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A pseudosubstrate peptide inhibits protein kinase C-mediated phosphorylation in permeabilized Rat-1 cells

Thomas Eichholtz, Jacqueline Alblas, Merian van Overveld, Wouter Moolenaar and Hidde Ploegh

Department of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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Activation of protein kinase C (PKC) in Rat-1 fibroblasts leads to rapid phosphorylation of an 80-kDa protein, a major substrate of PKC. Digitonin-permeabilized cells perfectly supported this early response. Introduction of a PKC pseudosubstrate peptide inhibited 80 kDa phosphorylation with an IC_{50} of 1 μM, while a control peptide had no effect. The results indicate that this semi-intact cell system can be used in combination with the inhibitory pseudosubstrate peptide to study the involvement of PKC in cellular processes.

Signal transduction; Protein kinase C; Protein, 80 kDa; Pseudosubstrate peptide; (Rat-1 cell)

1. INTRODUCTION

Protein kinase C (PKC) is a key enzyme involved in the control of a wide variety of physiological processes [1]. Among the more prominent of the short-term events induced upon activation of PKC is the phosphorylation of a ubiquitous acidic protein with an apparent molecular mass of 80 kDa [2-7], which has been cloned and sequenced recently [8].

Examination of the amino acid sequence of PKC revealed that the regulatory domain of the enzyme contains a sequence (residues 19-36) that fulfills most of the requirements for a PKC phosphorylation site. It contains a number of basic amino acid residues [9], but with an alanine residue instead of the phosphate acceptor serine. A synthetic peptide corresponding to this sequence was shown to inhibit PKC activity in in vitro assays [10], suggesting a possible role for this sequence in regulating PKC activity in vivo. This proposed mode of regulation of kinase activity may be widespread for serine/threonine kinases, as all members of this family contain pseudosubstrate sequences [11-18].

Little is known about the direct involvement of PKC in a number of processes in which the enzyme is supposed to play a role, like receptor down-regulation, cytosolic alkalinization and modulation of other components of the signal transduction machinery. These phenomena cannot be studied easily in cell-free systems. To assess the role of PKC under conditions that approximate the physiological situation, the pseudosubstrate peptide was used as an inhibitory probe in permeabilized cells [19,20]. We show that this peptide inhibits phosphorylation of the 80-kDa substrate induced by PKC activation, while a peptide capable of inhibiting cAMP-dependent protein kinase [13] has no effect.

Correspondence address: H. Ploegh, Department of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

2. MATERIALS AND METHODS

2.1. *Materials*

Tetradecanoyl phorbol acetate (TPA), digitonin and ATP were obtained from Sigma. [^{32}P]P_i and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham International. Peptides were synthesized as COOH-terminal amides using the Merrifeld procedure [21] on a Biosearch 9500 peptide synthesizer. Cell culture materials were from Gibco BRL.

2.2. *Cell culture*

Rat-1 cells (passage number 5-15) were grown to confluency in 35-mm dishes in DMEM/10% (v/v) fetal calf serum. To induce quiescence, cells were serum-starved for 24 h before experiments.

2.3. *Phosphorylation in intact cells*

Confluent quiescent Rat-1 cells were labeled with 0.5 mCi of carrier-free [^{32}P]P_i for 1 h at 37°C. 100 nM TPA was added for 15 min. After incubation cells were solubilized in SDS-sample buffer containing 4% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.0625 M Tris (pH 6.8), scraped off the dishes and heated to 100°C for 5 min.

2.4. *Phosphorylation in permeabilized cells*

Confluent and quiescent monolayers of Rat-1 cells were washed once in permeabilization buffer containing (in mM) 120 KCl, 30 NaCl, 1 $MgCl_2$, 1 K_2HPO_4, 10 sodium PIPES (pH 7.4), 1 EDTA and 0.037 $CaCl_2$. The final free-calcium concentration of this medium is 15 nM [22]. The assays were started by the addition of 200 μl permeabilization buffer containing 40 μM digitonin and 10 μM [γ-^{32}P]-ATP. TPA and peptides were added as described in the text. Cells were incubated at 37°C for 30 s or as indicated in the text. The assay was stopped as described for intact cells.

2.5. *Sample analysis*

Analysis of samples by two-dimensional gel electrophoresis was carried out as described buy O'Farrell [23]. Isoelectric focusing in the

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first dimension was performed using 1% ampholines 3.5–10 and 4% ampholines 3.5–5. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [24]. ^{32}P-labeled proteins were detected by autoradiography using Kodak XAR-5 films and DuPont intensifying screens. Gel slices were cut out and radioactivity in the slices was determined by liquid scintillation counting.

3. RESULTS AND DISCUSSION

Cells permeabilized by the addition of 40 μM digitonin were used to study PKC-mediated phosphorylation of the 80-kDa protein. Although this procedure eventually leads to a significant loss of PKC from the cells [19], PKC-mediated phosphorylation can still be detected, as was reported recently [20]. Fig.1 shows a comparison of the 80-kDa protein from intact cells labeled with ^{32}P[P]$_i$ and stimulated with 100 nM TPA for 15 min, and permeabilized cells incubated with [γ-^{32}P]ATP in the presence of 100 nM TPA for 5 min. Analysis of two-dimensional gel electrophoresis showed that the 80-kDa PKC substrate, as observed in permeabilized cells, can be identified as the well-known 80-kDa substrate seen in in vivo studies [2–7], judged by its electrophoretic behavior and PKC-dependent phosphorylation. In this semi-intact system, a marked stimulation of 80-kDa protein phosphorylation was detected already after 30 s (fig.2). At 30 s, the 80-kDa protein is the major phosphoprotein; at later time points, the complexity of the phosphoprotein pattern increased dramatically, as can be seen in fig.1. Phosphorylation of the 80-kDa protein was stimulated 3–5-fold by TPA, as compared to control levels.

The so-called pseudosubstrate peptide with the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val, derived from a part of the regulatory domain of PKC, was shown to be a potent inhibitor of PKC with an IC_{50} of 92 nM [9]. The preparation of the synthetic peptide actually used in the present study was found to inhibit purified rat brain PKC with an IC_{50} of approximately 100 nM, in accordance with the published data (not shown). This peptide was introduced into permeabilized cells in concentrations ranging from 0.3 to 300 μM. Addition of the pseudosubstrate peptide led to a marked decrease in phorbol ester-induced 80-kDa phosphorylation (fig.2). Half-maximal inhibition was observed at about 1 μM peptide (fig.2B). This value is considerably higher than the values obtained in in vitro studies. This discrepancy could be due to a higher affinity of PKC for the 80-kDa protein in permeabilized cells, as compared to a peptide substrate used in in vitro assays. An alternative explanation would be a lower effective peptide concentration in the permeabilized cells than actually administered in the extracellular medium. The phosphorylation of the 80-kDa substrate in unstimulated cells was already completely inhibited at 300 nM pseudosubstrate peptide (fig.2), indicating a greater availability of the unstimulated kinase for the peptide.

In fig.3, it is shown that a peptide with the sequence Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp, capable of inhibiting cAMP-dependent protein kinase [13], does not affect 80-kDa phosphorylation at concentrations ranging from 0.1 to 100 μM. Only at 300 μM can a slight inhibi-

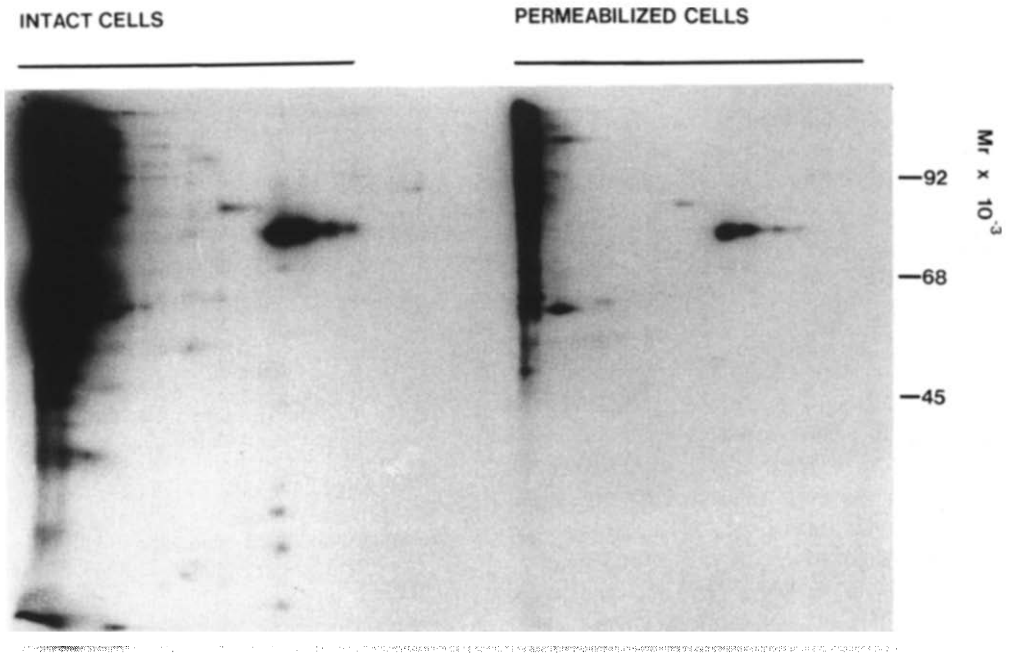

Fig.1. Comparison of the 80-kDa protein in intact and digitonin-permeabilized cells on two-dimensional gel electrophoresis. Intact cells were labeled with [^{32}P]P$_i$ for 1 h and stimulated with 100 nM TPA for 15 min. Permeabilized cells were incubated in the presence of digitonin, [γ-^{32}P]ATP and 200 nM TPA for 5 min, as described in section 2. In the first dimension, proteins were separated by isoelectric focusing from left (basic) to right (acidic); in the second, by SDS-polyacrylamide gel electrophoresis.

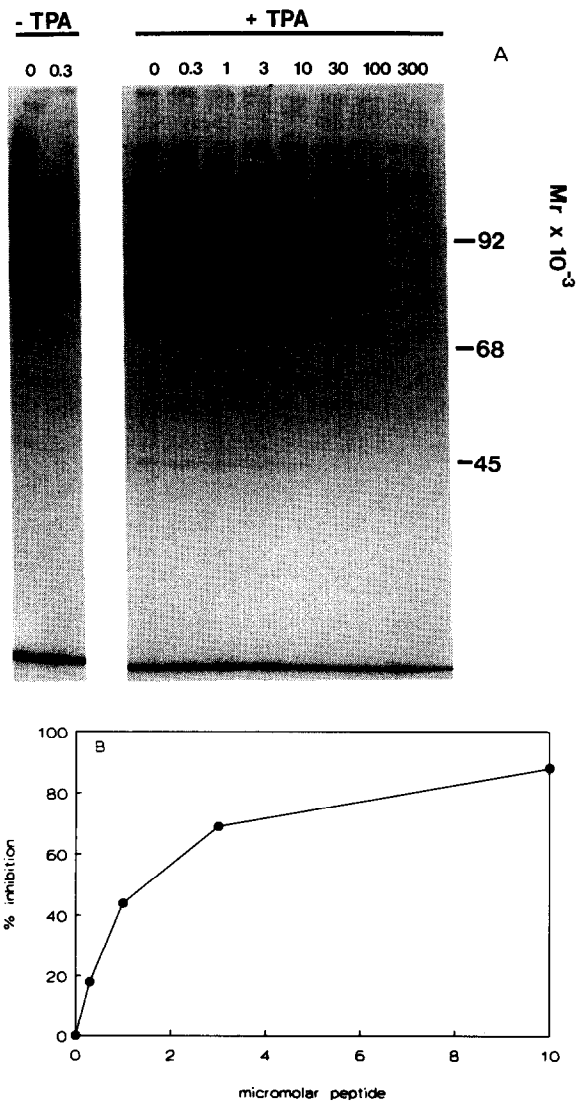

Fig.2. Inhibition of phorbol ester-induced 80-kDa protein phosphorylation in digitonin-permeabilized cells. (A) Cells were incubated for 30 s in the presence or absence of 100 nM TPA and pseudosubstrate concentrations in micromolar concentrations as indicated. (B) Quantification of the 80-kDa phosphorylation. Percentage inhibition of 80-kDa phosphorylation is plotted against pseudosubstrate peptide concentration.

tion of 80-kDa phosphorylation be seen. This effect is probably not specific, because at that concentration both peptides slightly inhibit background phosphorylation also.

Thus, it is demonstrated that the pseudosubstrate peptide can be used successfully as a specific inhibitor of PKC in permeabilized cells, as was also shown recently for phorbol ester-induced phosphorylation of the CD3 antigen in T lymphocytes [25]. Activation and inhibition of phospholipase C [26] and epidermal growth factor receptor down-modulation (unpublished observations) are supported in the semi-intact cell system. It will now be of interest to verify the proposed

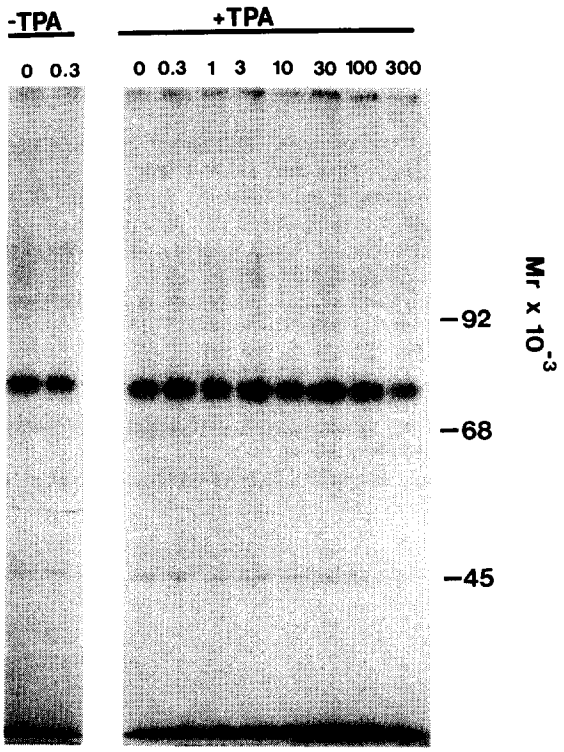

Fig.3. Introduction of control peptide into permeabilized cells. Cells were incubated for 30 s in the presence or absence of 100 nM TPA and peptide concentrations (micromolar) as indicated.

role of PKC in these processes. The approach reported here may provide a novel tool to study the involvement of PKC in cellular processes, in addition to more conventional approaches like PKC down-regulation by long-term pretreatment with phorbol ester [27] and the use of specific inhibitors such as staurosporin [28] and 1-*O*-alkyl-2-*O*-methylglycerol [29].

REFERENCES

[1] Nishizuka, Y. (1988) Nature 334, 661–665.
[2] Rozengurt, E., Rodriguez-Pena, A. and Smith, K.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7244–7248.
[3] Rozengurt, E., Rodriguez-Pena, A., Coombs, M. and Sinnett-Smith, J. (1984) Proc. Natl. Acad. Sci. USA 81, 5748–5752.
[4] Rozengurt, E. and Rodriguez-Pena, A. (1985) EMBO J. 4, 71–76.
[5] Rozengurt, E. and Rodriguez-Pena, A. (1986) EMBO J. 5, 77–83.
[6] Blackshear, P.J., Witters, L.A., Girard, P.R., Kuo, J.F. and Quamo, S.N. (1985) J. Biol. Chem. 260, 13304–13315.
[7] Blackshear, P.J., Wen, L., Glynn, B.P. and Witters, L.A. (1986) J. Biol. Chem. 261, 1459–1469.
[8] Stumpo, D.J., Graff, J.M., Albert, K.A., Greengard, P. and Blackshear, P.J. (1989) Proc. Natl. Acad. Sci. USA 86, 4012–4016.
[9] House, C. and Kemp, B.E. (1987) Science 238, 1726–1728.
[10] House, C., Wettenhall, R.E.H. and Kemp, B.E. (1987) J. Biol. Chem. 262, 772–777.
[11] Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M. and McCarthy, D. (1978) J. Biol. Chem. 253, 3997–4003.

[12] Lincoln, T.M., Flockhart, D.A. and Corbin, J.D. (1978) J. Biol. Chem. 253, 6002–6009.
[13] Cheng, H.-C., Kemp, B.E., Pearson, R.B., Smith, A.J., Misconi, L., Van Patten, S.M. and Walsh, D.M. (1985) J. Biol. Chem. 261, 989–992.
[14] Kennelly, P.J., Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987) J. Biol. Chem. 262, 11958–11963.
[15] Kemp, B.E., Pearson, R.B., Guerriero, V., Bagchi, I.C. and Means, A.R. (1987) J. Biol. Chem. 262, 2542–2548.
[16] Kelly, P.T., Weinberger, R.P. and Waxham, M.N. (1988) Proc. Natl. Acad. Sci. USA 85, 4991–4995.
[17] Pearson, R.B., Wettenhall, R.E.H., Means, A.R., Hartshorne, D.J. and Kemp, B.E. (1988) Science 241, 970–973.
[18] Hardie, G. (1988) Nature 335, 592–593.
[19] TerBush, D.R. and Holz, R.W. (1986) J. Biol. Chem. 261, 17099–17106.
[20] Erusalimsky, J.D., Friedberg, I. and Rozengurt, E. (1988) J. Biol. Chem. 263, 19188–19194.
[21] Hodges, R.S. and Merrifield, R.B. (1985) Anal. Biochem. 65, 241–272.
[22] Barrowman, M.M., Cockroft, S. and Gomperts, B.D. (1986) Nature 319, 5094–5097.
[23] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Alexander, D.R., Hexham, J.M., Lucas, S.C., Graves, J.D., Cantrell, D.A. and Crumpton, M.J. (1989) Biochem. J. 260, 893–901.
[26] Stutchfield, J. and Cockroft, S. (1988) Biochem. J. 250, 375–382.
[27] Rodriguez-Pena, A. and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. 120, 1053–1059.
[28] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Mozimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[29] Van Blitterswijk, W.J., Van der Bend, R.L., Kramer, IJ.M., Verhoeven, A.J., Hilkmann, H. and De Widt, J. (1987) Lipids 22, 842–846.