Volume 288, number 1,2, 37–40 FEBS 10037 August 1991

ADONIS 001457939100748F

Protein kinase C of a human megakaryoblastic leukemic cell line (MEG-01)

Analysis of subspecies and activation by diacylglycerol and free fatty acids

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Received 8 June 1991

Protein kinase C (PKC) from a human megakaryoblastic leukemic cell line (MEG-01) was resolved into two fractions by hydroxyapatite column chromatography, which are indistinguishable from the brain type II (βI/βII) and type III (α) subspecies, by biochemical and immunoblot analysis. In the presence of both phosphatidylserine and diacylglycerol, several free unsaturated fatty acids (FFA's), such as arachidonic, oleic, linoleic and linolenic acids, further enhanced the enzyme activation, and allowed the enzyme to exhibit almost full activity at nearly basal levels of Ca^{2+} concentration. The concentration of unsaturated FFA's giving rise to the maximum enzyme activation was around 2×10^{-5} M. Palmitic and stearic acids were inactive. The result implies that, in addition to diacylglycerol, the receptor-mediated release of unsaturated FFA's from membrane phospholipids may also take part in the activation of PKC.

Protein kinase C; Arachidonic acid; Megakaryocyte; MEG-01 cell

1. INTRODUCTION

The signal-dependent hydrolysis of inositol phospholipids, particularly phosphatidylinositol bisphosphate, catalyzed by phospholipase C is generally accepted to be a key event for initiating cellular responses [1,2]. Recent studies suggest that the receptor-mediated hydrolysis of phosphatidylcholine (PC) also plays a role in cellular responses, including cell proliferation and differentiation [3]. In fact, both phospholipase A_2 and phospholipase D appear to be involved in the PC hydrolysis, which is often observed at a relatively later phase of cellular responses [3–5]. It is becoming plausible that sustained activation of protein kinase C (PKC) is necessary, especially for long-term responses; for instance, T-lymphocyte activation [6] and HL-60 cell differentiation [7–9]. Since the activation of PKC due to the inositol phospholipid hydrolysis is normally transient, DAG [3] and unsaturated free fatty acids (FFA's) [10–15] which may be derived from PC hydrolysis have been postulated as second messengers for PKC activation.

In a human megakaryocytic cell line, MEG-01, the β-subspecies of PKC is shown to be a predominant form by immunoblot analysis [16]. A potential role of PKC in the differentiation of megakaryocytes has been suggested by Ogura et al. [17], who have shown that a tumor-promoting phorbol ester stimulates MEG-01 cells to differentiate into more mature megakaryocytes, in several morphological and biochemical criteria. In human platelets, on the other hand, there are two PKC subspecies, one of which is indistinguishable from the α-subspecies of the brain PKC, whereas the other is kinetically different from, but resembles, the β-subspecies [18]. To explore the potential role of PKC activation during differentiation of MEG-01 cells to undergo terminal maturation, the present studies were made to identify the PKC subspecies in this blastic cell line, and show that the enzyme can be activated by synergistic action of DAG and unsaturated FFA's.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

[γ-^{32}P]ATP was a product of New England Nuclear. Calf thymus H1 histone was prepared as described [19]. Phospholipids, DAG and all FFA's were purchased from Serdary Research Laboratories. MEG-01 cells were kindly donated by Dr. Saito, Nagoya University, Japan.

The data are taken in part from the dissertation that will be submitted by Y. Kitagawa to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Science.

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Abbreviations: PKC, protein kinase C; PS, phosphatidylserine; DAG, diacylglycerol; FFA, free fatty acid; EGTA, ethyleneglycol bis(2-aminoethylether) tetraacetic acid; EDTA, ethylenediaminetetraacetic acid

2.2. *Cell culture*

The human megakaryoblastic leukemic cell line, designated MEG-O1, has been established by Ogura et al. [20]. The cells were grown in RPMI 1640 medium (Flow Laboratories) supplemented with 10% fetal bovine serum (Whittaker), penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells, cultured for 24 h on dishes at 1-2 × 10^6/ml in a total of 100 ml medium, were used. The basal PKC activity did not vary with cell density over the range described.

2.3. *Preparation of PKC subspecies*

All procedures were carried out at 4°C. MEG-O1 cells (1-2 × 10^8 cells) were suspended in 4 ml of 20 mM Tris-HCl (pH 7.5) containing 1 mM EGTA, 1 mM EDTA, 0.25 sucrose, 1 mM phenylmethylsulfonyl fluoride and 0.4 mM leupeptin. The cells were lysed by sonication for 6 × 10 s, and the homogenate was centrifuged for 60 min at 100 000 × *g*. The supernatant was applied to a DEAE-cellulose column (column volume 4 ml, Whatman) which was equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA and 10 mM 2-mercaptoethanol (buffer A). After washing the column with 20 ml buffer A, PKC was eluted with 10 ml buffer A containing 0.5 M NaCl. The sample was applied to a packed hydroxyapatite column (0.78 × 15 cm, Koken Ltd, Tokyo), connected to an FPLC system (Pharmacia) that was equilibrated with 20 mM potassium phosphate (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol (buffer B). The column was washed with 24 ml buffer B, and PKC was eluted by application of a linear concentration gradient of potassium phosphate (20 mM to 200 mM) in 84 ml buffer B at a flow rate of 0.4 ml/min. Fractions of 1 ml each were collected, dialyzed against buffer A containing 10% (v/v) glycerol, and stored at -80°C. The rat brain PKC subspecies were prepared as described [12].

2.4. *Assay of PKC*

The enzyme activity was assayed by measuring the incorporation of $^{32}P_i$ into calf thymus H1 histone from [γ-^{32}P]ATP. The reaction mixture (final volume, 0.25 ml) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (0.5-1 × 10^2 cpm/pmol), 50 μg H1 histone and other chemicals including $CaCl_2$ (10^{-6} M), PS (8 μg/ml), DAG (0.8 μg/ml), FFA's (20 μM) and enzyme fraction unless otherwise indicated. PS and DAG were mixed first in chloroform and dried under nitrogen. The residue was then resuspended in 20 mM Tris-HCl (pH 7.5) by vortexing and sonication on ice for 3 × 1 min under nitrogen. FFA normally dissolved in ethanol was diluted with 20 mM Tris-HCl (pH 7.5), sonicated as above, and added to the reaction mixture directly. The reaction was started by the addition of enzyme. After incubation for 3 min at 30°C, the acid-precipitable materials were collected on a nitrocellulose filter. The radioactivity was quantitated using a scintillation spectrometer by Cerenkov counting as described [12]. One unit of PKC was defined as the amount of enzyme that incorporates 1 nmol of $^{32}P_i$ from [γ-^{32}P]ATP into H1 histone per min under the conditions described above.

2.5. *Immunoblot analysis*

Immunoblot analysis was carried out as described [21]. The PKC fractions of hydroxyapatite column chromatography were subjected to SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose filter. The filter was reacted with the PKC subspecies-specific antibodies, and stained by the avidin-biotin peroxidase complex method. The antibodies were raised against the synthetic oligopeptides which are parts of the deduced amino acid sequence of PKC [21]. The antibodies, CKpV1 β-a and CKpV5 α-a, reacted specifically with β (β1/β11)- and α-PKC, respectively.

3. RESULTS

3.1. *Isolation of PKC subspecies*

PKC from MEG-O1 cells was resolved into two distinct fractions (peaks a and b) upon hydroxyapatite column chromatography (Fig. 1A). The peak a and b

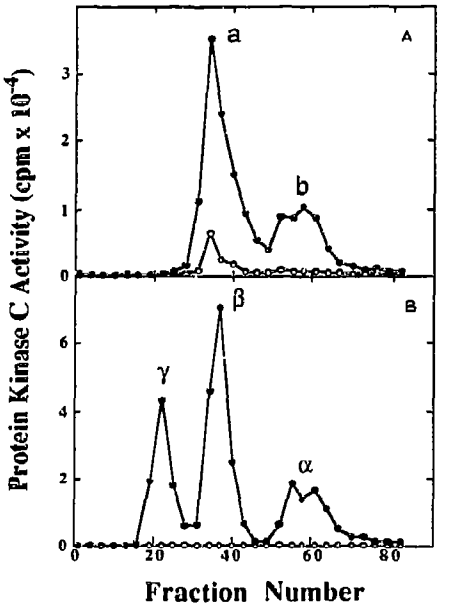

Fig. 1. Chromatographic profiles of PKC subspecies of MEG-O1 cells and rat brain. The sample from the DEAE-cellulose column was subjected to hydroxyapatite column chromatography and assayed as described in section 2. A. PKC of MEG-01 cells; B. PKC of the rat brain. (●—●), in the presence of 0.2 mM $CaCl_2$, 8 μg/ml PS and 0.8 μg/ml DAG; (○—○), in the presence of 2.5 mM EGTA instead of $CaCl_2$, PS and DAG.

enzymes were eluted at the positions corresponding to the rat brain type II (β) and type III (α) PKC, respectively (Fig. 1B). Both enzymes showed an approximate molecular weight of 80 kDa as estimated by SDS-polyacrylamide gel electrophoresis. Immunoblot analysis indicated that the peak a enzyme (fraction number 31-37) reacted with the antibody specific to the brain β-PKC, and the peak b enzyme (fraction number 52-61) reacted with the antibody specific to the brain α-PKC (Fig. 2). Both enzymes did not react with the antibodies specific to the PKC subspecies having the δ-, ε- or ζ-sequence.

3.2. *Synergistic action of DAG and arachidonic acid*

The peak a and b enzymes were activated by DAG in the presence of PS at the micromolar range of Ca^{2+}, and PS alone activated both enzymes only at higher concentrations of Ca^{2+}, around 10^{-4} M (Fig. 3A and B). DAG alone was inactive. The activity of both enzymes was greatly enhanced further by arachidonic acid in addition to DAG and PS (Fig. 3C and D). The stimulatory actions of DAG and arachidonic acid were synergistic, and more predominant at lower Ca^{2+} concentrations. Both peak a and b enzymes exhibited almost full activity at nearly basal levels of Ca^{2+} (Fig. 3C and D). In the absence of PS, arachidonic acid and DAG slightly activated both enzymes. The arachidonic acid alone showed some stimulatory effect on the peak b enzyme. The concentration of arachidonic acid giving

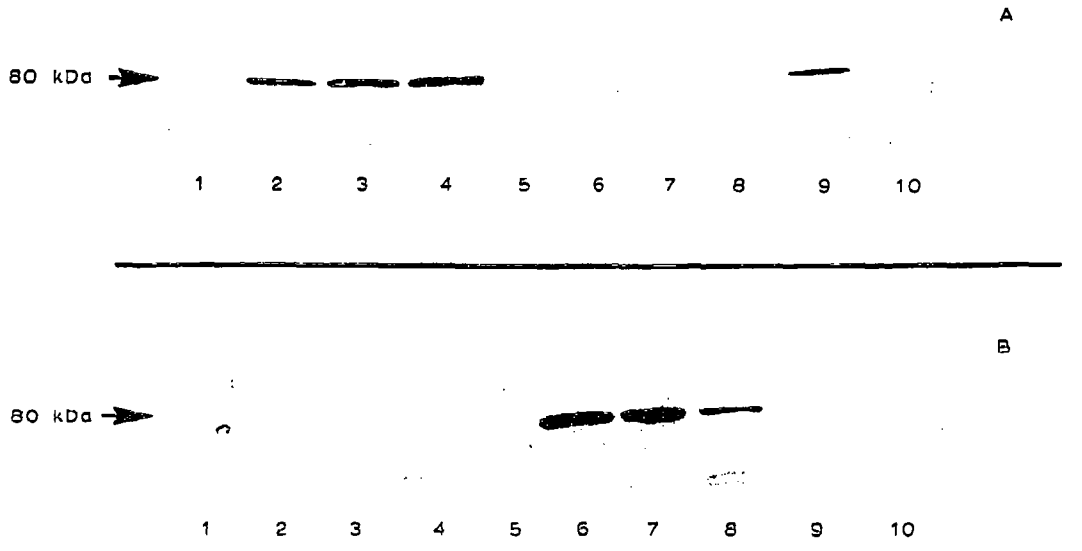

Fig. 2. Immunoblot analysis of PKC fractions of MEG-01 cells. The PKC fractions of hydroxyapatite column chromatography were subjected to immunoblot analysis as described in section 2. A. Immunoblot analysis with polyclonal antibody, CKpV1 β-a, which recognized specifically the β-subspecies. B. Immunoblot analysis with polyclonal antibody, CKpV5 α-a, which recognized specifically the α-subspecies. Lane 1, 2, 3, 4, 5; fraction number 28, 31, 34, 37, 43, respectively (peak a). Lane 6, 7; fraction number 52–56, 57–61, respectively (peak b). Lane 8, 9, 10; authentic samples of α-, β- and γ-PKC from the rat brain, respectively.

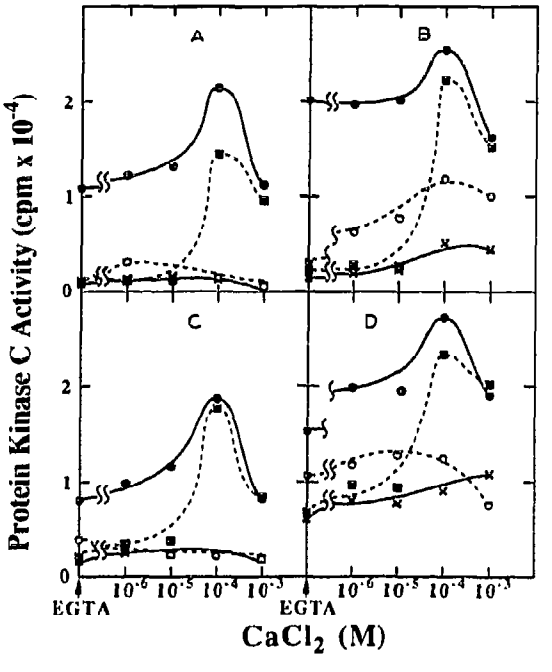

Fig. 3. Activation of PKC fractions by arachidonic acid in the presence of PS, DAG and various concentrations of Ca^{2+}. Each PKC fraction from MEG-01 cells (approximately 0.015 Unit) was assayed with H1 histone as substrate under the conditions described in section 2, except that various concentrations of $CaCl_2$ were added in the absence (A, B) or presence (C, D) of 20 μM arachidonic acid. Where indicated with arrows, 2.5 mM EGTA was added instead of $CaCl_2$. A and C, peak a; B and D, peak b. (●—●), in the presence of PS and DAG; (O···O), in the presence of DAG; (■···■), in the presence of PS; (×···×), in the absence of PS and DAG.

rise the maximum effect in the presence of PS and DAG was 2×10^{-5} M (Fig. 4). Higher concentrations of arachidonic acid were rather inhibitory.

3.3. *Specificity of fatty acid*

The synergistic action of DAG and arachidonic acid

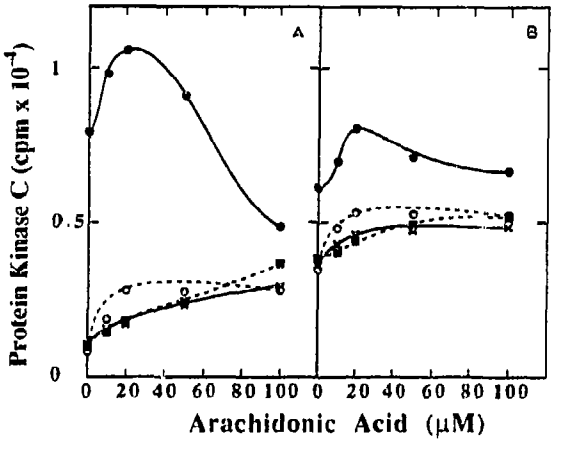

Fig. 4. Activation of PKC fractions by arachidonic acid at various concentrations in the presence of PS, DAG and Ca^{2+}. Each PKC fraction from MEG-01 cells (approximately 0.015 Unit) was assayed with H1 histone as substrate under the conditions described in section 2, except that various concentrations of arachidonic acid were added as indicated. A, peak a; B, peak b. (●—●), in the presence of PS and DAG; (O···O), in the presence of DAG; (■···■), in the presence of PS; (×···×), in the absence of PS and DAG.

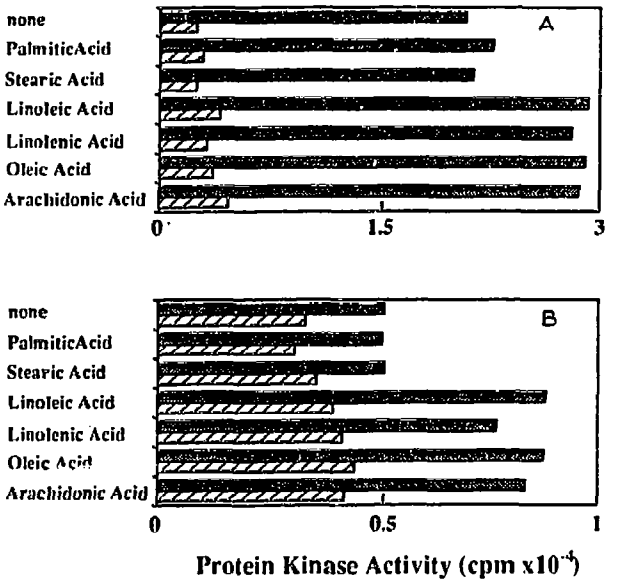

Fig. 5. Activation of PKC fractions by various FFA's in the presence of PS, DAG and Ca^{2+}. Each PKC fraction from MEG-01 cells (approximately 0.015 Unit) was assayed with H1 histone as substrate under the conditions described in section 2, except that various fatty acids (20 μM each) were added as indicated. A, peak a; B, peak b.

on the enzyme activation described above was observed also for many other unsaturated FFA's, such as linoleic, linolenic and oleic acids (Fig. 5). Palmitic and stearic acids were inactive. The stimulatory effect of DAG and unsaturated FFA was more remarkable for the peak b than for the peak a enzyme.

4. DISCUSSION

MEG-01 cells contain two PKC enzymes, corresponding the brain α- and β-subspecies. Kinetic analysis with H1 histone as phosphate acceptor reveals that both enzymes respond well to PS and DAG, and are sensitive to Ca^{2+}. On the other hand, human platelets do not appear to express the β-subspecies but contain, in addition to the α-subspecies, a structurally unidentified PKC subspecies which does not depend on Ca^{2+} but responds only partially to PS and DAG [18]. Presumably, these PKC subspecies play distinctly different roles during the differentiation of megakaryoblasts to platelets.

Both PKC enzymes found in MEG-01 cells were activated significantly by the simultaneous addition of DAG and unsaturated FFA's in the presence of PS. Unsaturated FFA's together with DAG increased an apparent affinity of the PKC for Ca^{2+}, and activated the enzyme almost fully at the basal level of Ca^{2+} concentrations. Although the physiological significance of this synergistic action of unsaturated FFA's and DAG remains to be explored further, it is possible that the PKC once activated by the hydrolysis of inositol phospholipids remains active even after the Ca^{2+} concentration returns to the basal level, when DAG and unsaturated FFA's are both available. Perhaps, several phospholipase activated in a signal-dependent manner may take part in cell growth and differentiation that apparently require sustained activation of PKC.

Acknowledgements: The skillful secretarial assistance of Ms. S. Nishiyama, Y. Kimura and Y. Yamaguchi are cordially acknowledged. This work was supported in part by research grants from the Specil Research Fund of Ministry of Education, Science and Culture, Japan; Muscular Dystrophy Association, U.S.; Juvenile Diabetes Foundation International, U.S.; Yamanouchi Foundation for Research on Metabolic Disorders; Sankyo Foundation of Life Science, Merck, Sharp and Dohme Research Laboratories; Biotechnology Laboratories of Takeda Chemical Industries; and New Lead Research Laboratories of Sankyo Company.

REFERENCES

[1] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[2] Nishizuka, Y. (1984) Nature 308, 693–698.
[3] Exton, J.H. (1990) J. Biol. Chem. 265, 1–4.
[4] Axelrod, J., Burch, R.M. and Jelsema, C.L. (1988) Trends Neurosci. 11, 117–123.
[5] Martin, T.W. and Michaelis, K. (1989) J. Biol. Chem. 264, 8847–8856.
[6] Berry, N., Ase, K., Kishimoto, A. and Nishizuka, Y. (1990) Proc. Natl. Acad. Sci. USA 87, 2294–2298.
[7] Ebeling, J.G., Vandenbark, G.R., Kuhn, L.J., Ganong, B.R., Bell, R.M. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 815–819.
[8] Morin, M.J., Kreutter, D., Rasmussen, H. and Sartorelli, A.C. (1987) J. Biol. Chem. 262, 11758–11763.
[9] William, F., Wagner, F., Karin, M. and Kraft, A.S. (1990) J. Biol. Chem. 265, 18166–18171.
[10] McPhail, L.C., Clayton, C.C. and Snyderman, R. (1984) Science 224, 622–625.
[11] Murakami, K. and Routtenberg, A. (1985) FEBS Lett. 192, 189–193.
[12] Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. and Nishizuka, Y. (1988) J. Biochem. (Tokyo) 103, 759–765.
[13] Seifert, R., Schachtele, C., Rosenthal, W. and Shultz, G. (1988) Biochem. Biophys. Res. Commun. 154, 20–26.
[14] El Touny, S., Khan, W. and Hannun, Y. (1990) J. Biol. Chem. 265, 16437–16443.
[15] Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. and Nishizuka, Y. (1991) Proc. Natl. Acad. Sci. USA, in press.
[16] Ito, T., Tanaka, T., Yoshida, T., Onoda, K., Ohta, H., Hagiwara, M., Itoh, Y., Ogura, M., Saito, H. and Hidaka, H. (1988) J. Cell Biol. 107, 929–937.
[17] Ogura, M., Morishima, Y., Okumura, M., Hotta, T., Takamoto, S., Ohno, R., Hirabayashi, N., Nagura, H. and Saito, H. (1988) Blood 72, 49–60.
[18] Tsukuda, M., Asaoka, Y., Sekiguchi, K., Kikkawa, U. and Nishizuka, Y. (1988) Biochem. Biophys. Res. Commun. 155, 1387–1395.
[19] Hashimoto, E., Takeda, M., Nishizuka, Y., Hamana, K. and Iwai, K. (1976) J. Biol. Chem. 251, 6287–6293.
[20] Ogura, M., Morishima, Y., Ohno, R., Kato, Y., Hirabayashi, N., Nagura, H. and Saito, H. (1985) Blood 66, 1384–1392.
[21] Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. and Nishizuka, Y. (1988) Biochem. Biophys. Res. Commun. 151, 973–981.