Volume 249, number 2, 264–266 FEB 07208 June 1989

Two isozymes of PKC found in HL-60 cells show a difference in activation by the phorbol ester TPA

Ingrid Beh, Rainer Schmidt and Erich Hecker

Institute of Biochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG

Received 1 April 1989

Cytosol from untreated cells and a detergent extract of the particulate fraction from TPA-treated HL-60 cells were analyzed for protein kinase C activity by consecutive column chromatography on Mono Q and hydroxyapatite . From both preparations two separate peaks of enzyme activity were obtained. The first peak, eluting at lower salt concentrations, is activated at lower TPA concentrations (3×10^{-9} M) than the other (10^{-7} M), which was eluted at higher salt concentrations.

Protein kinase C; Phorbol ester; (HL-60 cell)

1. INTRODUCTION

The human promyelocytic cell line HL-60 can be induced to differentiate to macrophage-like cells by treatment with the tumor-promoting phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) [1,2]. Protein kinase C (PKC), the putative receptor for TPA [3–5], is part of a cascade of second messengers resulting from phosphoinositide turnover. Hydrolysis of PIP_2 in response to a transmembrane signal, e.g. growth factor, leads to the formation of IP_3 and diacylglycerol (DAG) [6]. IP_3 stimulates the release of calcium from internal stores, whereas DAG activates PKC and causes translocation of PKC from cytosol to the membrane [7]. TPA can mimick DAG in activating PKC.

PKC comprises a family of serine/threonine-specific protein kinases. Thus far, a number of subspecies (α, βI/II, γ [8] and δ, ϵ, ξ [9]) have been identified by molecular cloning. Separation of PKC from mouse brain cytosol into two subtypes on a hydroxyapatite column was reported by Schmidt and Hecker [10].

Here, the separation of PKC activity from untreated and TPA-treated HL-60 cells into two PKC subtypes on hydroxyapatite column chromatography and their different activation by TPA are described.

Correspondence address: E. Hecker, Institute of Biochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG

Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol acetate; DAG, diacylglycerol; DTE, dithioerythritol; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Chemicals*

[γ-^{32}P]ATP (spec. act. 2–10 Ci/mmol) was obtained from NEN Research Products, Boston. Chemicals of analytical or purest obtainable grade were from Sigma, Taufkirchen. Materials for cell culture were from Boehringer Mannheim and Gibco (Eggenstein, FRG).

2.2. *Cell culture*

HL-60 cells obtained from Dr Braun (Institute of Immunology, Heidelberg) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine and penicillin (100 U/ml)/streptomycin (10 μg/ml) and incubated at 37°C in an atmosphere of 5% CO_2. Cells were seeded at a density of 3×10^5 cells/ml and grown for 3 days. Treatment of cells with TPA was performed by addition from a stock solution of 10^{-4} M TPA in acetone to a final concentration of 10^{-7} M, 30 min before cells were harvested.

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2.3. *Preparation of cytosol and particulate fractions*

Cells were centrifuged, washed with cold PBS, resuspended in homogenization buffer (10 mM EGTA, 5 mM EDTA, 2 mM DTE, 20 mM Tris, 0.1 mM PMSF, 10 μg/ml leupeptin) and disrupted with a Dounce homogenizer (tight fitting pestle). The homogenate was centrifuged at 110000 × *g* for 60 min at 2°C. The supernatant was used as the cytosol fraction. The pellet was resuspended in homogenization buffer containing 0.5% Triton X-100 and rehomogenized using the Dounce homogenizer. After centrifugation at 110000 × *g* for 60 min, the resulting supernatant was used as the extract of the membrane fraction (Detergent extract) for further purification by chromatography.

2.4. *Mono Q anion-exchange column*

Cytosol and detergent extracts of the particulate fraction were analyzed by chromatography at 2°C on a Mono Q column (Pharmacia, Freiburg) equilibrated in buffer A (20 mM Tris, 2 mM EDTA, 2 mM DTE). PKC activity was eluted with a linear gradient of 0–500 mM NaCl (30 ml). 1-ml fractions were collected.

2.5. *Hydroxyapatite column*

Pooled fractions from the Mono Q column containing PKC activity were applied to a hydroxyapatite column (HPHT, Bio-Rad, Munich) equilibrated at 2°C in buffer B (10 mM potassium phosphate, 0.3 mM $CaCl_2$, 2 mM DTE, 0.1 mM PMSF, 1 μg/ml leupeptin) and enzyme activity was eluted with a linear gradient of 10–500 mM potassium phosphate (high-salt buffer containing 2 μM $CaCl_2$). 1-ml fractions were collected.

2.6. *PKC assay*

PKC activity was detected by measuring the incorporation of phosphate from [γ-^{32}P]ATP into histone. The assay mixture (125 μl) consisted of histone type III (Sigma no. H-5505) at 240 μg/ml, 4 mM $MgCl_2$, 50 mM mercaptoethanol, 180 μg/ml phosphatidylserine and 1 mM EGTA. 10-μl aliquots of each column fraction were used. TPA was used at a final concentration of 10^{-7} M; [γ-^{32}P]ATP was at 20 μM. The mixture was incubated for 10 min at 30°C, aliquots of 50 μl spotted onto phosphocellulose paper sheets (P81 Whatman, Bender und Hobein, Munich), washed with four changes of water, then acetone and petroleum ether and counted in a liquid scintillation counter.

2.7. *Dose-response experiments*

PKC was assayed as described in section 2.6 with the exception that TPA was added in final concentrations from 10^{-10} to 10^{-6} M.

3. RESULTS

3.1. *Distribution of PKC in HL-60 cells*

Under the conditions of homogenization described in section 2, PKC activity in untreated cells was found almost exclusively in the cytosolic fraction. It eluted from Mono Q at salt concentrations between 150 and 180 mM NaCl. Treatment of HL-60 cells with 10^{-7} M TPA for 30 min resulted in almost complete translocation of PKC activity from the cytosol to the particulate fraction (not shown, see also [11,12]).

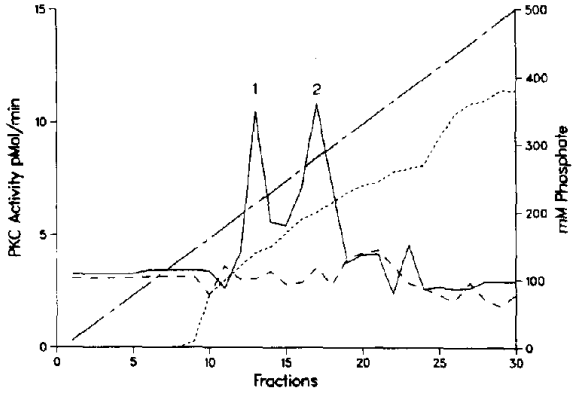

Fig.1. PKC profile on hydroxyapatite column of cytosol from untreated HL-60 cells. Pooled PKC activity [determined as described in section 2; (——) TPA, (– – –) acetone control] from the Mono Q gradient were loaded on the column and enzyme activity was eluted with a linear gradient of 10–500 mM potassium phosphate [gradient: (——) nominal, (· · · · ·) determined with conductometer]. No enzyme activity was found in the isocratic wash.

3.2. *PKC subtypes on hydroxyapatite column chromatography*

PKC activity from untreated as well as TPA-treated cells pooled from a Mono Q gradient and analyzed on a hydroxyapatite column consistently showed two peaks of enzyme activity. The first peak appeared at salt concentrations between 120 and 140 mM, the other at 160–200 mM phosphate (fig.1).

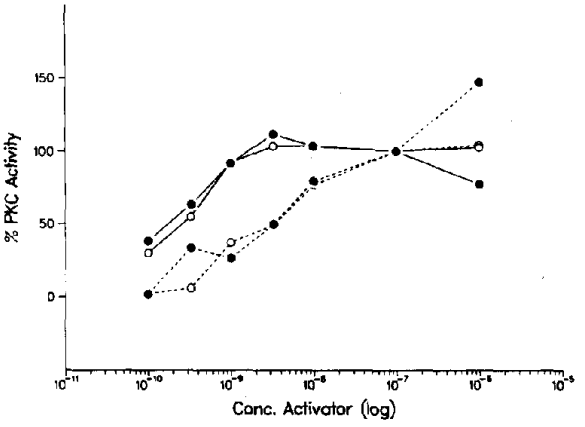

Fig.2. Dose-response curve for activation by TPA of PKC subtypes [peak 1 (——), peak 2 (– – –)] from cytosol of untreated cells (●) and detergent extract of particulate fraction of TPA-treated HL-60 cells (○). TPA was tested in the range 10^{-10}–10^{-6} M. Enzyme activity is expressed in percent of the activity at 10^{-7} M TPA which is taken as 100%. Data are shown for one out of two experiments with comparable results.

3.3. *Dose response of PKC activation by TPA*

In fig.2, typical dose-response curves for separated PKC activity from cytosol of untreated Hl-60 cells by TPA are shown. Peak 1 (eluting at lower salt) is maximally activated at lower TPA concentrations (3×10^{-9} M) than peak 2 (10^{-7} M). The same results were obtained for the two PKC subtypes from the detergent extract of the particulate fraction from TPA-treated cells (fig.2).

4. DISCUSSION

In cytosol of mouse brain, two PKC subtypes were isolated on a hydroxyapatite column [10]. Huang et al. [13] described the isolation of three isozymic forms (PKC type I/II/III) from rat brain on hydroxyapatite. They also showed that type I PKC is encoded by the γ gene, type II by the β gene and type III by the α gene [14]. In HL-60 cells we observed two forms of PKC separable on hydroxyapatite column chromatography. This is in agreement with the data of Huang et al. [14] who showed the existence of PKC types II and III in HL-60 cells by immunoblot analysis. In dose-response experiments with TPA the two isozymic forms isolated from HL-60 cells were activated differently, peak 1 requiring lower TPA concentrations for activation than peak 2. This result is interesting in the context of recent observations by Warren et al. [15] of a dose-dependent difference in the phosphorylation pattern of HL-60 cells after treatment with PDBu and bryostatin, both known activators of PKC. Moreover, bryostatin is an inhibitor of the effect of TPA as a promoter in skin [16] or as an inducer of differentiation in HL-60 cells [17]. Shearman et al. [18] reported the specific activation of a γ subspecies of PKC from bovine cerebellum by micromolar concentrations of arachidonic acid in the absence of phospholipid and diacylglycerol. Taken together, these data show that different and sometimes inhibitory effects of activators of PKC may arise from selective and (eventually dose-dependent) activation of PKC isozymes followed by phosphorylation of specific substrates. This aspect has recently been discussed in studies of the 'classic' PKC subforms [19–21] and of the calcium-independent PKC ϵ [22] and was reviewed by Nishizuka [23]. Thus, to explain the mechanism of TPA-induced differentiation of HL-60 cells it seems important to clarify the role of the two PKC subtypes, their activation and endogenous substrates depending on different stimuli (e.g., TPA, OAG and bryostatin).

Acknowledgement: This work was part of the thesis of I.B., University of Heidelberg (1989).

REFERENCES

[1] Rovera, G., Santoli, D. and Damsky, C. (1979) Proc. Natl. Acad. Sci. USA 76, 2779–2783.
[2] Nakayasu, M., Terada, M., Adolf, W., Opferkuch, H.J., Schmidt, R., Hecker, E. and Sugimura, T. (1982) J. Cancer Res. Clin. Oncol. 103, 17–29.
[3] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257 (13), 7848–7851.
[4] Hecker, E. (1985) Arzneimittelforschung 35 (II), 12a, 1890–1903.
[5] Blumberg, P. (1988) Cancer Res. 48, 1–8.
[6] Berridge, M. (1987) Biochim. Biophys. Acta 907, 33–45.
[7] Kraft, A. and Anderson, W.B. (1983) Nature 301, 621–623.
[8] Parker, P.J., Coussens, L., Tatty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) Science 233, 853–859.
[9] Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igaraski, K. and Nishizuka, Y. (1987) FEBS Lett. 226, 125–128.
[10] Schmidt, R. and Hecker, E. (1984) Hoppe Seylers Z. Biol. Chem. 365, 1057–1058.
[11] Vandenbark, G.R. and Niedel, J.E. (1984) J. Nat. Cancer Inst. 73, 1013–1019.
[12] Kiss, Z., Deli, E., Girard, P.R., Pettit, G.R. and Kuo, J.F. (1987) Biochem. Biophys. Res. Commun. 146, 208–215.
[13] Huang, K.-P., Nakabayashi, H. and Huang, F.L. (1986) Proc. Natl. Acad. Sci. USA 83, 8535–8539.
[14] Huang, F.L., Yoshida, Y., Nakabayashi, H., Knopf, J.L., Young, W.S. and Huang, K.P. (1987) Biochem. Biophys. Res. Commun. 149, 916–952.
[15] Warren, S.B., Kamano, Y., Pettit, G.R. and Blumberg, P.M. (1988) Cancer Res. 48, 5984–5988.
[16] Hennings, H., Blumberg, P.M., Pettit, G.R., Herald, C.L., Shores, R. and Yuspa, S.H. (1987) Carcinogenesis 8, 1343–1346.
[17] Kraft, A., Smith, J.B. and Berkow, R.L. (1986) Proc. Natl. Acad. Sci. USA 83, 1334–1338.
[18] Shearman, M.S., Naor, Z., Sekiguchi, K., Kishimoto, K. and Nishizuka, Y. (1989) FEBS Lett. 243, 177–182.
[19] Azhar, S., Butte, Y. and Reaven, E. (1989) Int. J. Biochem. 21, 209–218.
[20] Dale, I.L. and Gescher, A. (1989) Int. J. Cancer 43, 158–163.
[21] Wheeler, M.B. and Veldhuis, J.D. (1989) Mol. Cell. Endocrinol. 61, 117–122.
[22] Schaap, D., Parker, P.J., Bristol, A., Kriz, R. and Knopf, J. (1989) FEBS Lett. 243, 351–357.
[23] Nishizuka, Y. (1988) Nature 334, 661–665.