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Downregulation of protein kinase C-γ is independent of a functional kinase domain

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Prolonged activation of protein kinase C (PKC) types α and β by tumor-promoting phorbol esters leads to desensitization of the phorbol ester response, downregulation of protein kinase C activity and depletion of the protein kinase C polypeptide. When the γ isoenzyme of PKC is transiently expressed in COS-1 cells and exposed to phorbol esters, PKC-γ is downregulated in COS cells although these cells do not normally express this subtype. A point mutation in the putative ATP-binding site (Lys-380→Met-380) of the protein kinase C γ isoenzyme which results in a kinase-deficient enzyme does not interfere with this downregulation. Our results suggest that autophosphorylation or constitutive signalling through the protein kinase C-γ kinase domain is not a prerequisite for downregulation of PKC activity.

Protein kinase C; Downregulation; Baculovirus expression; Kinase-deficient mutant; Phorbol ester

1. INTRODUCTION

Protein kinase C (PKC) comprises a family of four structurally closely related subtypes α, β_1, β_2, and γ which are all dependent on calcium and phospholipid for their kinase activity and are activated by diacylglycerol and tumour-promoting phorbol esters (reviewed in [1–3]). Based on structural homology at least three additional members, δ, ε and ζ, have been added to the PKC family which differ from the latter group in that they do not require calcium for phorbol ester binding or kinase activity or do not appear to bind phorbol esters at all [4–7].

The members of the PKC family are thought to be part of the signal transduction pathway which initiates with the turnover of phosphatidylinositol, although the role of the individual isoenzymes in this pathway has not yet been elucidated. It is conceivable that all PKC subtypes, which bind phorbol ester in vitro, contribute to the multiple effects of phorbol esters in the animal model as well as in cell culture [8] and binding of phorbol esters appears to be mediated by a cysteine-rich zinc-finger structure proposed for all PKC isoenzymes in the N-terminal part of the molecule [9].

The action of phorbol esters on PKC has been extensively studied in vitro and in vivo. In vivo, biologically active phorbol esters induce association of the PKC molecule with the particulate fraction which is concomitant with activation [10]. Upon prolonged exposure to phorbol esters the PKC molecule is degraded with a faster rate than it is synthesized leading to 'downregulation' or depletion of immunologically detectable PKC [11–13].

The degradation process has been claimed to involve calpain [14] and the isoenzymes show differential susceptibility to proteolysis by calpain in vitro [15]. In addition, it has been postulated that proteolytic activation of PKC might be a physiological process generating an activated kinase fragment involved in the cellular signal transduction process [16]. Hence downregulation may be viewed as an exaggeration of a physiological process which leads to cofactor-independent phosphorylation by the PKC polypeptide.

In an attempt to answer the question whether downregulation of PKC is a consequence of constitutive signalling through the PKC kinase domain and whether the phosphotransferase activity of the kinase is required for this process, we have introduced a point mutation into the putative ATP-binding site of bovine PKC-γ and studied its effect on the properties of the enzyme. The mutant polypeptide was expressed in insect cells from a Baculovirus vector to examine its enzymatic behaviour and was analysed for its response to phorbol ester after transient expression in COS-1 cells. In vitro phosphorylation assays show that the mutation leads to a kinase-negative polypeptide which downregulates in response to phorbol esters to the same extent as wild-type PKC-γ. This behaviour suggests that downregulation of PKC-γ occurs independently of the autophosphorylating and signalling capacity of the polypeptide. It has been proposed that autophosphorylation might enhance the susceptibility of PKC-βII towards proteases and thereby indirectly

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promote downregulation of the PKC polypeptide [17].

Our results are in contrast to a recent study on downregulation of PKC-α which shows that a kinase-deficient mutant of PKC-α is not able to downregulate in the presence of phorbol ester after stable introduction into rat fibroblasts under the control of a glucocorticoid-inducible promoter [18]. This apparently different effect of the phosphotransferase activity of the isoenzymes α and γ may hint at a very distinct behaviour of the isoenzymes in response to phorbol ester.

2. MATERIALS AND METHODS

2.1. *Mutagenesis of PKC-γ*

A 259 base pair *SacI/PstI* fragment was subcloned from pAcC4-PKC-γ [19,20] into M13mp18 and mutagenized with an oligonucleotide-directed site-specific mutagenesis kit (Amersham) using a mutant oligonucleotide of the sequence 5'-CTTCAGGATC$\underline{A}$-TGATGGCG-3' which changes lysine-380 into methionine and abolishes an *XhoII* restriction site. Successful mutagenesis was confirmed by restriction analysis and by sequencing the complete mutant phage DNA insert.

The mutated fragment was re-introduced into the pAcC4-PKCγ plasmid. From there the complete coding region of bovine PKC-γ was inserted via *Eco*RI linkers into the *Eco*RI site of the vector PMT-2 [21], kindly provided by Dr John Knopf (Genetics Institute, Cambridge, MA).

2.2. *Expression of mutant PKC-γ from a Baculovirus vector*

The mutated PKC-γ cDNA carrying a Lys-380→Met-380 replacement was inserted into the baculovirus expression vector pAcC4, kindly provided by Dr Frank McCormick, as described [20]. Recombinant virus was isolated according to published protocols [22], and used to infect *Spodoptera frugiperda* (Sf9) insect cell cultures. Expression of the recombinant PKC-γ polypeptide was detected by immunoblotting whole insect cell lysates.

2.3. *Measurement of PKC activity and autophosphorylation*

3×10^6 insect cells were infected with recombinant Baculovirus, harvested after 2 days, lysed in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 0.1% Triton X-100 and centrifuged at $10000 \times g$ for 10 min at 4°C.

Aliquots of the supernatant were used undiluted or diluted in PKC activity assays using histone IIIS (Sigma) as substrate. PKC activity assays were performed for 5 min at 30°C as described [20] and contained in a volume of 40 μl: 50 mM Tris-HCl, pH 7.4, 1.25 mg/ml histone, 12.5 mM $MgCl_2$, 750 μM $CaCl_2$, 0.25% Triton X-100, 125 μM [γ-^{32}P]ATP with or without 625 μg/ml phosphatidylserine (Lipid Products, Surrey, UK)/2 μM phorbol 12-myristate 13-acetate (PMA) (Sigma).

For autophosphorylation, histone substrate was omitted in the assay and the reaction was stopped after 5 min by boiling with SDS-sample buffer [24].

PKC polypeptides were immunoprecipitated with the 0442 antibody after dilution of the samples as described earlier [20] and phosphorylated proteins were identified by SDS-polyacrylamide electrophoresis and autoradiography.

2.4. *Transfection of COS-1 cells*

Transfection of COS-1 cells on 60 mm or 100 mm dishes was carried out as described [23]. Cells were harvested after 48–54 h by lysis in boiling hot SDS-sample buffer [24] with or without previous exposure to PMA for 16 h at a final concentration of 400 nM.

2.5. *Measurement of PDB binding*

Sf9 insect cells were seeded at 2.5×10^6 cells on 60 mm dishes, and infected with recombinant Baculovirus. At 48 h post-infection cells were washed twice with PBS and incubated with 0.5 μCi/ml (11 nM) [^{3}H]PDB (Amersham) for 30 min at 37°C. After incubation cells were washed twice with PBS and cell-associated radioactivity was counted in a liquid scintillation counter.

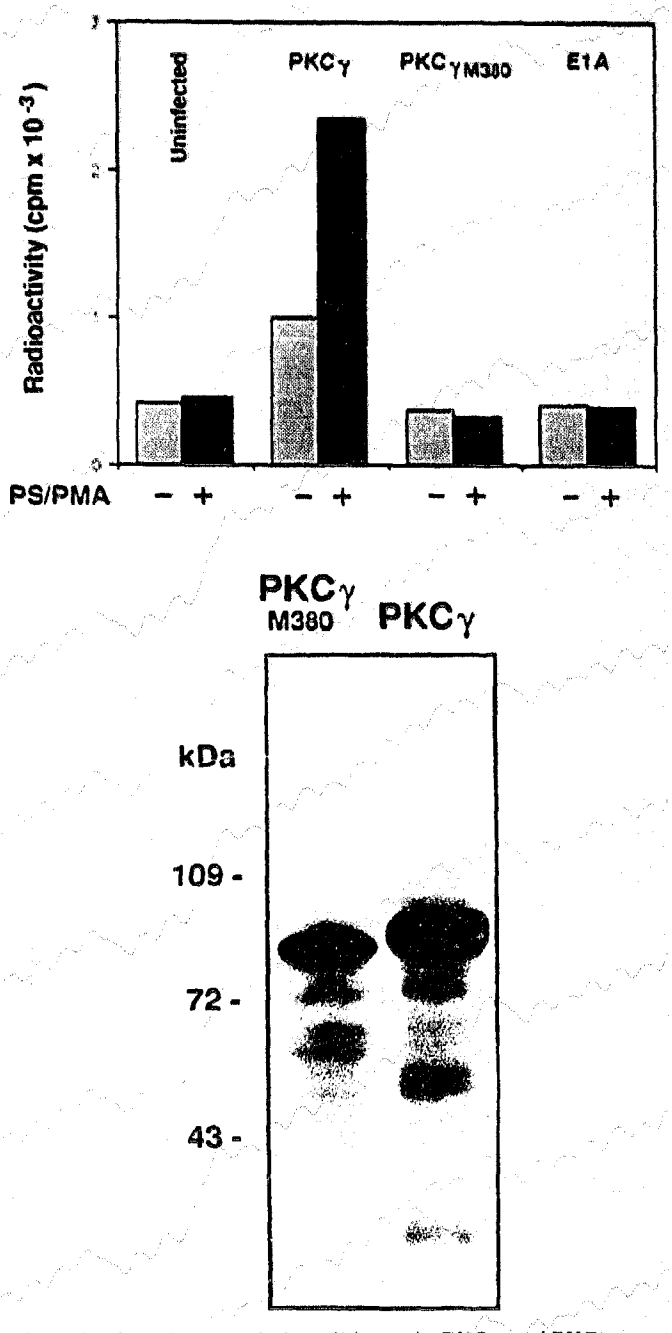

Fig. 1. In vitro phosphorylation of histone by PKC-γ and PKC-γ_{M380}. (A) 3×10^6 insect cells were infected with recombinant Baculoviruses expressing PKC-γ, PKC-γ_{M380}, Adenovirus E1A [31] as indicated. At 2 days post-infection cells were harvested, lysed and identical aliquots of extracts were analysed for PKC activity in the presence (black bars) or absence (hatched bars) of phosphatidylserine/PMA. (B) Aliquots of extracts used in (A) were immunoblotted with the antibody 0442 to verify the presence of PKC polypeptides. Note the apparent lower molecular weight of PKC-γ_{M380}.

2.6. *Other methods*

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [24]. Immunoblotting and immunoprecipitation was performed as described earlier [12,20].

3. RESULTS

3.1. *Replacement of lysine-380 of PKC-γ by methionine abolishes kinase activity but does not alter phorbol ester binding*

The putative ATP binding site in PKC-γ has been located to the C-terminal half of the molecule according to the presence of a stretch of residues conserved in all protein kinases [25]. These residues include the conserved lysine residue (lysine-380) which has been shown to make direct contact with ATP in other kinases [26]. Substitution of this residue in a number of tyrosine kinases results in loss of kinase activity and alteration of associated functions (e.g. [27–30]).

We have mutated lysine-380 in bovine PKC-γ to methionine (PKC-γ_{M380}) and analysed the effect of such a point mutation on the kinase activity of PKC-γ after expression in insect cells from a recombinant Baculovirus. The mutated PKC cDNA was expressed in insect cells since endogenous PKC activity is not detectable in these cells, which facilitates the analysis of exogenously introduced PKC [20].

Kinase activity was measured in cell extracts from cells infected with recombinant Baculovirus expressing wild-type PKC-γ, mutated PKC-γ_{M380} and the Adenovirus E1A protein [31] as a negative control (Fig. 1A). Cells expressing wild-type PKC-γ showed elevated histone phosphorylation which was specifically increased in the presence of PKC activators. Cell extracts containing PKC-γ_{M380} showed no detectable PKC kinase activity above control cells expressing the unrelated product of the Adenovirus E1A gene which is a transcriptional activator with no associated protein kinase activity (Fig. 1A). The presence of wild-type and mutated protein in the assays was verified by immunoblotting aliquots of the kinase assays with the PKC-specific antiserum 0442 [25] (Fig. 1B).

Mutation of lysine-380 also abolished PKC autophosphorylation as shown in Fig. 2. Mutation of the kinase domain did not, however, affect phorbol ester binding. Wild-type PKC-γ and PKC-γ_{M380} exhibited similar phorbol-dibutyrate binding activities with the kinase-negative polypeptide showing slightly higher PDB binding in two separate experiments (Table I). The Adenovirus E1A product served as a negative control.

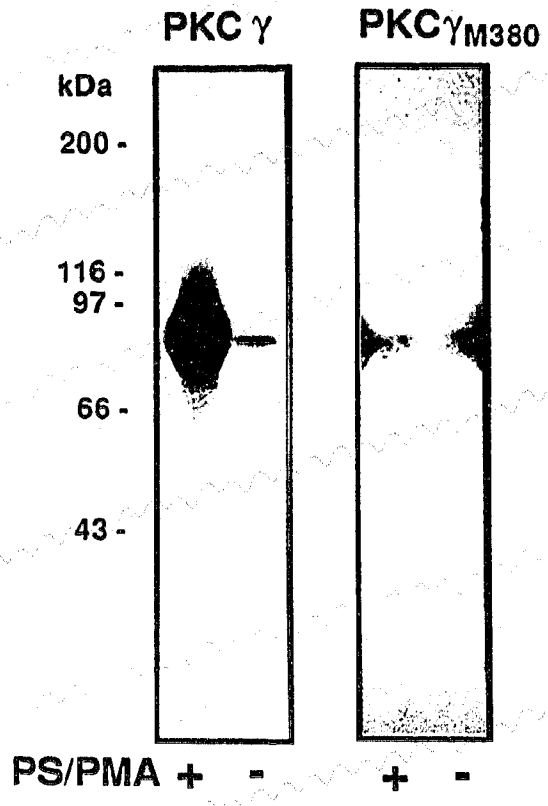

Fig. 2. Autophosphorylation of PKC-γ and PKC-γ_{M380}. 3×10^6 insect cells were infected with PKC-γ and PKC-γ_{M380} Baculoviruses and harvested after 2 days. Cell extracts were incubated in autophosphorylation assays with or without phosphatidylserine/PMA as indicated. After the phosphorylation reaction PKC polypeptides were immunoprecipitated with the 0442 antibody and labelled proteins were identified by autoradiography. The left half of the gel was exposed for 4 days, the right half for 6 days.

3.2. *Phorbol ester causes downregulation of wild-type and kinase-deficient PKC-γ*

In order to analyse the reaction to phorbol esters in vivo, both wild-type and kinase-deficient PKC-γ polypeptides were expressed in COS-1 cells after transfection of the respective constructs. Constitutive expression of polypeptides was driven by the Adenovirus major late promoter, in these constructs.

Fig. 3A shows that PKC-γ can be detected in immunoblots of whole cell extracts from transfected COS-1 cells with a polyclonal antibody that recognizes the α, β and γ isoenzymes of PKC and tubulin ([12], and unpublished data).

If transfected cells are exposed to the phorbol ester PMA for 16 h depletion of immunologically reactive

Table I

PDB binding of PKC-γ and PKC-γ_{M380}

Cells expressing	pmoles PDB bound per 2.5×10^6 cells	
	Expt. 1	Expt. 2
PKC γ	2,93 ± 0.1	3.26 ± 0.01
PKC γ_{M380}	3.62 ± 0.06	3.82 ± 0.02
E1A	0.25 ± 0.03	0.36 ± 0.06

2.5×10^6 insect cells were infected with recombinant Baculoviruses expressing the polypeptides as indicated. [^{3}H]PDB binding was measured as cell-associated radioactivity.

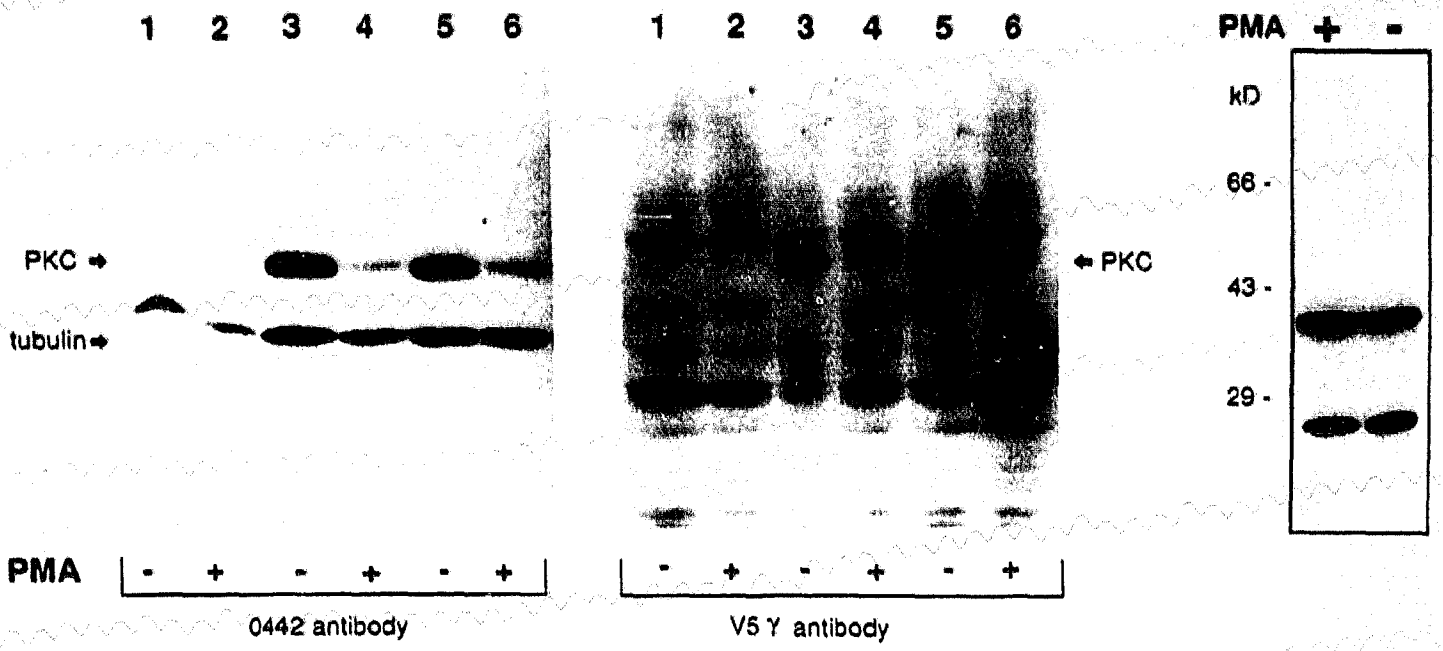

Fig. 3. Downregulation of PKC-γ and PKC-γ_{M380} in COS-1 cells by PMA. (A) COS-1 cells were transfected with vector alone (1,2), PKC-γ(3,4) or PKC-γ_{M380} (5,6). At 32 h post-transfection 400 nM PMA was added to the cultures (+) for 16 h. Cells were harvested in boiling hot sample buffer and analysed by immunoblotting with the antibody 0442 (left half) which detects PKC α, β, γ (80 kDa) and tubulin (55 kDa). Right half: identical samples as in (A) were immunoblotted with a PKC-γ-specific antibody V5-γ [32]. (B) The unrelated protein kinase c-mos (37 kDa) was expressed in COS-1 cells from the same vector and treated with (+) or without (-) 400 nM PMA for 16 h. Cell extracts were immunoblotted with an antiserum against a C-terminal peptide of the c-mos protein. The immunoreactive protein at 27 kDa is a truncated c-mos product derived from an internal translational start.

PKC is observed for PKC-γ and the mutant PKC-γ_{M380} (Fig. 3A).

In order to confirm the identity of the downregulated species detection was performed with a different antiserum specific for PKC-γ, which is directed against the C-terminal 15 amino acids of PKC-γ [32]. Use of this antibody (V5γ) confirms that PKC-γ and PKC-γ_{M380} are downregulated to the same extent by the phorbol ester PMA.

3.3. *Downregulation of transfected PKC-γ in COS cells is not due to promoter effects*

Disappearance of endogenous PKC-α polypeptide after prolonged exposure to phorbol esters has been shown to be due to an increased rate of degradation of the protein and not due to alterations at the transcriptional level [13]. In order to rule out that phorbol ester treatment affected the transcription of the transfected construct which is expressed from the Adenovirus major late promoter, expression of an unrelated protein from the same vector under the same conditions was tested. The unrelated product of the mouse c-mos proto-oncogene [33] was expressed from the same vector and the presence of the c-mos polypeptide was detected with an antiserum against the C-terminal 10 amino acids of the v-mos protein (R.S. Paules and G.F. Vande Woude, manuscript in preparation). Fig. 3B shows that the levels of c-mos expression in transfected COS cells are not afffected by exposure to PMA for 16 h, therefore excluding transcriptional shut-down as a cause for the downregulation of PKC-γ and PKC-γ_{M380}.

4. DISCUSSION

We have shown that substitution of lysine-380 in the putative ATP binding site of PKC-γ results in a kinase-deficient mutant which is no longer able to phosphorylate histone in vitro or autophosphorylate, whereas the extent of phorbol ester binding is not affected by the mutation. After transfection into COS cells wild-type and mutant PKC-γ are expressed as 80 kDa polypeptides in these cells which contain endogenous PKC-α. Although COS cells only express the α type of PKC we show here that transfected PKC-γ is also downregulated in these cells by exposure to the phorbol ester PMA. The same is true for the kinase-negative mutant of PKC-γ. These results suggest that the process of downregulation does not require an active kinase domain in the downregulated molecule. Some indirect effect of activated endogenous PKC-α on the downregulation of PKC-γ cannot be excluded.

While the experiments reported here were in progress a report by Suzuki and coworkers [18] showed that rabbit PKC-α stably introduced into rat fibroblasts under a dexamethasone-inducible promoter was downregula-

tion-resistant when lysine-368 which is part of the ATP binding site of PKC-α was changed to arginine resulting in loss of kinase activity.

Surprisingly, these results reported for PKC-α are in apparent contrast to our results on PKC-γ. Although it is formally possible that the two isoenzymes show such a different behavior, it cannot be ruled out completely that different results may be obtained in stably transfected lines where expression of the exogenous enzyme is induced by steroids. This possibility is supported by the observation that a kinase-deficient mutant of bovine PKC-α appears to downregulate after transient expression in COS cells (C. Pears and P. Parker, personal communication). Another potentially important feature is the nature of the endogenous PKC isoenzyme. Where as Suzuki and coworkers have carried out their studies on exogenously introduced PKC-α, which is the same type as the endogenous enzyme, we have studied the behaviour of an isoenzyme foreign to these cells. However, the observation that exogenously introduced PKC-γ is downregulated in COS cells by PMA convinced us to consider this system as appropriate to study the effect of a mutant kinase domain in this system. In addition, expression of a PKC isoenzyme different from the endogenous type allows immunological distinction between the exogenous and endogenous PKC isoenzymes.

From our results we would therefore conclude that the downregulation of the PKC-γ polypeptide proceeds in the same way whether the kinase domain is functional or not. This implies that downregulation or proteolytic degradation is a direct consequence of perhaps conformational changes induced by phorbol ester binding to the regulatory domain which in turn makes the polypeptide susceptible to proteolytic attack.

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