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Proteolytic activation of protein kinase C in the extracts of cells treated for a short time with phorbol ester

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A 10 min treatment of human neutrophils with phorbol 12-myristate 13-acetate (PMA) has been reported to induce accumulation of the proteolytically activated Ca^{2+}/phospholipid-independent catalytic fragment of protein kinase C in the cytosol of intact cells [(1986) J. Biol. Chem. 261, 4101–4105]. We investigated the proteolytic conversion of protein kinase C to the Ca^{2+}/phospholipid-independent form in the cytosol and membrane fractions of pig neutrophils. The activity of protein kinase C was measured with its specific oligopeptide substrate Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide designed previously. In our experiments the short-term treatment of neutrophils with PMA did not induce the accumulation of the proteolytically activated form of protein kinase C in the cytosol of intact cells. However, treatment of cells with PMA enhanced the limited proteolysis of protein kinase C during the preparation of cell extracts.

Protein kinase C; Phorbol ester; Proteolytic activation; Synthetic peptide substrate; (Neutrophil, Lymphocyte, Hepatocyte)

1. INTRODUCTION

The transient activation of the Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) by diacylglycerol produced in the cell membrane is one of the key events in initiating cellular responses to certain extracellular signals. The tumor promoter compound phorbol 12-myristate 13-acetate (PMA) can substitute for diacylglycerol to activate protein kinase C and is widely used to stimulate a variety of cells (reviews [1,2]). Under in vitro conditions protein kinase C also can be activated irreversibly by limited proteolysis [3–6], liberating the C-terminal catalytic domain of the enzyme from the N-terminal regulatory domain [7]. The fragment containing the catalytic domain is active in the absence of Ca^{2+} and phospholipid. The conversion of protein kinase C to the Ca^{2+}/phospholipid-independent proteolytic fragment has been reported to occur also in intact human platelets [8] and neutrophils [9,10] treated for 10 min with a tumor-promoting phorbol ester.

Tumor-promoting phorbol esters are considered to induce their effects directly by intercalating into the cell membrane and permanently activating protein kinase C [1,11]. The activated membrane-bound protein kinase C phosphorylates a number of membrane proteins but cytoplasmic substrates of the enzyme are also known [2,12]. The mechanism of phosphorylation of cytosolic substrates by membrane-bound protein kinase C is not known. In human neutrophils treated for 10 min with PMA the proteolytic cleavage of membrane-bound protein kinase C by the Ca^{2+}-requiring thiol proteinase calpain and the liberation of the catalytic fragment into the cytosol have been proposed to account for the

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phosphorylation of cytosolic or nuclear proteins [9].

While the proteolytic degradation of protein kinase C seems to play a role in the down-regulation of the phorbol receptor [13,14], the physiological significance of the proteolytic activation of protein kinase C is an open question. The aim of the present studies was to investigate the proteolytic activation of protein kinase C in pig neutrophils treated for a short time with PMA. The catalytic activity of the enzyme was measured with the synthetic oligopeptide substrate designed previously [15,16]. In the absence of calcium and phospholipid the proteolytically activated form of protein kinase C has been found to be the single enzyme which phosphorylates this oligopeptide under standard conditions [15,16]. However, we could not observe accumulation of the catalytic fragment of protein kinase C in PMA-treated intact cells. The catalytic fragment was also demonstrated in extracts of non-treated cells and seemed to be an artifact of the extraction procedure. The circumstances which may be responsible for the enhancement of limited proteolysis of protein kinase C during extraction of PMA-treated cells were investigated in detail.

2. EXPERIMENTAL

2.1. *Preparation of neutrophil extracts*

Neutrophils were prepared from pig blood according to [17] with the modification that instead of Hank's solution neutrophils were suspended in a medium (medium A) containing 0.01 M sodium phosphate (pH 7.4), 5 mM KCl, 120 mM NaCl, 24 mM $NaHCO_3$ and 5 mM glucose [9]. The cell population obtained consisted of 96–97% polymorphonuclear cells, 1–2% lymphocytes and 1–2% monocytes. Living cells accounted for more than 97% of the cell population. The cells (5×10^6 cell/ml) were treated with PMA (30 ng/ml) for 10 min at 37°C according to [9] with the modification that in our experiments the concentration of PMA was higher. After 10 min incubation the cells were centrifuged at 400 × *g*, then washed and suspended in a medium ('lysing medium') comprising 20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 2 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM mercaptoethanol and 0.02% leupeptin. The suspension (5×10^6 cells/ml) was cooled in an ice bath and lysed by sonication with a Sonic 300 Dismembrator (Artek) using 4 × 10 s bursts. The suspension was centrifuged for 30 min at 100000 × *g*; the supernatant produced was referred to as the cytosol fraction. The pellet was suspended in 1 ml lysing buffer containing 0.2% Triton X-100. (The lysing buffer was completed again with 2 mM PMSF just before the pellet was suspended.) The suspension was incubated at 0°C for 60 min and centrifuged at 11000 × *g* for 15 min. The supernatant yielded by this procedure was referred to as the particulate fraction.

2.2. *Hepatocyte isolation*

Hepatocytes were isolated from the liver of male mice essentially by the collagenase perfusion method [18] as detailed in [19]. Isolated hepatocytes (about 10^7 cells/ml) were incubated for 10 min in Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 1% albumin, 8.5 mM glucose, 5 mM pyruvate, all amino acids necessary for protein synthesis (1 mM each), under constant bubbling of gas (O_2:CO_2, 95:5, v/v) at 37°C in the absence or presence of 200 ng/ml PMA. Lymphocytes were obtained as a side product during the isolation of pig neutrophils. They were treated with PMA exactly as the neutrophils. The preparation of cell extracts from hepatocytes or lymphocytes was performed as in the case of neutrophils.

2.3. *Assay of the activity of protein kinase C*

The assay was carried out in an assay mixture comprising 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.01 mM [^{32}P]ATP (500000–800000 cpm per reaction mixture) and the synthetic peptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide (0.75 mM) in a final volume of 0.4 ml and 0.05-ml samples of the extracts were assayed. The activity of the proteolytic fragment was measured in the presence of 1.25 mM EGTA, while the total activity of protein kinase C was determined in the presence of 1.25 mM $CaCl_2$/20 μg/ml phosphatidylserine/25 ng/ml PMA. Incubation was carried out at 37°C for 10 min. Radioactivity incorporated into the oligopeptide substrate was measured as in [14]. Assays were performed in duplicates.

2.4. *Separation of the proteolytic fragment from the intact protein kinase C*

Cell were sonicated in lysing buffer in the presence of 0.01 mM cAMP, the cytosol fraction being diluted 10 times with distilled water containing 0.01 mM cAMP and 2 mM PMSF and applied immediately onto a DEAE-Sephacel column (1 × 10 cm). Chromatography was carried out as in [14].

3. RESULTS AND DISCUSSION

Extracts of pig neutrophils were prepared in the presence of the usual protease inhibitors. Leupeptin (0.02%) or pepstatin (30 μg/ml) did not influence significantly the conversion of protein kinase C to the proteolytically activated form but PMSF was an effective inhibitor of limited proteolysis in neutrophil extracts. However, in spite of the presence of PMSF, in freshly prepared extracts of pig neutrophils the calcium/phospholipid-independent activity accounted for 25–30% of the total protein kinase C activity measured with the synthetic oligopeptide substrate (table 1). Storage of the cytosol fraction at 0°C for 2–3 h resulted in the complete loss of the intact enzyme.

The intact and proteolytically activated forms of protein kinase C were demonstrated by DEAE-cellulose chromatography (fig.1). The proteolytically activated form of the enzyme was eluted in the known position, at higher ionic strength than the intact enzyme [3,8,9,15,16] and was inhibited by calcium/phosphatidylserine as mentioned previously [16].

Treatment of cells with PMA (30 ng/ml) for 10 min caused translocation of protein kinase C.

Table 1

The activity of protein kinase C and its proteolytic fragment in subcellular fractions of pig neutrophils

Subcellular fractions	Peptide kinase activity (pmol/min per 10^7 cells) (mean ± SE; n = 7) in the presence of	
	EGTA	Ca^{2+}/PS/PMA
Cytosolic	44 ± 6	190 ± 9
Particulate	56 ± 4	85 ± 14

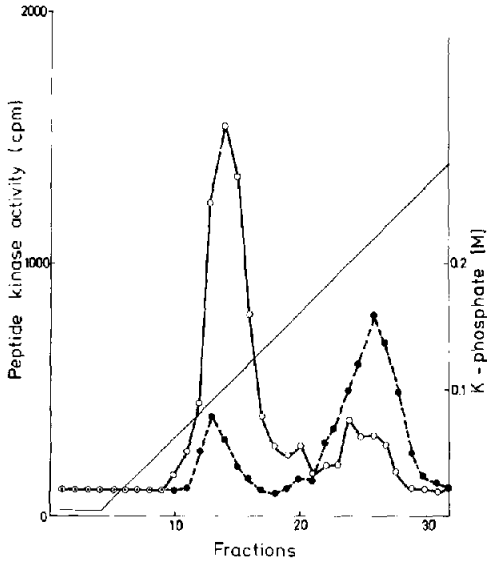

Fig.1. Separation of intact and proteolytically activated forms of protein kinase C by DEAE-cellulose chromatography. Cytosol fraction was prepared from 5 × 10^6 neutrophils and chromatography was performed as described in the text. About 4-ml fractions were collected and 0.1-ml samples were assayed for peptide kinase activity in the presence of 1 mM EGTA (●---●) or $CaCl_2$ (1 mM)/phosphatidylserine (20 μg/ml)/PMA (25 ng/ml) (○—○).

The intact enzyme which disappeared from the cytosol was found in the particulate fraction (fig.2A,B). It is important to note that in the present experiments the concentration of Triton X-100 was 0.025% in the assay mixture for the particulate fraction. At this concentration the inhibitory effect of Triton on the activation of intact enzyme was slight. Accumulation of the proteolytically activated fragment of protein kinase C in the cytosol of intact PMA-treated neutrophils was not observed (fig.2). More exactly, in 3 out of 7 similar experiments performed with different batches of neutrophils the amount of proteolytic fragment did not increase at all in extracts of PMA-treated cells (fig.2A,B), while in the other 4 cases a small (never more than the 15% of the total protein kinase C activity) increase in calcium/phospholipid-independent activity was detected but exclusively in the particulate fraction. We can state that in pig neutrophils the PMA-induced translocation of protein kinase C was not accompanied by liberation of the proteolytically

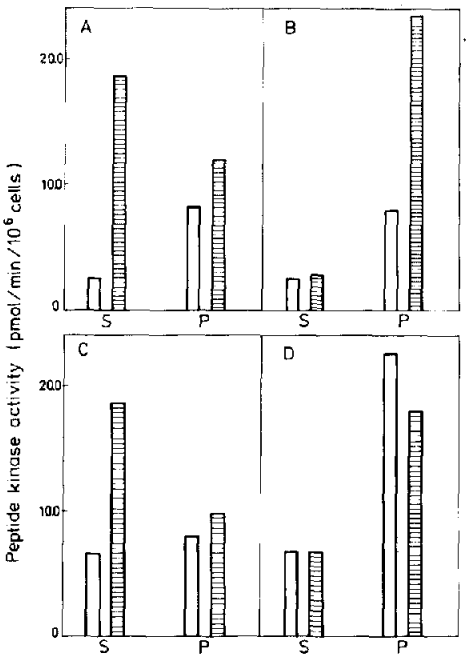

Fig.2. Translocation of protein kinase C from the cytosol into the particulate fraction of pig neutrophils on the effect of PMA. Neutrophils were incubated in the absence (A,C) or presence of 30 ng/ml PMA (B,D). Cells were sonicated in lysing medium, and membranes were solubilised in lysing medium containing 0.2% Triton X-100. In A,B this medium was completed again with 2 mM PMSF immediately before the membrane pellet was suspended. In C,D lysing medium was not completed again with PMSF before the membrane was suspended. A,B and C,D show results obtained from different batches of neutrophils. Peptide kinase activity was measured in the presence of 1.25 mM EGTA (open columns) or in the presence of 1.25 mM $CaCl_2$/20 μg/ml phosphatidylserine/25 ng/ml PMA (hatched columns). S, cytosol fraction; P, particulate fraction. The peptide kinase activity of the extracts was measured immediately after preparation. Data are means of duplicate determinations. A,B and C,D each represent one out of 7 and 4 similar experiments, respectively.

activated form into the cytosol, at variance with the result in [9].

Since we were unable to reproduce the phenomenon demonstrated in [9] we investigated the conditions which differed in our experiments from those reported in [9]. According to the descriptions of the experimental procedures of [9] in a part of the experiments demonstrating accumulation of the catalytic fragment in the cytosol of intact PMA-treated neutrophils, protease inhibitors were not used at all during the course of the preparation of the cellular extracts. Therefore, we performed a series of experiments without inhibition of the extracellular proteolysis of protein kinase C. When PMSF was present in the lysing medium but the medium used for the solubilisation of the particulate fraction was not completed with PMSF just before the membrane pellet was suspended, the treatment of cells with PMA induced not only the translocation of protein kinase C from the cytosol into the membrane but the enzyme found in the particulate fraction was also converted completely to the proteolytically ac-

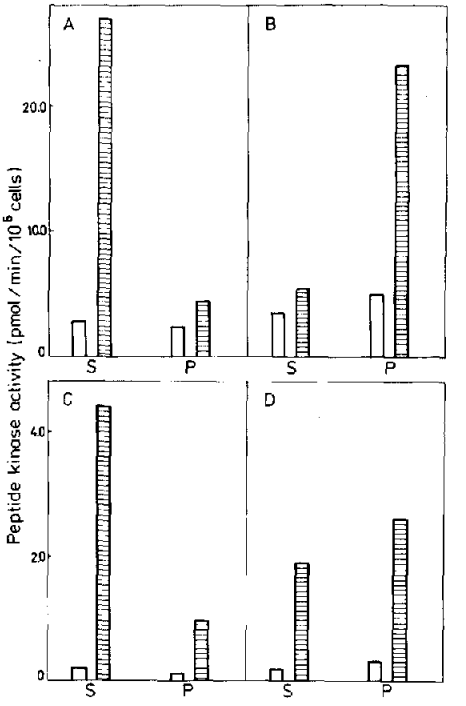

Fig.3. Effect of treatment of lymphocytes and hepatocytes with PMA on the activity of protein kinase C in extracts of cells. Pig lymphocytes were incubated in the absence (A) or presence of 30 ng/ml PMA (B). Isolated mouse hepatocytes were incubated in the absence (C) or presence (D) of 200 ng/ml PMA. Extracts were prepared in the presence of 2 mM PMSF and 0.02% leupeptin. Peptide kinase activity was measured in the presence of 1.25 mM EGTA (open columns) or in the presence of 1.25 mM $CaCl_2$/20 μg/ml phosphatidylserine/25 ng/ml PMA (hatched columns). S, cytosol fraction; P, particulate fraction. Data are means of duplicate determinations.

tivated form (fig.2C,D). Exactly the same phenomenon has been described in the case of PMA-treated thrombocytes [8]. When PMSF was present during the sonication but the sonicated cell suspension was stored for several minutes at room temperature before centrifugation, the amount of the proteolytic fragment of protein kinase C was significantly higher in the cytosol fraction of PMA-treated cells than in control cells (not shown).

It is known that the reversible activation of protein kinase C by phospholipid and diacylglycerol stimulates in vitro proteolysis of the enzyme by the calcium-dependent thiol proteinase calpain I, because calpain I reacts preferentially with the active form of protein kinase C [6]. The active form of the enzyme produced by treatment of cells with PMA also seems to be more susceptible than the inactive form to in vitro attack of some serine proteinases of the neutrophils. Our results led us to the conclusion that in pig neutrophils the proteolytic activation of protein kinase C has no physiological significance in the PMA-induced effects during the first 10 min.

In neutrophil extracts protection of the intact protein kinase C against in vitro proteolysis was very difficult, however, this was not easy either in the extracts of other cell types. We also investigated the PMA-induced translocation of protein kinase C in pig lymphocytes and isolated mouse hepatocytes. Hepatocytes were treated with 200 ng/ml PMA instead of 30 ng/ml, and at variance with neutrophil and lymphocyte extracts the presence of leupeptin (0.02%) was essential for preservation of the activity of protein kinase C in hepatocyte extracts. In spite of careful inhibition of the extracellular proteolysis, in the extracts of both cell types a small amount of the proteolytically activated fragment was observed and a small enhancement of proteolytic conversion in extracts of PMA-treated cells could be detected (fig.3). In a single case out of 5 similar experiments the proteolytic fragment was not found in the extracts of hepatocytes, either of the control or of PMA-treated cells. Our results suggest that data concerning the proteolytic activation of protein kinase C in intact cells on the effect of a short-term treatment with PMA can be evaluated only under conditions where extracellular proteolysis is completely inhibited. The synthetic oligopeptide substrate of protein kinase C may provide a tool to check the methods used for the protection of intact protein kinase C during the preparation of cellular extracts.

REFERENCES

[1] Nishizuka, Y. (1984) Nature 308, 693–698.
[2] Nishizuka, Y. (1986) Science 233, 305–312.
[3] Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603–7609.
[4] Kikkawa, U., Minakuchi, R.T., Takai, Y. and Nishizuka, Y. (1983) Methods Enzymol. 99, 288–298.
[5] Takai, Y., Kishimoto, A., Iwasa, Y., Kawakasa, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695.
[6] Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
[7] Parker, P.P., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) Science 233, 853–859.
[8] Tapley, P.M. and Murray, A.W. (1985) Eur. J. Biochem. 151, 419–423.
[9] Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B. and Horecker, B.L. (1986) J. Biol. Chem. 261, 4101–4105.
[10] Pontremoli, S. and Melloni, E. (1986) Annu. Rev. Biochem. 55, 455–481.
[11] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[12] White, J.R., Huang, C.-K., Hill, J.M., Naccache, P.H., Becher, E.L. and Sa'afi, R.I. (1984) J. Biol. Chem. 259, 8605–8611.
[13] Chida, K., Kato, N. and Kuroki, T. (1986) J. Biol. Chem. 261, 13013–13018.
[14] Stabel, S., Rodriguez-Pena, A., Young, S., Rozengurt, E. and Parker, P.J. (1987) J. Cell. Physiol. 130, 111–117.
[15] Romhányi, T., Sepródi, J., Antoni, F., Mészáros, G. and Faragó, A. (1985) Biochim. Biophys. Acta 827, 144–149.
[16] Romhányi, T., Sepródi, J., Antoni, F., Mészáros, G., Buday, L. and Faragó, A. (1986) Biochim. Biophys. Acta 888, 325–331.
[17] Shoog, W.A. and Beck, W.S. (1956) Blood 11, 436–441.
[18] Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506–520.
[19] Mandl, J., Garzó, T., Mészáros, K. and Antoni, F. (1979) Biochim. Biophys. Acta 586, 560–567.