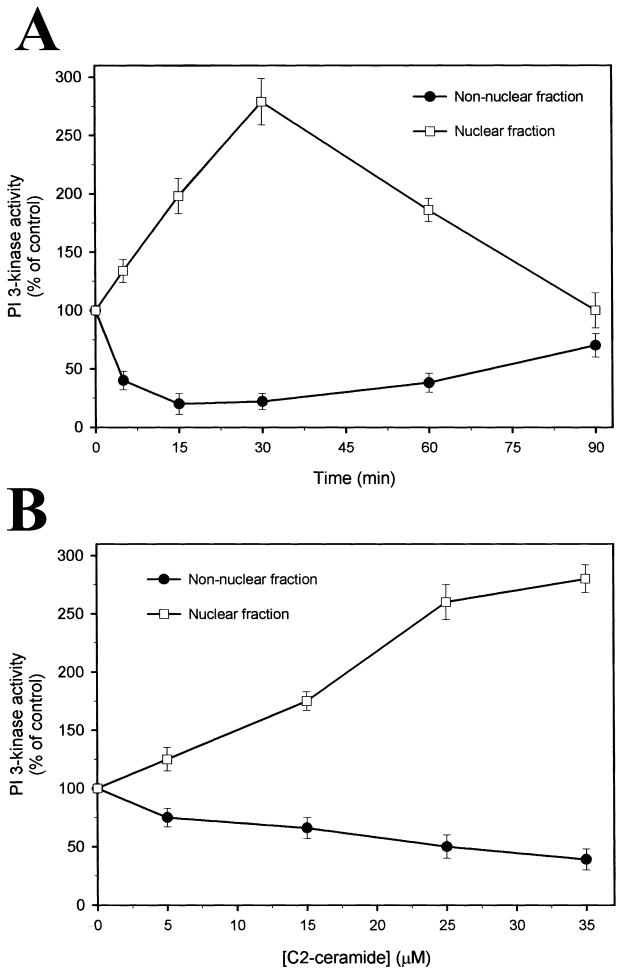

Fig. 3. Effect of C2-ceramide on nuclear PI3-kinase activity in rat hepatocytes. Isolated hepatocytes were incubated with 35 μM C2-ceramide for different times (A) or different concentrations of C2-ceramide for 90 min (B). The nuclear fraction was separated and PI3-kinase activity was measured. Basal values were: 615 and 1169 cpm/mg protein for nuclear and non-nuclear fractions respectively. Results are expressed as percentage of control (100) and are means ± S.E.M. of three experiments.

fact that nuclear PI3-kinase activation is required for C2-ceramide-induced PKC ζ translocation to the nucleus may indicate a link for the communication between cytosolic and nuclear signal pathways.

Acknowledgements: We thank Dr. M. Guzmán and J.G. Villafranca for their valuable contribution, and Miss M.V. Mora-Gil for valuable assistance. This investigation was supported in part by research grants from the DGICYT (PM97-0004), Fundación Ramón Areces, and the Universidad Complutense de Madrid, Spain.

References

[1] Barenholz, Y. and Gatt, S. (1982) Sphingomyelin: metabolism, chemical synthesis and physical properties, in: Phospholipids (Hawthorne, J.N. and Aussel, G.B., Eds.), pp. 129–177, Elsevier, Amsterdam.
[2] Spiegel, S., Foster, D. and Kolesnick, R. (1996) Curr. Opin. Cell Biol. 8, 159–167.
[3] Im, D.S., Fujioka, T., Katada, T., Kondo, Y., Ui, M. and Okajima, F. (1997) Am. J. Physiol. 272, G1091–G1099.
[4] Albi, E. and Viola Magni, M.P. (1997) Biochem. Biophys. Res. Commun. 236, 29–33.
[5] Nishizuka, Y. (1995) FASEB J. 9, 484–496.
[6] Hannun, Y.A. (1994) J. Biol. Chem. 269, 3125–3128.
[7] Lozano, J., Berra, E., Municio, M.M., Díaz-Meco, M.T., Domínguez, L., Sanz, L. and Moscat, J. (1994) J. Biol. Chem. 269, 19200–19202.
[8] Galve-Roperh, I., Haro, A. and Díaz-Laviada, I. (1997) FEBS Lett. 415, 271–274.
[9] Lee, J.Y., Hannun, Y.A. and Obeid, L.M. (1996) J. Biol. Chem. 271, 13169–13174.
[10] Tanabe, F., Cui, S.-H. and Masatriko, I. (1998) Biochem. Biophys. Res. Commun. 242, 129–133.
[11] Manzoli, F.A., Muchmore, I.H., Bonora, B., Sabioni, A. and Stefoni, S. (1972) Biochim. Biophys. Acta 277, 251–255.
[12] Neitcheva, T. and Peeva, D. (1995) Int. J. Biochem. Cell Biol. 27, 995–1001.
[13] Catalán, R.E., Miguel, B.G., Calcerrada, M.C., Ruiz, S. and Martínez, A.M. (1997) Biochem. Biophys. Res. Commun. 238, 347–350.
[14] Rakhit, S., Conway, A.T., Tate, R., Bower, T., Pyne, N.S. and Pyne, S. (1999) Biochem. J. 338, 643–649.
[15] Ibitayo, A.I., Tsunoda, Y., Nozu, F., Owyang, C. and Bitar, K.N. (1998) Am. J. Physiol. 275, G705–G711.
[16] Leevers, S.J., Vanhaesebroeck, B. and Waterfield, M.D. (1999) Curr. Opin. Cell Biol. 11, 219–225.
[17] Bae, Y.S., Cantley, L.G., Chen, C.S., Kim, S.R. and Rhee, S.G. (1998) J. Biol. Chem. 273, 4465–4469.
[18] Rameh, L.E., Rhee, S.G., Spokes, K., Kalauskar, A., Cantley, L.C. and Cantley, L.G. (1998) J. Biol. Chem. 273, 23750–23757.
[19] Falasca, M., Logan, S.K., Lehto, V.P., Baccante, G., Lemmon, M.A. and Schlessinger, J. (1998) EMBO J. 17, 414–422.
[20] Takeda, H., Matozaki, T., Takada, T., Noguchi, T., Yamao, T., Tsudo, M., Ochi, F., Fukunaga, K., Inagaki, K. and Kasuga, M. (1999) EMBO J. 18, 386–395.
[21] Lavoie, L., Band, C.J., Kong, M., Bergeron, J.J.M. and Posner, B.I. (1999) J. Biol. Chem. 274, 28279–28285.
[22] Mizukami, Y., Hirata, T. and Yoshida, K. (1997) FEBS Lett. 401, 247–251.
[23] Lu, P.-J., Hsu, A.-L., Wang, D.-S., Yan, H.Y., Yin, H.L. and Chen, C.-S. (1998) Biochemistry 37, 5738–5745.
[24] Berry, M.N., Barritt, G.J. and Edwards, A.M. (1991) Isolated hepatocytes. Preparation, properties and applications, in: Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 21 (van der Vliet, P.C. and Pillai, S., Eds.), Elsevier, Amsterdam.
[25] Miguel, B.G., Calcerrada, M.C., Mata, F., Aller, P., Clemente, R., Catalán, R.E. and Martínez, A.M. (2000) Biochem. Biophys. Res. Commun. 274, 596–602.
[26] Fields, A.R., Pettit, G.R. and May, N.S. (1988) J. Biol. Chem. 263, 8253–8260.
[27] Calcerrada, M.C., Miguel, B.G., Catalán, R.E. and Martínez, A.M. (1999) Neurosci. Res. 33, 229–232.
[28] Lowry, O.H., Rosebrough, N.J., Farr, A. and Randall, N.I. (1955) J. Biol. Chem. 193, 265–275.
[29] Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y. and Yuzuru, M. (1993) J. Biol. Chem. 268, 25846–25855.
[30] Catalán, R.E., Martínez, A.M., Aragonés, M.D., Lombardía, M. and Garde, E. (1995) Eur. J. Pharmacol. 290, 183–188.
[31] Dang, P.M.C., Rais, S., Hakim, J. and Périanin, A. (1999) Biochem. Biophys. Res. Commun. 212, 664–672.
[32] Clerk, A., Bogoyevitch, M.A., Andersson, M.B. and Sugden, P. (1994) J. Biol. Chem. 269, 32848–32857.
[33] Puceat, M., Hilal-Dandan, R., Strulovici, B., Bonton, L.L. and Brawn, J.H. (1994) J. Biol. Chem. 269, 16938–16944.
[34] Zhou, G., Wooten, M.W. and Coleman, E.S. (1994) Exp. Cell Res. 214, 1–11.
[35] Wagle, A., Jivraj, S., Garlock, G.L. and Stapleton, S.R. (1998) J. Biol. Chem. 273, 14968–14974.
[36] Neri, L.M., Milani, D., Bertolaso, L., Stroscio, M., Bertagnolo, V. and Capitani, S. (1994) Cell. Mol. Biol. 40, 619–626.
[37] Neri, L.M., Martelli, A.M., Borgatti, P., Colamussi, M.L., Marchisio, M. and Capitani, A.S. (1999) FASEB J. 13, 2299–2310.