Volume 296, number 2, 166–168 FEBS 10589 January 1992

Oleoylamine and sphingosine stimulation of phosphatidylserine synthesis by LA-N-2 cells is protein kinase C independent

Indrapal N. Singh[1], Giuseppe Sorrentino[2], Raphael Massarelli[3] and Julian N. Kanfer[1]

[1]*Department of Biochemistry, University of Manitoba, Winnipeg, Canada,* [2]*Instituto Di Scienze Neurologiche, Napoli, Italy* and [3]*CNRS Centre de Neurochimie, Strasbourg, France*

Received 14 November 1991

The presence of sphingosine and oleoylamine in the culture medium of LA-N-2 cells stimulated the incorporation of [^{3}H]serine into its corresponding phospholipid, phosphatidylserine (PtdSer). The optimum stimulation for sphingosine and oleoylamine were 50 μM and 100 μM, respectively. Oleoylamine increased the incorporation of [^{3}H]serine over 6-fold while sphingosine increased the incorporation of [^{3}H]serine over 2.5-fold. The amount of radioactivity found in water-soluble components and in protein was similar to that found with control LA-N-2 cells. The incorporation of [^{3}H]choline and [^{3}H]ethanolamine into their corresponding phospholipids were decreased in the presence of either oleoylamine or sphingosine. A protein kinase C (PKC) activator, DiC8, and a PKC inhibitor, H7, did not influence the enhanced phosphatidylserine formation by sphingosine and oleoylamine. In addition, there were no differences in the stimulatory effect of sphingosine and oleoylamine discernable between PKC down-regulated cells or controls. These observations indicate that this oleoylamine and sphingosine mediated enhanced phosphatidylserine synthesis is PKC-independent.

Serine base exchange enzyme; Phosphatidylserine; Sphingosine; Oleoylamine; LA-N-2 cell; PKC-independent

1. INTRODUCTION

Interest in the possible functions of long-chain bases has received impetus because of their myriad effects found in vitro and with various cell cultures [1–3]. Free long-chain bases structurally related to sphingosine are potent inhibitors of protein kinase C (PKC) activity both with intact cells and in vitro [2–4]. However, there are numerous examples of non-PKC related effects of sphingosine [5–12].

We have recently found that sphingosine can increase the serine base exchange enzyme activities of rat brain membrane particles without effecting either the ethanolamine or choline base exchange enzyme activities [13]. We have also shown that TPA increases the incorporation by LA-N-2 cells of radioactive choline as well as radioactive ethanolamine and L-serine into their corresponding phospholipids [14]. In contrast, Kiss et al. reported that TPA treatment of HL 60 cells inhibited the incorporation of radioactive serine into phosphatidylserine by 30% [15]. Stimulation of Jurkat T cells with anti-CD3 or anti-TCR antibodies of PHA decreased the incorporation of radioactive serine into phosphatidylserine [16]. This report demonstrates that the oleoylamine and sphingosine stimulated phosphatidylserine synthesis in LA-N-2 cells is by a PKC-independent process.

Correspondence address: J.N. Kanfer, Department of Biochemistry and Molecular Biology, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Canada R3E 0W3.

2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

L-[^{3}H(G)]serine (29.8 Ci/mmol) and [^{3}H-methyl]choline chloride (81.8 Ci/mmol) were purchased from DuPont-New England Nuclear, Boston, MA. [1-^{3}H]Ethan-1-ol-2-amine hydrochloride (28.8 Ci/mmol) was purchased from Amersham Canada Limited, Ontario, Canada. Falcon T-25 flasks, Leibovitz's L-15 medium, and heat-inactivated fetal calf serum were obtained from Flow Labs. Serine-free L-15 medium was prepared in this laboratory. D-sphingosine, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), L-1,2-dioctanoylglycerol (DiC8) were obtained from Sigma Chemical Co., St.Louis, MO. Oleoylamine was obtained from Fluka Chemical Co., Switzerland. Lipid standards were from Serdary Research Laboratories, London, Ontario.

2.2. *Cell culture*

The human neuroblastoma cell line, LA-N-2 (passage 81) was obtained from Dr. Robert Seeger, University of California, Los Angeles. The maintainance and growth conditions for this cell line were as previously described by Singh et al. [17].

2.3. *Cell labeling and analyses of radiolabeled lipid and water-soluble components*

Confluent monolayer cultures of LA-N-2 cells were incubated in triplicate in serine-free L-15 medium with [^{3}H]serine (1.2 μCi/ml) in the absence or presence of various agents for 1 h as listed in the individual tables and figure. The cells were harvested, rinsed and the cell pellets were subjected to the Folch extraction procedure [18] to obtain hydrophilic and hydrophobic components. The insoluble material was taken as proteinaceous. Lipids were separated on silica gel G 60 plates with a solvent system composed of chloroform/methanol/conc. ammonium

Published by Elsevier Science Publishers B.V.

hydroxide (65:25:4, v/v/v) employing authentic phospholipid standards. The plates were air-dried and processed for autoradiography using Kodak-X-O-mat film. Each experiment was conducted on at least two seperate occasions.

2.4. *PKC down-regulation*

Confluent LA-N-2 cell cultures were incubated overnight in the presence of 100 nM TPA in serum-free L-15 medium. This medium was removed and the cells were rinsed three times with L-15 medium and utilized for the experiments as described in Table II.

2.5. *Protein determination*

Protein was determined according to the method of Lowry et al. [19] using bovine serum albumin as the standard.

2.6. *Statistical analysis*

Statistical significance was assessed using the Student's *t*-test.

3. RESULTS

3.1. *The effects of sphingosine and oleoylamine on the incorporation of [^{3}H]serine into phosphatidylserine*

LA-N-2 cell cultures were incubated with [^{3}H]serine in the presence of varying concentrations of oleoylamine and sphingosine. There was a progressive dose-dependent increase in the incorporation of radioactive serine into phosphatidylserine as compared to control cultures (Fig. 1). A maximal 6-fold increase ($P<0.001$) of [^{3}H]serine incorporation into phosphatidylserine was found at 100 μM oleoylamine. A maximal 2.5-fold increase ($P<0.025$) of [^{3}H]serine incorporation into phosphatidylserine was found to be at 50 μM sphingosine.

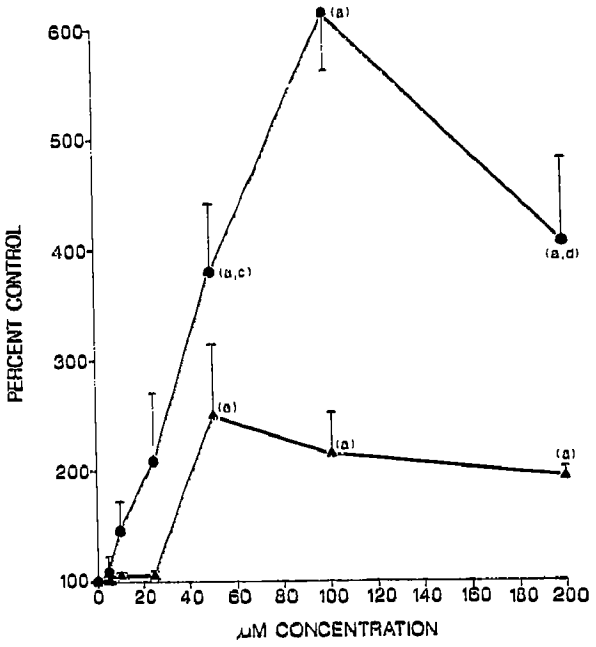

Fig. 1. The effect of varying the concentrations of oleoylamine (circles) and sphingosine (triangles) upon the incorporation of [^{3}H]serine into phosphatidylserine by LA-N-2 cell cultures. The letter designations are: (a) vs 5 μM, P = 0.001; (b) vs 5 μM, P = 0.005; (c) vs 100 μM, P = 0.005; (d) vs 100 μM, P = 0.01; (e) vs 5 μM, P = 0.025.

Radioautograms of TLC of the lipids showed that the principal component was phosphatidylserine with traces of phosphatidylethanolamine. There were no other radioactive compounds detected.

3.2. *Comparative effects of oleoylamine and sphingosine on the incorporation of [^{3}H]choline and [^{3}H]ethanolamine into various constituents*

It seemed useful to determine if the effect of oleoylamine and sphingosine under these experimental circumstances on the LA-N-2 cells was restricted only to the incorporation of [^{3}H]serine into phosphatidylserine. These two amines were incubated with [^{3}H]ethanolamine and [^{3}H]choline and the results obtained on the incorporation into their corresponding phospholipids, and of [^{3}H]ethanolamine into proteins are presented in Table I. Sphingosine inhibited the incorporation of [^{3}H]choline and [^{3}H]ethanolamine into their corresponding phospholipids by 13% and 64%, respectively. Oleoylamine inhibited the incorporation of [^{3}H]choline and [^{3}H]ethanolamine into their corresponding phospholipids by 32% and 56%, respectively.

3.3. *Possible PKC involvement in this phenomenon*

Some of the effects of sphingosine may be a PKC-dependent phenomenon and we wished to determine if the oleoylamine stimulation of [^{3}H]serine appearance in phosphatidylserine was PKC-dependent. This was investigated by exposing LA-N-2 cells to 100 nM TPA overnight, an accepted condition for down-regulation of PKC activity [5]. TPA pretreatment did not effect the incorporation of [^{3}H]serine into phosphatidylserine by LA-N-2 cells either in the presence or absence of either sphingosine or oleoylamine compared to untreated cells

Table I

Effect of 50 μM sphingosine and 100 μM oleoylamine upon the incorporation of labeled serine, ethanolamine and choline into cellular constituents by LA-N-2 cell culture*

Conditions				
Labeled precursor	Sphingosine	Oleoylamine	Lipids**	Proteins**
[^{3}H]Choline	–	–	70,733± 2,518	–
	+	–	61,652± 4,963	–
	–	+	48,246± 2,081	–
[^{3}H]Ethanolamine	–	–	1,902,499±506,262	1,231,908±142,037
	+	–	697,185± 21,598	1,999,499±223,298
	–	+	836,156±172,561	1,606,213±443,062
[^{3}H]Serine	–	–	93,717± 2,629	397,976± 46,957
	+	–	145,417± 18,806	461,666±165,107
	–	+	452,138± 34,454	298,646± 71,931

*Where indicated either 1.5 μCi/ml for [^{3}H]choline, 1.2 μCi/ml of [^{3}H]ethanolamine or [^{3}H]serine were present.

**Values expressed as dpm/mg protein/h. Each value is the average ± SD for 3 separate flasks of LA-N-2 cells and represent a typical experiment.

Table II

Effect of PKC down-regulation, a PKC inhibitor and a PKC activator upon the oleoylamine and sphingosine stimulation of labeled serine incorporation into phosphatidylserine by LA-N-2 cell culture*

Treatment	Lipids**	Proteins**
Series A		
Control cells, no addition	91,473 ± 20,031	896,015 ± 128,736
Control cells + 100 μM oleoylamine	277,646 ± 49,784	450,316 ± 34,986
PKC down-regulated, no addition	112,024 ± 27,846	1,243,824 ± 72,337
PKC down-regulated + 100 μM oleoylamine	251,319 ± 55,497	796,442 ± 94,984
Control cells + 100 μM DiC_8	71,514 ± 18,278	713,340 ± 59,720
Control cells + 100 μM DiC_8 + 100 μM oleoylamine	188,680 ± 42,410	495,844 ± 57,537
Control cells + 20 μM H7	82,829 ± 18,452	728,569 ± 113,425
Control cells + 20 μM H7 + 100 μM oleoylamine	206,030 ± 57,336	450,353 ± 77,538
Series B		
Control cells, no additions	82,158 ± 21,916	678,759 ± 45,402
Control cells + 50 μM sphingosine	151,020 ± 30,750	507,467 ± 94,109
PKC down-regulated, no additions	55,267 ± 15,605	918,419 ± 53,605
PKC down-regulated + 50 μM sphingosine	125,803 ± 22,098	600,215 ± 91,024
Control cells + 100 μM DiC_8	54,634 ± 14,915	478,649 ± 52,846
Control cells + 100 μM DiC_8 + 50 μM sphingosine	146,728 ± 58,839	295,982 ± 96,905
Control cells + 20 μM H7	81,571 ± 20,928	743,323 ± 64,815
Control cells + 20 μM H7 + 50 μM sphingosine	165,487 ± 18,857	349,313 ± 115,927

*PKC downregulated cells remained for 18 h in medium containing 100 nM TPA.
**All values expressed as DPM/mg protein/h and are derived from 2 independent experiments with each treatment carried out in triplicate.

(Table II). A PKC activator 1,2-dioctanoylglycerol (DiC8) and a PKC inhibitor H7 were utilized in order to confirm that this was a PKC-independent process. These compounds did not effect the incorporation of [^{3}H]serine into phosphatidylserine either in the presence or absence of either sphingosine or oleoylamine. However, there were some reductions in protein labeling.

4. DISCUSSION

This investigation with LA-N-2 cultures has provided experimental evidence demonstrating: (1) sphingosine and oleoylamine stimulated the incorporation of [^{3}H]serine into phosphatidylserine (Fig. 1); (2) these amines do not have a similar effect upon [^{3}H]choline or [^{3}H]ethanolamine incorporations into their respective phospholipids (Table I); (3) this sphingosine and oleoylamine-mediated increased synthesis of phosphatidylserine occurs by a PKC-independent process (Table II).

It is conceivable that some of the non-PKC dependent phenomenon described by others [5–12] provoked by sphingosine may be mediated through enhanced phosphatidylserine formation because of enhanced serine base exchange enzyme activity. Indeed, it is also conceivable that some of the PKC-dependent events provoked by sphingosine might also be partly ascribable to enhanced formation of phosphatidylserine, the preferred PKC activator.

Acknowledgements: Supported by grants from the Medical Research Council of Canada.

REFERENCES

[1] Hannun, Y. and Bell, R.M. (1987) Science 236, 670–674.
[2] Hannun, Y. and Bell, R.M. (1989) Science 243, 500–507.
[3] Merril Jr., A.H. and Stevens, V.L. (1989) Biochim. Biophys. Acta 1010, 131–139.
[4] Hannun, Y., Loomis, C.R., Merril Jr., A.H. and Bell, R.M.(1986) J. Biol. Chem. 261, 12604–12609.
[5] Zhang, Z., Buckley, N.E., Gibson, K. and Spiegel, S. (1990) J. Biol. Chem. 265, 76–81.
[6] Faucher, M., Girones, N., Hannun, Y., Bell, R.M. and Davis, R.J. (1988) J. Biol. Chem. 263, 5319–5327.
[7] Davis, R.J., Girones, N. and Faucher, M. (1988) J. Biol. Chem. 263, 5373–5379.
[8] Jefferson, A.B. and Schulman, H. (1988) J. Biol. Chem. 263, 15241–15244.
[9] Winicov, I. and Gershengorn, M.C. (1988) J. Biol. Chem. 263, 12179–12182.
[10] Krishanmurti, S., Patel, Y. and Kakkar, V.V. (1989) Biochim. Biophys. Acta 1010, 258–264.
[11] Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K. and Racker, E. (1989) Biochemistry 28, 6796–6800.
[12] Fiore, S., Nicolaou, K.C., Caulfield, T., Kataoka, A.H. and Serhan, C.N. (1990) Biochem. J. 266, 25–31.
[13] Kanfer, J.N. and McCartney, D.G., FEBS Lett., in press.
[14] Singh, I., McCartney, D.G., Massarelli, R. and Kanfer, J.N. (1991) International Symposium and NATO Workshop on 'Phospholipids and Signal Transmission', Wiesbaden, Germany, S31, 1991.
[15] Kiss, Z., Deli, E. and Kuo, J.F. (1987) Biochem. J. 248, 649–656.
[16] Pelassy, C., Mary, D. and Aussel, C. (1991) Cellular Signalling 3, 35–40.
[17] Singh, I.N., Sorrentino, G., McCartney, D.G., Massarelli, R. and Kanfer, J.N. (1990) J. Neurosci. Res. 25, 476–485.
[18] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
[19] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.H. (1951) J. Biol. Chem. 193, 265–275.