Volume 284, number 1, 120–122 FEBS 09810 June 1991

ADONIS 001457939100518 8

Down-regulation of a kinase defective PKC-α

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Received 12 April 1991

A kinase defective mutant of PKC-α down-regulates in response to phorbol esters as effectively as the wild-type protein when introduced into COS-1 cells. This demonstrates that intramolecular autophosphorylation is not a prerequisite for down-regulation.

Protein kinase C; Down regulation; Kinase deficient mutant

1. INTRODUCTION

Down-regulation of protein kinase C (PKC) has been effected in various cell types through chronic exposure to phorbol esters. While this phenomenon has been of use in implicating this pathway in agonist responses, the precise underlying mechanisms have not been clearly defined. We have previously shown that down-regulation of PKC-α in a glioma cell line is a direct consequence of an increased rate of proteolysis with no change in synthetic rate [1]. The induction of down-regulation appears then to be a consequence of activation of PKC and as such it remains to be determined whether the increased proteolysis reflects: (i) a conformational effect on the PKC itself making it a better protease substrate as observed in vitro [2]; (ii) a secondary consequence of activation, namely autophosphorylation; (iii) activation of a protease; or (iv) some combination of the above. In order to address the issue of autophosphorylation in relation to down-regulation, a kinase defective form of PKC-α which still binds phorbol ester has been assessed for its ability to be down-regulated.

2. MATERIALS AND METHODS

2.1. *Mutagenesis, subcloning and expression in COS-1 cells*

PKC-α kin⁻ was obtained by site-directed mutagenesis of lysine 368 of PKC-α to a methionine residue. Similar mutagenesis of this conserved residue of the ATP binding site has previously been shown to abolish the activity of other kinases (e.g. [3,4]). Wild-type PKC-α and PKC-α kin⁻ were subcloned into the mammalian expression vector pMT-2 under the control of the adenovirus major late promoter [5] to generate pMT-PKC-α and pMT-PKC-α kin⁻. These constructs were used to transfect COS-1 cells as described previously [6]. The transfected cells were incubated in the presence of 0.5% fetal calf serum and 400 nM 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) was added after various time intervals as indicated in the text or figures; all cells were harvested 48 h after transfection.

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2.2. *Expression using recombinant baculovirus*

Recombinant baculovirus containing the coding sequences for PKC-α kin⁻ was isolated as previously [7]. Sf9 cells, seeded at 10^6 cells per 6 cm dish, were infected with recombinant baculovirus containing PKC-α, PCK-α kin⁻, or as a control a recombinant virus containing E1A (kindly provided by G. Patel and N. Jones, London) and harvested 3 days post-infection.

2.3. *Phorbol ester binding*

COS-1 cells (48 h after transfection) or Sf9 cells (3 days post-infection) were lysed in buffer containing 1% (v/v) Triton X-100, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 50 mM sodium fluoride, 25 μg/ml leupeptin, 50 μg/ml phenylmethylsulphonylfluoride, 10 mM benzamidine and 0.3% (v/v) β-mercaptoethanol. After removal of insoluble material by centrifugation at 12000 rpm, 4°C for 10 min, binding of [^{3}H]phorbol dibutyrate (PDBu) was determined as in [8].

2.4. *Other methods*

Western analysis on whole cell extracts was performed using antisera raised against a C-terminal peptide of PKC-α [9]. Protein kinase C activity was assayed using either histone III S (1.25 mg/ml) or a peptide based on the pseudosubstrate site of PKC-α (0.125 mM) as substrate [9].

3. RESULTS

In order to compare the down-regulation of PKC-α kin⁻ wild-type PKC-α functional expression was tested by transfection of COS-1 cells. Transient expression of both PKC-α and PKC-α kin⁻ led to an increase in extractable phorbol ester binding activity (Table I). However, while introduction of PKC-α led to an increase in kinase activity, PKC-α kin⁻ did not (Table I). In order to further establish this distinction, recombinant baculovirus vectors were used to introduce PKC-α and PKC-α kin⁻ into Sf9 cells. As with COS-1 expressed constructs, both wild-type and mutant protein showed phorbol binding activity but only the wild-type PKC-α showed kinase activity (Table I).

To determine the sensitivity to down-regulation, PKC-α kin⁻ was transiently expressed in mammalian

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Table I

Activities of expressed PKC-α and PKC-α kin$^-$

Protein	COS-1 cells		Sf9 cells	
	PDBu bound (nmol/10^6 cells)	Kinase activity (cpm)	PDBu bound (nmol/10^6 cells)	Kinase activity (cpm)
Control	9.6	5560	15	–
PKC-α	20	19416	35	20746
PKC-α kin$^-$	31	3507	43	–

Control cells are either COS-1 cells transfected with pMT2 vector or Sf9 cells infected with a recombinant baculovirus expressing the adenovirus E1A protein as appropriate. Cells were lysed in 1% (v/v) Triton X-100 and insoluble material removed by centrifugation before determination of the binding of [^{3}H]phorbol ester dibutyrate ([^{3}H]PDBu) to the soluble fraction as described in Methods. Kinase activity was determined directly in cell extracts of Sf9 cells or following ammonium sulphate fractionation of COS-cell extracts. Activities represent phospholipid/TPA-dependent histone kinase activity using standard assay conditions [9]. Shown are data from one of two similar series of experiments.

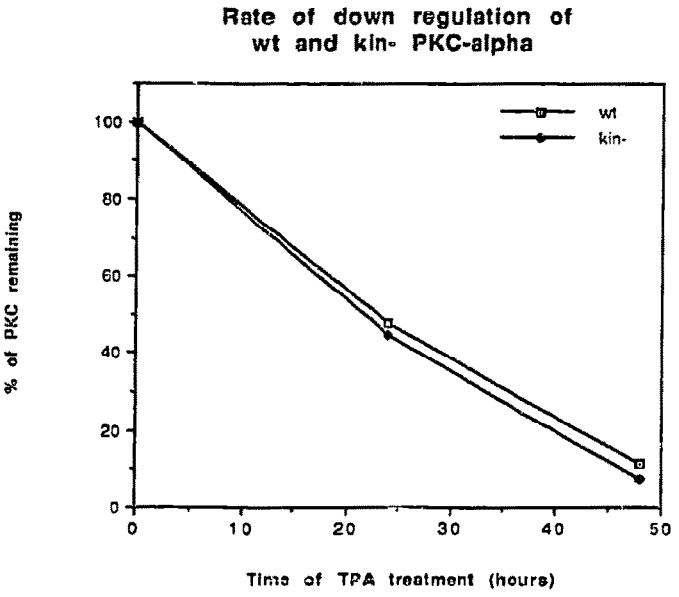

Fig. 2. A quantitative comparison of PKC-α and PKC-α kin$^-$ down-regulation. Shown is a time course of down-regulation of PKC-α and PKC-α kin$^-$ in COS-1 cells. After autoradiography of a Western blot following the time-dependent loss of PKC antigen, the radioactive bands were excised from the nitrocellulose and the ^{125}I-protein A counted. The percentage of PKC-α antigen remaining is plotted against the time of incubation of the cells with TPA.

COS-1 cells. The cells were then exposed to either TPA or vehicle and subsequently analysed for PKC-α expression by Western analysis (Fig. 1). It is evident that this mutant PKC-α is readily down-regulated in these cells. The time course of down-regulation is consistent with that observed for wild-type PKC-α (Fig. 2). To control for the effect of the promoter on the TPA-induced effects, an adenovirus major late promoter – chloramphenicol acetyl transferase (CAT) construct – was also introduced into COS-1 cells and the expressed CAT activity was found not to be reduced by TPA treatment; in fact, a small increase is observed (190% of control; mean of triplicate observations).

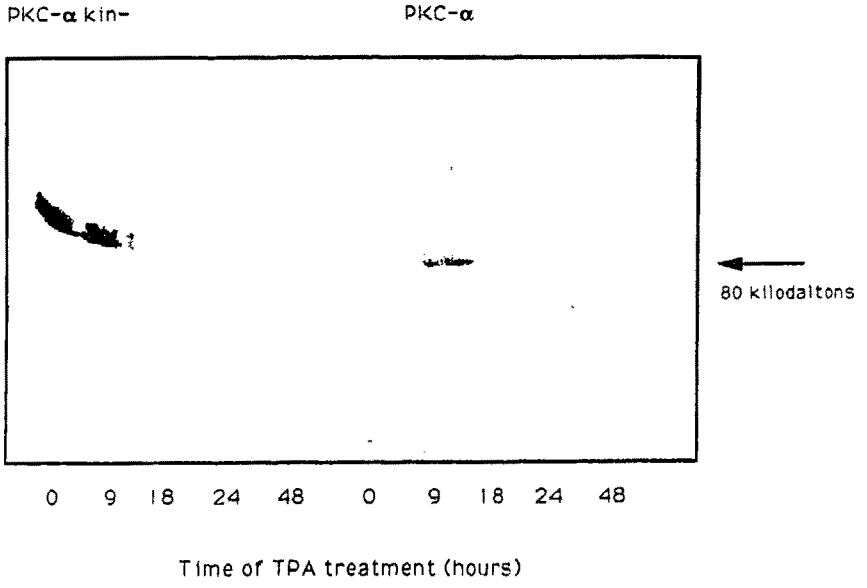

Fig. 1. Western analysis of the TPA-induced down-regulation of PKC-α and PKC-α kin$^-$ expressed in COS-1 cells. COS-1 cells were transfected with pMT-PKC-α or pMT-PKC-α kin$^-$ and incubated for 48 hours in medium containing 0.5% fetal calf serum. TPA (400 nM) was added at the indicated times before harvesting. Western analysis was performed using an antibody raised against the C-terminal peptide of PKC-α [9]. This is one of several similar time courses.

4. DISCUSSION

The results show that in COS-1 cells, the PKC-α kin$^-$ mutant is susceptible to TPA-induced down-regulation. This demonstrates that intramolecular phosphorylation is not a prerequisite for down-regulation in COS-1 cells. This does not rule out the possibility that the endogenous COS-1 cell PKC-α can trigger down-regulation through phosphorylation of the introduced PKC-α kin$^-$ protein. However, it should be noted that the down-regulation of the PKC-α kin$^-$ protein does not 'recover' at a time when in control cultures the endogenous PKC-α becomes down-regulated. It would appear likely therefore that the induced down-regulation is a function of altered conformation and/or activation of a cellular protease pathway.

This conclusion is at odds with the observations of Ohno and colleagues [10], who expressed a similar PKC-α mutant in 3YI fibroblasts, but were unable to induce down-regulation. It is difficult to reconcile their results with those presented here and it can only be concluded that the difference is a vector/context-dependent effect (in the previous studies there could be a compensatory increase in synthesis of the mutant protein that masks the increase in proteolysis). The results presented here are similar to those observed for a PKC-γ kin$^-$ mutant which will also down-regulate (S. Stabel, personal communication), suggesting that this is not an exceptional case.

Previously it has been demonstrated that the initial step in down-regulation is cleavage in the V_3 region of PKC-α [11] although the precise mechanism has yet to be defined. While trypsin will cleave at one of four defined V_3-region basic residues in vitro [11], these are not targets for the endogenous protease, since mutation of these does not prevent down-regulation (C.P., unpublished). Calpain by contrast has been shown to cleave at distinct sites in vitro [2] and evidence has been presented to suggest that this pathway may be operative in vivo [12]. If indeed Calpain is primarily responsible for the increased proteolysis, it remains to be determined whether there is a TPA-induced increase in Calpain activity or whether the conformational change in PKC wholly accounts for the observed down-regulation with Calpain playing a passive role.

In conclusion, the results demonstrate that intramolecular autophosphorylation of PKC-α is not a prerequisite for phorbol ester-induced down-regulation.

Acknowledgements: We should like to thank Dr. S. Stabel for communication of results prior to publication, Dr. R.M. Marais and Dr. C. Dickson for critical comments and Amanda Wilkinson for typing the manuscript.

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