Volume 253, number 1,2, 71-75 FEB 07459 August 1989

A protein kinase C-related enzyme activity in *Dictyostelium discoideum*

M.E. Eva Ludérus, Robbert G. Van der Most+, Arie P. Otte* and Roel Van Driel

E.C. Slater Institute for Biochemical Research, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam and **Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*

Received 12 June 1989

In crude cell lysates of the cellular slime mould *Dictyostelium discoideum* we identified a protein kinase C (PKC)-like enzyme activity. This activity, measured as phosphorylation of a synthetic EGF-receptor-derived peptide [(1987) J. Biol. Chem. 262, 772-777], was regulated by Ca^{2+}, phosphatidylserine (PS), 1,2-dioleoyl-rac-glycerol (DG) and the phorbol ester PMA. PS and DG stimulated the enzyme in a synergistic manner. The stimulation by these lipids was, in contrast to what has been found for 'classical' mammalian PKC, not dependent on Ca^{2+}. The *D. discoideum* enzyme was strongly stimulated by nanomolar concentrations of PMA, and inhibited by PKC-inhibitor staurosporine.

Protein kinase C; Chemotactic receptor; cAMP receptor; (*Dictyostelium discoideum*)

1. INTRODUCTION

Protein kinase C (PKC), as first described by Nishizuka and co-workers [1,2], is a phospholipid- and Ca^{2+}-dependent serine/threonine protein kinase. In vivo, the enzyme is activated by diacylglycerol [3]. This second messenger is transiently produced by the breakdown of membrane phospholipids in response to extracellular signals. Tumor-promoting phorbol esters, like phorbol 12-myristate 13-acetate (PMA), are able to mimick diacylglycerol in activating PKC [4].

Correspondence address: M.E.E. Ludérus, E.C. Slater Institute for Biochemical Research, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands

+ *Present address:* Veterinary Department, State University of Utrecht, Yalelaan 1, 3508 TD Utrecht, The Netherlands

Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; EGF-R-peptide, synthetic EGF-receptor-derived peptide Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-NH_2; DG, 1,2-dioleoyl-rac-glycerol

PKC is believed to play a major role in cellular regulation. Its function has been implicated in many cellular processes, including cell growth, differentiation, gene expression, and regulation of cell surface receptors. Thus far, identification of the enzyme has been restricted to tissues and organs of mammals and other higher eukaryotes [5–7].

For the lower eukaryote *Dictyostelium discoideum*, effects of the phorbol ester PMA on regulation of chemotactic cAMP receptors have been described [8–10], suggesting the involvement of a PKC-related enzyme.

Our present study confirms this suggestion. Using a synthetic oligopeptide as a substrate, we were able to identify a PKC-related kinase activity in *D. discoideum* cells. The enzyme activity was found to be regulated by Ca^{2+}, phosphatidylserine (PS), 1,2-dioleoyl-rac-glycerol (DG) and PMA. PS and DG stimulated the enzyme in a synergistic manner. Stimulation by these lipids was, in contrast to the regulation of 'classical' PKC, not dependent on Ca^{2+}.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2. MATERIALS AND METHODS

2.1. *Culture conditions and cell lysis*

D. discoideum cells (strain AX2) were grown in HL-5 medium [11], that contained maltose instead of glucose. Cells were harvested in the late logarithmic growth phase at a density of approximately 5×10^6 cells/ml. Cells were washed with lysis buffer (20 mM Tris-Cl, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 5 mM β-mercaptoethanol, 2 mM PMSF) and suspended at a density of 1×10^8 cells/ml in lysis buffer containing 0.15 M sucrose and the following protease inhibitors: 10 μg/ml leupeptin, 20 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, 2 μg/ml antipain, 5 mM benzamidin (final concentrations). Subsequently, cells were lysed by nitrogen cavitation [12].

2.2. *Partial purification of an EGF-receptor-peptide kinase*

The crude cell lysate (1 ml, 1×10^8 cell equivalents/ml) was treated with Nonidet P-40 (1% final concentration) for 2 min on ice, and applied to a DE52-cellulose (Whatman, England) column (0.4 ml) pre-equilibrated with lysis buffer. The column was washed with 3 bed volumes of lysis buffer and eluted with a NaCl step-gradient (0–200 mM). Fractions were collected and immediately assayed for kinase activity. Immediate assaying was essential because the enzyme activity, after cell lysis, was quite unstable. All procedures were carried out at 4°C and fractions were kept on ice until assaying.

2.3. *Assay of protein kinase activity*

Protein kinase activity was assayed (at 30°C for 7 min) by measuring the incorporation of $^{32}P_i$ from [γ-^{32}P]ATP into the synthetic EGF-receptor-derived peptide Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-NH_2 (EGF-R-peptide; Auspep, Australia; [13]). The assay conditions were adapted from Kikkawa et al. [6]. The reaction mixture (80 μl) contained: DE52 column fraction (5 μg of protein), 20 mM Tris-Cl, pH 7.4, 7.5 mM $MgCl_2$, 0.1 mg/ml EGF-receptor peptide, 10 μM ATP, 0.5 μCi [γ-^{32}P]ATP (5×10^5 cpm), 2 mg/ml BSA (fatty acid free), 5 mM NaF, protease inhibitors as listed above, and, when indicated in the text, 96 μg/ml PS (lipid products, England), 3.2 μg/ml DG (Sigma, USA), PMA (Sigma, USA; concentrations between 1 and 500 nM), 1 mM EGTA, and/or 10 mM Ca^{2+}/EGTA buffer yielding free Ca^{2+} concentrations between 10^{-6} and 10^{-3} M [14].

PS and DG were stored under nitrogen at −20°C, solubilized in chloroform/methanol (1:1). Before use, the chloroform/methanol was vaporized under nitrogen and the lipid residue was suspended in 20 mM Tris-Cl, pH 7.4, and sonicated for 3 min. When both PS and DG were to be used in the assay, both activators were mixed before the chloroform/methanol was removed. PMA (stored as 8.1 mM stock solution in DMSO) was mixed with sonicated lipid before being added to the reaction mixture, according to Castagna et al. [4]. The final concentration of DMSO in the reaction mixture was maximally 0.01%. This concentration of DMSO did not affect kinase activity.

The phosphotransferase reaction was stopped by addition of 120 μl of 25% ice-cold TCA. The mixture was kept on ice for at least 30 min. Subsequently, the TCA-precipitated protein was sedimented by centrifugation at $10000 \times g$ for 2 min in a microfuge at 4°C. Portions (150 μl) of the supernatant, containing the EGF-R-peptide substrate, were spotted onto phosphocellulose P81 strips (Whatman, England), according to the procedure described by Roskoski [15]. Strips were washed 3 times for 3 min in a large volume of 75 mM phosphoric acid. Subsequently, ^{32}P was measured by liquid scintillation spectrometry.

Background ^{32}P-labeling of proteins other than the peptide substrate was determined in assays lacking the substrate. For each assay condition, this background value (between 300 and 500 cpm) was measured and subtracted from the total ^{32}P-incorporation, to give the substrate-specific labeling. The substrate-specific labeling was generally 3 to 6 times the background labeling.

3. RESULTS AND DISCUSSION

Protein kinase C (PKC) has proven to be a ubiquitous enzyme in mammalian cells, pivotal in various control processes. Recently, also several PKC-related enzymes have been characterized [16–21]. Properties of these enzymes are similar, but not identical to those of the classical enzyme.

Indirect evidence has been presented that the lower eukaryote *D. discoideum* also contains PKC or a PKC-related enzyme. We have reported effects of PMA, Ca^{2+} and ATP on the regulation of chemotactic cAMP receptors in lysates of *D. discoideum* [8]. In addition, Thiery et al. [9] recently showed that PMA modulates the cAMP-induced light-scattering response of intact *D. discoideum* cells, probably by reducing the number of cAMP receptors. These findings suggest a role of a PKC-like activity in cAMP-receptor regulation of *D. discoideum*.

Our present work was aimed at identifying such an activity in crude cell lysates of *D. discoideum*. As substrate for this putative kinase we used a synthetic oligopeptide of the EGF receptor, known to be a specific substrate of mammalian PKC in vitro [13]. The peptide corresponded to a PKC phosphorylation site sequence of the intact EGF-receptor molecule. We studied regulation of the putative enzyme activity by Ca^{2+}, phospholipid and phorbol ester, being characteristic regulators of classical mammalian PKC.

Fig.1 shows the fractionation of a protein kinase activity from *D. discoideum* cell lysates by DE52-cellulose chromatography. The column fractions were assayed for kinase activity with the EGF-R-peptide as substrate. The kinase activity, measured as ^{32}P incorporation into the substrate, was determined in the absence and presence of Ca^{2+}, PS and DG. In the absence of Ca^{2+} and lipid

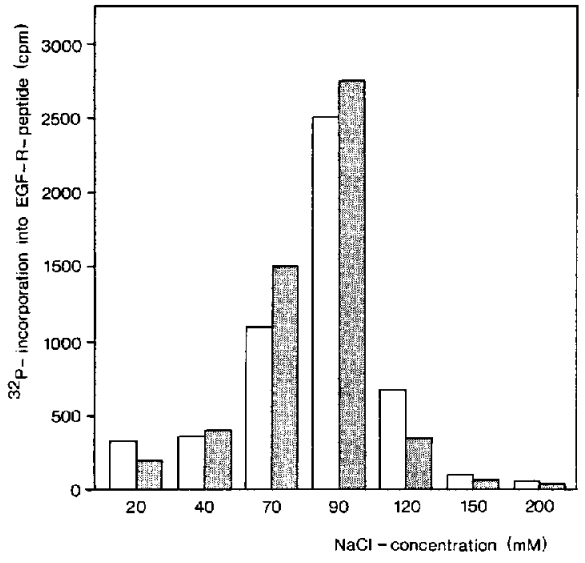

Fig.1. DE52 elution pattern of EGF-R-peptide kinase activity. Protein kinase activity was assayed as described in section 2.3, in the presence of either 10^{-4} M Ca^{2+} (open bars), or 96 μg/ml PS plus 3.2 μg/ml DG (filled bars). Kinase activity is expressed as the amount (cpm) of ^{32}P incorporation into the EGF-R-peptide. Shown is a typical experiment.

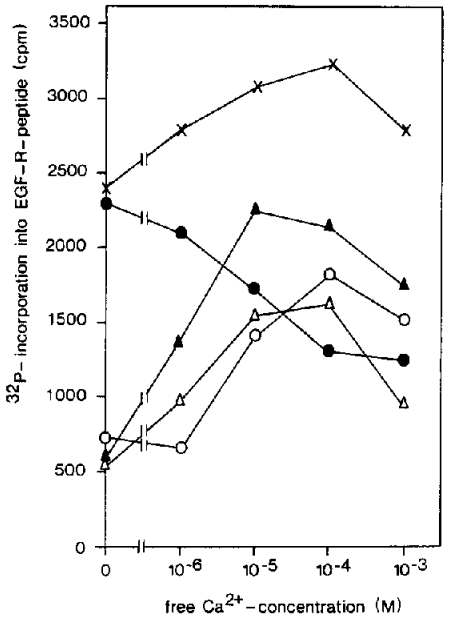

Fig.2. Regulation of EGF-R-peptide kinase activity by Ca^{2+}, PS, DG and PMA. Kinase activity was measured in the 40–90 mM NaCl DE52-column fraction as a function of the free Ca^{2+} concentration, in the presence of 96 μg/ml PS (▲), 3.2 μg/ml DG (○), 96 μg/ml PS plus 3.2 μg/ml DG (●), 96 μg/ml PS plus 500 nM PMA (×), or in the absence of lipid (△).

(i.e. in the presence of 1 mM EGTA), no significant EGF-R-peptide kinase activity was seen in any of the column fractions (results not shown). In the presence of either 10^{-4} M Ca^{2+}, or PS plus DG, considerable activity was observed in the 70 mM and 90 mM NaCl fractions. When, however, all three activators, i.e. Ca^{2+}, PS and DG, were present together, the activity in these fractions was reduced (results not shown in fig.1).

We conclude that *D. discoideum* lysates contain a protein kinase that is able to phosphorylate a synthetic PKC substrate. The kinase activity is regulated by Ca^{2+} and lipid, and elutes from a DE52-cellulose column at pH 7.4 between 40 mM and 90 mM NaCl. A similar elution pattern has been found for several members of the PKC family, such as classical PKC [6], nPKC [17] and PAK II [16].

In fig.2 the Ca^{2+}/lipid regulation of *D. discoideum* EGF-R-peptide kinase was studied in more detail. In the absence of PS and DG, as well as in the presence of either one of these lipids alone, the kinase activity was Ca^{2+}-dependent. In the absence of Ca^{2+}, a basal activity was measured; when the free Ca^{2+} concentration was increased up to 10^{-4} M, the activity increased 2–3-fold. Further elevation of the Ca^{2+} concentration up to 10^{-3} M reduced the activity again. Furthermore, the *D. discoideum* EGF-R-peptide kinase was greatly stimulated by the synergistic action of PS and DG. The stimulation by these lipids was maximal in the absence of Ca^{2+}, and decreased when the Ca^{2+} concentration was elevated.

In conclusion, like classical PKC, the *D. discoideum* EGF-R-peptide kinase is regulated by Ca^{2+}, PS and DG. The type of regulation is, however, somewhat different from that of the classical PKC activity. In the absence of lipid, the *D. discoideum* enzyme is strongly stimulated by Ca^{2+}, while mammalian PKC under these conditions only shows a minor Ca^{2+} dependency [22]. Furthermore, in the presence of PS plus DG, the activity of the *D. discoideum* enzyme is inhibited by Ca^{2+}, while the PS/DG stimulation of mammalian PKC is potentiated by Ca^{2+}. In this respect, the *D. discoideum* kinase more closely resembles a group of PKC-related enzymes that have been

shown to be phospholipid-dependent, but Ca^{2+}-independent [17,18,20] or Ca^{2+}-inhibited [16,19].

We found that the *D. discoideum* kinase, like mammalian PKC, was strongly stimulated by phorbol ester PMA (fig.2). PMA was added together with PS. The enzyme was already considerably activated in the absence of Ca^{2+}, but its activity was further increased by addition of Ca^{2+}. The dose-response curve in fig.3 shows half-maximal stimulation at approx. 40 nM PMA. Similar PMA concentrations have been shown to affect cAMP receptors in *D. discoideum* cell lysates [8]. Somewhat lower PMA concentrations have been found to activate mammalian PKC [2]. In the absence of PS, the *D. discoideum* kinase was stimulated by PMA to some extent.

The microbial alkaloid staurosporine is a potent inhibitor of classical mammalian PKC [23]. Fig.4 shows that, like mammalian PKC, the *D. discoideum* EGF-R-peptide kinase was inhibited by staurosporine. Both the lipid-induced activity and the Ca^{2+}-induced activity were almost completely inhibited by 100 nM staurosporin. The basal, i.e. Ca^{2+}/lipid-independent kinase activity was not affected by the inhibitor. It is possible that the Ca^{2+}/lipid-dependent and -independent kinase activities reflect different enzymes. Although the specificity of staurosporine has been questioned [24], we consider the results of fig.4 as additional evidence for the presence of a PKC-like activity in *D. discoideum*.

In summary, our present work indicates that the cellular slime mould *D. discoideum* contains an EGF-R-peptide kinase activity with properties typical for protein kinases of the PKC family in higher eukaryotes. Like classical mammalian PKC, the *D. discoideum* enzyme (i) phosphorylates the synthetic EGF-R-peptide; (ii) is regulated by Ca^{2+}, PS and DG; (iii) is stimulated by phorbol ester; and (iv) is inhibited by staurosporine. Unlike classical PKC, which is a strictly Ca^{2+}-dependent enzyme, the lipid-activated form of the *D. discoideum* enzyme is inhibited by Ca^{2+}. Earlier work has suggested that the *D. discoideum* enzyme may be involved in regulation of the chemotactic cAMP receptor.

To our knowledge, this is the first time that a PKC-related activity is identified in a lower eukaryote. This finding further strengthens the notion [25,26] that transmembrane signalling mechanisms in higher and lower eukaryotes are very similar.

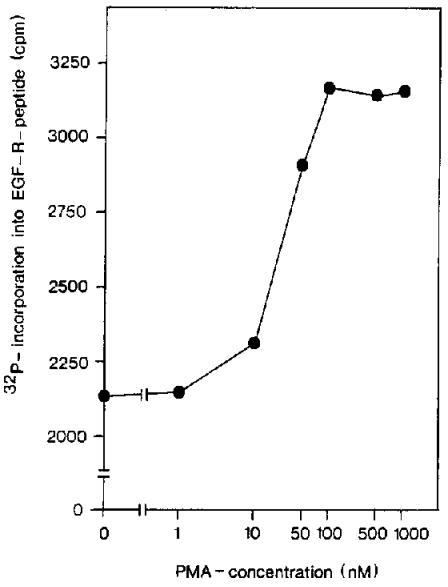

Fig.3. Effect of PMA on EGF-R-peptide kinase activity. Kinase activity was measured in the 40–90 mM NaCl DE52-column fraction, in the presence of 96 μg/ml PS, 10^{-4} M free Ca^{2+}, plus different concentrations PMA.

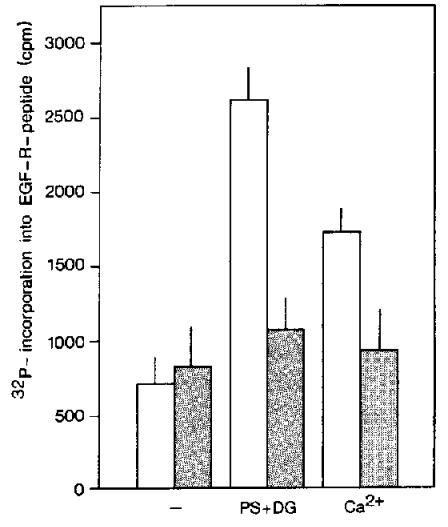

Fig.4. Effect of staurosporine on EGF-R-peptide kinase activity. Kinase activity was measured in the 40–90 mM NaCl DE52-column fraction, in the absence (open bars), or presence (filled bars) of 100 nM staurosporine, with further additions as indicated in the figure. Final concentrations: PS, 96 μg/ml; DG, 3.2 μg/ml; Ca^{2+}, 10^{-4} M.

Acknowledgements: We thank Dr G.T. Snoek for the gift of purified rat brain PKC, that we used in control experiments.

REFERENCES

[1] Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276.
[2] Nishizuka, Y. (1984) Nature 308, 693–698.
[3] Bell, R.M. (1986) Cell 45, 631–632.
[4] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[5] Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610–7616.
[6] Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341–13348.
[7] Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7039–7043.
[8] Ludérus, M.E.E., Reymond, C.D., Van Haastert, P.J.M. and Van Driel, R. (1988) J. Cell Sci. 90, 701–706.
[9] Thiery, R., Klein, R. and Tatischeff, I. (1988) FEBS Lett. 241, 149–153.
[10] Van Haastert, P.J.M., De Wit, R.J.W. and Van Lookeren Campagne, M.M. (1988) Biochem. Biophys. Res. Commun. 128, 185–192.
[11] Watts, D.J. and Ashworth, J.M. (1970) Biochem. J. 119, 171–174.
[12] Janssens, P.J.M., Van der Geer, P.L.J., Arents, J.C. and Van Driel, R. (1985) Mol. Cell. Biochem. 67, 119–124.
[13] House, C., Wettenhall, R.E.H. and Kemp, B.E. (1987) J. Biol. Chem. 262, 773–777.
[14] Bartfai, T. (1979) Adv. Cyclic Nucleotide Res. 10, 219–241.
[15] Roskoski, R., jr (1983) Methods Enzymol. 99, 3–6.
[16] Gonzatti-Haces, M.I. and Traugh, J.A. (1986) J. Biol. Chem. 261, 15266–15272.
[17] Ohno, S., Akita, Y., Konno, Y., Imajoh, S. and Suzuki, K. (1988) Cell 53, 731–741.
[18] Ryves, W.J., Garland, L.G., Evans, A.T. and Evans, F.J. (1989) FEBS Lett. 245, 159–163.
[19] Farago, A., Farkas, G., Meszaros, G., Buday, L., Antoni, F. and Seprodi, J. (1989) FEBS Lett. 243, 328–332.
[20] Schaap, D., Parker, P.J., Bristol A., Kriz, R. and Knopf, J. (1989) FEBS Lett. 243, 351–357.
[21] Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932.
[22] Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276.
[23] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[24] Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) Biochem. Biophys. Res. Commun. 159, 548–553.
[25] Janssens, P.M.W. and Van Haastert, P.J.M. (1987) Microbiol. Rev. 51, 396–418.
[26] Janssens, P.M.W. (1987) Trends Biochem. Sci. 12, 456–459.