The broad specificity of dominant inhibitory protein kinase C mutants infers a common step in phosphorylation

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Dominant negative properties are conferred on protein kinase (PK) C α by mutation of the phosphorylation site in the activation loop of the kinase domain. To address the universality and/or specificity of such mutations, analogous alterations were introduced in other members of the PKC family and tested for their effects on the function of co-transfected activated PKC. For all three subclasses of the PKC family, mutations of the predicted activation loop phosphorylation sites resulted in dominant negative properties. These properties were not restricted to the cognate

PKC isotypes, but were effective across the different subclasses. For example, two PKC ζ mutants (atypical isotype) inhibited both PKC α (classical isotype) and PKC ϵ (novel isotype). For all these mutants, inhibition correlated with an ability to prevent the accumulation of phosphorylated PKC α , consistent with the expected mode of action. In the case of the PKC α mutant, it was shown that inhibition required the full-length mutant protein. The results provide evidence for the involvement of a common step in the phosphorylation of all PKC isotypes.

INTRODUCTION

Protein kinase (PK) C is a family of serine/threonine protein kinases that are regulated through agonist-induced hydrolysis of membrane phospholipids and are considered to play critical roles in many signal-transduction pathways [1–3]. This enzyme family comprises eleven members divided into three groups: (i) classical (c)PKC (α , β_1 , β_2 - and γ), which are Ca²⁺- and phorbol-ester/diacylglycerol (DAG)-dependent; (ii) novel (n)PKC (ϵ , δ , η , θ and μ /PKD), which are phorbol-ester-/DAG-dependent but do not require Ca²⁺; and atypical (a)PKC (ζ and λ/ι), which do not respond to Ca²⁺ or phorbol ester/DAG, although PKC ζ can be activated by phosphatidylinositol 3,4,5-trisphosphate [4], as can other PKC family members [5–7].

Several reports have revealed an additional and highly efficient mode of control of PKC involving its phosphorylation. This may involve both autophosphorylation [8] and transphosphorylation [9,10], as well as activation-induced dephosphorylation [11,12]. Consistently with a critical role in function, PKC α expression in bacteria yields an unphosphorylated precursor that does not require phosphorylation for the formation of a functional enzyme–substrate complex, but does appear to require phosphorylation for catalytic activity [13]. In eukaryotes, pulse–chase experiments demonstrate that PKC α undergoes a series of phosphorylations within the first hour post-synthesis that account for its conversion into an active, albeit effector-dependent, protein kinase [14].

Cazaubon and colleagues [15,16] have shown that the phosphorylation of a threonine residue present in the activation loop within the catalytic domain is involved in controlling PKC α activity. Similar observations have been made for PKC β [17]. In PKC α , the substitution of Thr-497 $\rightarrow$ Ala results in the production of an unphosphorylated and inactive 76 kDa PKC α form. Co-expression in COS-1 cells of an activation loop PKC α mutant and wild-type (wt) PKC β_1 leads to the expression of a fast migrating PKC β_1 protein that is similar in size to the unphosphorylated primary translation product of PKC α [15]. Thus this

PKC α mutant appears to have dominant negative properties that can affect PKC β .

Recent studies have suggested that other relevant phosphorylations take place in PKC α . Phosphorylation at Thr-638 at the C-terminus of PKC α controls the duration of activation by regulating the rate of dephosphorylation and, consequently, inactivation of PKC α [18]. The phosphorylation at the equivalent site of PKC β_1 (Thr-642) and PKC β_2 (Thr-641) may be essential for PKC β activity [19,20], although these proteins may also be hypersensitive to dephosphorylation and inactivation (discussed in [14]). Further studies on PKC α define the Ser-657 site of phosphorylation as controlling the accumulation of phosphate at other sites on PKC, as well as contributing to the maintenance of the phosphatase-resistant conformation [14]. Sites equivalent to Thr-638 and Ser-657 have been shown to be phosphorylated in baculovirus-expressed PKC β [19,21]. Recent studies have shown that in PKC β_1 , the C-terminal 642 site also controls Ca²⁺ affinity [22].

Although priming phosphorylations have been well-characterized in the cPKC isotypes PKC α and PKC β , little is known of these events in the novel and atypical classes of PKC. In order to investigate priming phosphorylation in the non-conventional PKCs and the specificity of these processes, activation loop mutations were derived for novel and atypical PKCs. Here we show that putative phosphorylation-site mutation in the activation loops of PKC δ , PKC ϵ and PKC ζ confer dominant negative properties on these proteins *in vivo*. Furthermore, the results show that these inhibitory properties operate in a heterologous fashion, providing evidence for a common step in the processing of all these PKC isotypes.

MATERIALS AND METHODS

Construction of PKC α , δ , ϵ and ζ mutant cDNA expression vectors

PKC mutants were constructed using bovine PKC α , rat PKC δ , mouse PKC ϵ and rat PKC ζ cDNAs [23–26]. Exchange of the

Abbreviations used: DAG, diacylglycerol; FACS, fluorescence-activated cell sorting; PK, protein kinase; PKM, catalytic fragment of PKC; Ψ SS, pseudo-substrate site; TRE, TPA (or PMA) response element; wt, wild-type.

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Table 1 Oligonucleotides employed in the generation of the T-loop PKC mutants

The mutagenic oligonucleotides (with mismatches underlined), the changed amino acid (aa) residue numbers and the oligonucleotides employed for screening are shown for the PKC δ , PKC ϵ and PKC ζ proteins.

Mutant	Changed aa	Mutagenic oligonucleotide	Screening oligonucleotide
$\delta(T/A)_1$	505	AACCGGGCCAGCGCTTTCTGCGGC	$-\delta$ sense: 5'-GGGACTGGCCAAATGAGC-3'
$\delta(ST/A)_2$	504/505	GAGAACCGGGCGGCGCCTTCTGCGGCACT	antisense: 5'-TTACAGGGACCTCAAGC-3'
$\epsilon(T/A)_1$	566	AATGGTGTGACAACCTACCGCCTTCTGTGGG	$-\epsilon$ sense: 5'-TGCCAGCCATCATCTC-3'
$\epsilon(T/A)_4$	563/564/565/566	ATGAATGGTGTGGCGGCGCGCCTTCTGTGGGACT	antisense: 5'-ATGGAGTGATCTACAGG-3'
$\zeta(T/A)_1$	410	CCCGGCGACACAACAGCGCTTTTGTGGA	$-\zeta$ sense: 5'-GTCAAAGGGGAGCGCC-3'
$\zeta(T/A)_4$	407/408/409/410	GGCCCCGGCGACGCGCGCGCCTTTGTGGAACC	antisense: 5'-GGATCATCTACCGGAC-3'

threonine residue(s) with (an) alanine residue(s) was made using the Altered Sites *in vitro* Mutagenesis System (Promega). Briefly, PKC δ *EcoRI*, PKC ϵ *XhoI* and PKC ζ *XbaI* fragments containing the full coding sequence of each respective PKC were cloned into the pAlter-1 vector and mutagenized with appropriate oligonucleotides (Table 1). The introduced mutations created several new restriction sites: *HaeII* in $\delta(T/A)_1$ and $\zeta(T/A)_1$ and *NotI* in $\delta(ST/A)_2$, $\epsilon(T/A)_4$ and $\zeta(T/A)_4$; these sites were utilized during the screening. The mutated sequence was verified by sequence analysis using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH, U.S.A.). The *EcoRI* digestion of the pAlter-1 plasmids containing the different PKC δ mutants yielded a 2.8 kbp fragment that was subcloned into pcDNA3 for expression in COS-1 cells. The *SphI/KpnI* digestion fragment of pAlter-1 containing the PKC ϵ mutants was subcloned in the wrong sense into pSL301. From this vector, a 3.1 kbp *KpnI/SalI* fragment was subcloned into pcDNA3. PKC ζ mutants were digested with *SalI/EcoRI* from pAlter-1 and recloned using an *EcoRI/XhoI* fragment into pcDNA3. The PKC $\alpha(T/A)_3$ mutant was as described previously [15].

The constitutively active PKC α and ϵ mutants were expressed using the pMT2 vector. These PKCs are full-length α and ϵ constructs with a deletion in their inhibitory pseudo-substrate sequences within the regulatory domain. Mutants in the regulatory domain were generated by deletion of amino acids 22–28 in the pseudo-substrate site (ψ SS) of PKC α [27] and 154–163 in PKC ϵ (ψ SS) [28]. The PKC α regulatory domain was generated by taking the *SacI* fragment from pKS-1 PKC α . The 3'-overhang was removed by T4 DNA polymerase and the fragment was cut with *EcoRI* at the 5'-end, followed by ligation into pcDNA3 restriction-digested with *EcoRI/XbaI* (blunted). For the α mutant of the catalytic fragment of PKC (PKM; the PKM α mutant), the *BstXI/XhoI* fragment of PKC α was subcloned into a *BstXI/XhoI*-restriction-digested His₆-tagged PKM α (wild-type) construct (F. Bornancin and P. J. Parker, unpublished work). For the construction of the membrane-targeted CD2-PKM(T/A)₃ mutant, the catalytic domain of PKC $\alpha(T/A)_3$ (from bp 972 to the 3'-end) was amplified by PCR using the following oligonucleotide primers: 5'-GCGCTGCGGCCGCAAGCTTGGC-CCTGCCGGCAACA 3'-GTAGGACGTCTCGCGCCATAC-TGAGCTCGATCC. A *NotI* restriction site was introduced at the 5'-end, for ligation into pcDNA3/*BamHI*-rCD2/*NotI* (kindly provided by Doreen Cantrell, ICRF, London) restriction-digested with *NotI* and *XhoI*.

To demonstrate that the newly generated construct CD2-PKM- $\alpha(T/A)_3$ in pcDNA3 encoded the correct PKM fusion protein, the DNA was transiently transfected by electroporation into COS-1 cells. Western blot analysis with rCD2 mAb, Ox34, revealed the presence of the expected approx. 70 kDa species. In

parallel with Western blot analysis, cell-surface expression of the CD2-PKC $\alpha(T/A)_3$ chimera was demonstrated by flow cytometry (or fluorescence-activated cell sorting; FACS), employing Ox34.

Reporter construct

The TPA (or PMA) response element (TRE)-luciferase reporter plasmid contains three copies of TRE (TGACTCA) upstream of the luciferase structural gene and it was constructed as follows: a *ScaI/BglII* fragment from PBL-chloramphenicol acetyltransferase (CAT)2 containing the TK promoter was inserted into the luciferase reporter gene PGL-2 plasmid (Promega), where the simian virus 40 (SV40) promoter is downstream of the luciferase structural gene.

Assay for luciferase activity

COS-1 cells were maintained in Dulbecco's modified Eagle's medium with 10 % (v/v) foetal-calf serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml) at 37 °C in 10 % CO₂ in humidified air. One day before transfection, COS-1 cells were plated at a density of 0.3×10^5 cells per 60-mm culture dish. Calcium phosphate DNA precipitates [29] were prepared with 5 μ g of TRE-luciferase, 0.5 μ g of the constitutively active mutant and 1 μ g of each plasmid expressing an individual PKC-isotype mutant or the control vector pcDNA3 or 5 μ g of TRE-luciferase plus 1.5 μ g of empty vector. An equal amount of transfection mix was added to two separate plates for each transfected sample. In some experiments, these plates were harvested separately for luciferase assays and for electrophoresis and Western blot analysis. The cells were washed three times with Dulbecco's modified Eagle's medium 12–15 h after transfection, and then fresh culture medium with 2 % (v/v) foetal-calf serum was added to the dishes. The dishes were washed twice with PBS 48 h after transfection. Then 300 μ l of lysis buffer [0.65 % Nonidet P40/10 mM Tris/HCl (pH 8.0)/1 mM EDTA/150 mM NaCl] was added; the lysate was transferred to a tube when the intact nuclei were visible under the microscope. After centrifugation, 20 μ l of the supernatant was transferred to a polystyrene luminometer cuvette with 350 μ l of luciferase reaction buffer [25 mM glycylglycine (pH 8.0)/5 mM ATP (pH 8.0)/15 mM MgSO₄]. Luciferase activity was determined by addition of 33 μ l of 3 mM luciferin (Sigma) as described previously [30]. The background measurement was subtracted from each duplicate, and experimental values are expressed as the percentages of the total activity found in extracts from cells transfected with constitutively active mutants. Some of these plates were harvested with 200 μ l of

Laemmli sample buffer [31] and samples were subjected to SDS/PAGE and Western blot analysis (see text and Figure legends).

Western blot analysis

COS-1 cells were transfected as above, except for the data in Figure 3 where 10% (v/v) foetal-calf serum was employed. After 48 h of transient expression, the cells were washed with ice-cold PBS and lysed in 300 μ l of an ice-cold lysis buffer containing 20 mM Tris/HCl, pH 7.5, 10 mM EGTA, 10 mM EDTA, 1% (v/v) Triton X-100, 2 mM 2-mercaptoethanol, 5 mM benzamide, 50 μ g/ml leupeptin, 50 μ g/ml aprotinin, 50 μ g/ml trypsin inhibitor, 5 μ g/ml pepstatin, 1 mM PMSF, 20 mM NaF, 1 mM Na_3VO_4 and 1 mM *p*-nitrophenyl phosphate. This procedure, and all others subsequently, were performed at 4 °C. The suspension was subjected to 50 strokes in a Dounce homogenizer and centrifuged at 12000 r.p.m. (14000 *g*) for 30 min at 4 °C. 100 μ l of 4 $\times$ SDS sample buffer was added to the supernatant (Triton-soluble extract) and the pellet was resuspended in 400 μ l of 1 $\times$ SDS sample buffer (insoluble fraction). Cell lysates were sonicated, boiled for 3 min, separated on a running gel containing 7.5% (v/v) acrylamide and 0.06% (v/v) bisacrylamide, and then subjected to immunoblot assay with polyclonal antibodies specific to each PKC as described previously [24,26,32,33].

Other methods

Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.), with BSA as the standard.

RESULTS

To assess the dominant negative potential of PKC activation loop phosphorylation-site mutants, two of these were tested for their ability to block reporter gene activation by their cognate activated forms, mutated at their inhibitory pseudo-substrate sites [27]. This latter mutation confers ligand-independent activity on the proteins, but does not by-pass any requirement for post-translational modification. As illustrated in Figure 1 (left panel), activated PKC α co-transfected with a TRE-luciferase construct induced an approx. 10-fold increase in luciferase activity, consistently with previous data [27,34,35]. Co-expression of the mutant PKC α (T/A)₃ with activated PKC α leads to a complete block in luciferase induction. Thus this mutant PKC α protein has dominant negative properties with respect to PKC α .

In a similar fashion, the effect of two mutant PKC ϵ constructs was tested on the ability of activated PKC ϵ to induce luciferase from a TRE-dependent reporter construct. Unlike wtPKC α , wtPKC ϵ overexpression alone is sufficient to elicit luciferase induction (Figure 1, right panel). A similar 'basal' activity of wtPKC ϵ has been noted on transfection of this construct into RAW cells [35]. Expression of the activated PKC ϵ produces a more effective (approx. further 2-fold) increase in luciferase. This induction is inhibited by both the (T/A)₁ and (T/A)₄ PKC ϵ mutants (65% and 75% inhibition respectively), although their efficacy is less than that observed for the PKC α mutant acting on PKC α .

The above data demonstrate that both the PKC α and PKC ϵ mutants have dominant negative effects on their active counterparts. To determine the specificity of action of these mutants, as well as similar mutant forms of PKC δ and PKC ζ , co-transfection experiments were carried out with activated PKC α , a TRE-luciferase reporter and each of the mutant PKC isotypes (or the appropriate vector control). As illustrated in Figure 2, all the

Figure 1 Effect of the dominant negative mutants of PKC α and PKC ϵ on the TRE-luciferase activity stimulated by their constitutively active counterparts

COS-1 cells were co-transfected with 5 μ g of TRE-luciferase reporter plasmid together with: (left panel) empty vector (pKS1) plus wtPKC α or PKC α -activated mutant (α^*), plus either empty vector or PKC α (T/A)₃. Right panel: empty vector plus either wtPKC ϵ or PKC ϵ constitutively active (ϵ^*), or PKC ϵ constitutively active plus either dominant negative mutant PKC ϵ (T/A)₁ or PKC ϵ (T/A)₄. After 12–15 h, transfected cells were washed, cultured in 2% (v/v) foetal-calf serum/Dulbecco's modified Eagle's medium for 48 h and harvested for luciferase assay. The data shown are the means of 9–12 independent transfections performed in duplicate. The luciferase activity is expressed as a percentage of the luciferase activity induced by the constitutively active mutants after subtraction of the activity induced by the empty vector (< 15%).

mutants tested inhibit activated PKC α induction of luciferase. In each case, Western blot analyses were performed to determine (i) the expression of the dominant negative constructs for δ , ϵ and ζ (Figure 2, inset) and (ii) the expression of the activated PKC α (lower panel). Although each mutant was expressed, evidently PKC ϵ (T/A)₄ and PKC ζ (T/A)₄ were poorly expressed compared with their (T/A)₁ counterparts (there was no consistent difference in the electrophoretic migration of the mutants of an individual isotype). Nevertheless, these (T/A)₄ mutant constructs proved to be more effective as inhibitors than the (T/A)₁ mutants. Table 2 presents a summary of the inhibitory effects of these constructs on luciferase induction by activated PKC α and activated PKC ϵ . Over the entire series of experiments, all dominant negative mutants showed a mean inhibition of at least 50%. However, there are relative differences: thus PKC α (T/A)₃ is a more effective inhibitor than PKC ζ (T/A)₄ of activated PKC α , whereas the reverse is true for activated PKC ϵ . Similarly, PKC ϵ (T/A)₁ is a poorer inhibitor of activated PKC α than PKC δ (T/A)₁, whereas the opposite is true for activated PKC ϵ . The data indicate that these PKC mutants are not specific for the individual isotypes, although they do display relative differences in specificity.

The Western blots of activated PKC α (Figure 2) provide evidence that all the mutants modulate PKC α phosphorylation. This is consistent with previous studies on the PKC α (T/A)₃ mutant [15]. The active phosphorylated form of PKC α is the 80 kDa species and it is evident from the data shown that all mutants reduce the amount of this form in a manner that approximately corresponds to their ability to inhibit luciferase induction. Thus the weakest inhibitor in this series of experiments [PKC ϵ (T/A)₁] also causes the smallest loss of the 80 kDa PKC α species. These shifts in the 80 kDa species of activated PKC α are consistent with inhibitory effects of the mutant PKC isotypes on phosphorylation. However, owing to the constitutive accumulation of dephosphorylated, activated PKC α , it is impossible to assess increased accumulation of this dephosphorylated form (ligand-activated, open-conformation PKC α has been shown to be a much better substrate for protein phosphatases than the

Figure 2 Inhibition of constitutively active PKC α -induced TRE-luciferase activity by dominant negative mutants of PKC δ , PKC ϵ and PKC ζ

COS-1 cells were transfected with a TRE-luciferase reporter construct together with either an empty vector (white) or with the PKC α constitutively active, α^* (black) or the PKC α constitutively active together with the different mutants for PKC δ , ϵ and ζ (dotted). The induced luciferase activity is shown in arbitrary units (au). This is one of seven independent experiments (see summary of data in Table 2). The inset is a Western blot showing the expression of the dominant negative mutants. Lower panel: the expression of constitutively active PKC α , where the horizontal bars indicate migration of the 80 kDa and 76 kDa forms of PKC α . The transfections, luciferase activity determination, SDS/PAGE and the Western blots were carried out as described in the Materials and methods section.

Table 2 Inhibition of constitutively active PKC α or PKC ϵ by different PKC dominant negative mutants

The experiments were carried out as indicated in Figure 2. The data shown are the mean for seven independent transfections, each performed in duplicate. The luciferase activity is expressed as a percentage of the luciferase activity induced by each constitutively active mutant alone after subtraction of the activity induced by the empty vector.

Dominant negative mutants	Activated mutants	
	Luciferase activity (% of the control)	
	α^*	ϵ^*
Vector	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
α (T/A) ₃	0.00 $\pm$ 0.0	18.2 $\pm$ 7.3
δ (T/A) ₁	30.5 $\pm$ 7.3	44.2 $\pm$ 11
δ (T/A) ₂	20.6 $\pm$ 4.2	20.6 $\pm$ 2.5
ϵ (T/A) ₁	49.4 $\pm$ 4.7	32.4 $\pm$ 8.8
ϵ (T/A) ₄	47.8 $\pm$ 8.9	23.8 $\pm$ 6.8
ζ (T/A) ₁	19.6 $\pm$ 5.4	23.1 $\pm$ 3.2
ζ (T/A) ₄	12.4 $\pm$ 6.3	0.00 $\pm$ 0.0

inactive protein [11], consistent with the observed accumulation of the dephosphorylated activated form). In order to determine the effect on dephosphorylation in a more suitable context,

Figure 3 Unphosphorylated PKC α accumulates in response to dominant negative PKC mutants

COS-1 cells co-transfected with either wtPKC α plus empty vector or wtPKC α plus the different mutants were extracted and fractionated into cytosol and Triton-X-100-soluble fraction (S) and Triton-X-100-insoluble (I) fraction (as described in the Materials and methods section). Equivalent samples from each fraction were separated in SDS/PAGE and PKC α was detected by Western blot analysis. Upper and lower horizontal bars indicate the migration of phospho- and dephospho-PKC respectively. This is a representative of 2–4 independent experiments for each PKC isotype.

dominant negative mutants were tested on wtPKC α , which accumulates largely as an 80 kDa species (i.e. fully phosphorylated). To evaluate the effects of the mutants on the phosphorylation state of wtPKC α , co-transfections were carried out as described above for the constitutively activated forms. As shown in Figure 3, each of the mutants leads to the accumulation of dephosphorylated wtPKC α and a substantial proportion of this dephosphorylated protein is characteristically in the Triton X-100-insoluble fraction. At these ratios of wt:mutant PKC plasmid, the inhibitory effect is incomplete: at higher ratios, the inhibition can be virtually complete (for example, see [15]); the data shown here reflect the concentrations employed with the activated PKC isotypes. For PKC ϵ (T/A)₁, longer exposure of the Western blot was required to reveal the insoluble PKC α that accumulated; this is consistent with the weaker effect of this mutant (see Table 2).

The broad specificity of these dominant negative PKC mutants and their action indicates that they might each suppress phosphorylation by a non-productive interaction with a shared binding protein (e.g. a common PKC kinase). However, it is possible that inhibition is exerted through regulatory domain interactions with effectors, in the context of compromised protein kinase

Figure 4 Regulatory domain and catalytic domain constructs of PKC α do not have dominant properties

(A) COS-1 cells were transfected with in combination with regulatory (α_{wt} -RD) or mutant catalytic [PKM- $\alpha(T/A)_3$] domains (right panel). For comparison, wtPKC α (α_{wt}), PKC $\alpha(T/A)_3$ [$\alpha_{(T/A)_3}$] and their combination are shown (left panel). Whole extracts were subjected to Western blot analysis employing PKC α antisera. Molecular mass markers are as indicated. (B) COS-1 cells were transfected and analysed as above, except that a CD2-PKM- $\alpha(T/A)_3$ construct was employed in combination with wtPKC α . For comparison, single transfections of PKC $\alpha(T/A)_3$ and wtPKC α are shown. The Western blot was exposed for longer in (B) such that endogenous PKC α is revealed in the PKC $\alpha(T/A)_3$ transfection. The results shown are a representative from three independent experiments.

activity. To test this, a PKC α regulatory domain construct was co-expressed with wtPKC α . However, this deletion mutant had no effect on PKC α phosphorylation, as judged by its migration at 80 kDa (Figure 4A). In order to assess the minimum requirement for inhibition, the kinase domain of PKC α containing the (T/A) $_3$ mutation was also investigated. This construct was found not to inhibit PKC α phosphorylation (Figure 4). Furthermore, membrane targeting of the PKC $\alpha(T/A)_3$ as a CD2-fusion construct was also found not to inhibit wtPKC α phosphorylation (Figure 4B), despite its expression at the membrane as determined by FACS analysis (results not shown). The results indicate that effective competition requires an intact mutant kinase.

DISCUSSION

The results presented here have a number of implications for PKC function and properties. It is established that mutations in the activation loops of PKC δ , ϵ and ζ have dominant negative properties on expression in COS-1 cells. It is shown also that these dominant negative properties operate in a heterologous fashion, e.g. PKC $\zeta(T/A)_4$ inhibits activated PKC α . These dominant negative mutants act by blocking the accumulation of phosphorylated PKC, as evidenced by studies with PKC α , indicating that there is a common titratable component that promotes the priming phosphorylations of all these PKC isotypes. Furthermore, this competitive interaction is shown to require an intact mutant protein and is not supported by either the

regulatory or mutant catalytic domains alone. It is concluded that phosphorylation of the wtPKCs requires appropriate localization and is effected through at least one overlapping step.

Previous studies from this laboratory have defined Thr-497 as a critical site of phosphorylation for PKC α [16]. Similar data have also been reported for PKC β_2 [17]. The Thr-497 site resides in the predicted activation loop of PKC α between kinase domains VII and VIII; the equivalent region is found to be phosphorylated in many other protein kinases [36]. Expression of cPKC isotypes in bacteria has led to the idea that phosphorylation in this region requires some other mammalian protein, the simplest interpretation being the action of a 'PKC-kinase'. The finding that a PKC α mutant modified in the activation loop has dominant negative properties was consistent with the view of competition with such a saturable 'PKC-kinase'. Here we investigated the behaviour of cPKC, nPKC and aPKC isotypes mutated in this region. Like the original PKC α mutant, it is established that all have dominant negative properties and that this dominance shows no absolute specificity towards particular PKC isotypes. Thus both activated cPKC and nPKC isotypes are functionally inhibited by mutants derived from all three (cPKC, nPKC and aPKC) subclasses. It is concluded that phosphorylation of all these PKC isotypes in their activation loops occurs *in vivo*, accounting for the observed behaviour of the mutants. Evidence derived from the use of site-specific antisera on PKC isotypes expressed in eukaryotic cells indicates that this is indeed the case (P. J. Parker, unpublished work). Thus although it has been argued that activation loop (Thr-505) phosphorylation of nPKC δ is not required for activity [37], eukaryotes nevertheless phosphorylate this site.

These results clearly demonstrate that this type of mutant cannot be employed to define PKC isotype specificity *in vivo*. More interestingly, however, this lack of specificity defines a common step in the processing/activation of all these PKC isotypes. The simplest conclusion is that the mutants compete with a PKC kinase; however, inhibition could be effected at either a prior or parallel step. Studies on PKC α phosphorylation indicate that all these mutants are able to inhibit the normal accumulation of a fully phosphorylated PKC α protein (Thr-497, Thr-638 and Ser-657 sites [14]), coincidentally with an increased recovery of PKC α in a Triton-X-100-insoluble fraction. This insolubility is characteristic of the accumulated dephosphorylated form, which is derived normally from activation-induced dephosphorylation (discussed in [14]). It has been observed that there is a distinction between the behaviour of the low concentration of unphosphorylated PKC α primary translation product, which is soluble, and the dephosphorylated protein, which is insoluble. This distinction may reflect the normal operation of a PKC α chaperone that binds to the primary translation product and is needed to maintain conformation, and hence solubility, before the protein's phosphorylation [14]. In light of the observations here, it is plausible that the common saturable step in the processing of all these PKC isotypes is at the level of such a chaperone, i.e. it is this step that is blocked by the action of the dominant negative mutants. It is noteworthy that there are subtle differences in efficacy between these PKC mutants acting on different activated PKC isotypes. Thus although a common kinase and/or chaperone requirement may be involved in the priming phosphorylations, it is possible that this requires not one, but a series, of related proteins that have overlapping specificity but differing affinities for the PKC isotypes. Characterization of these priming events and the identity of the kinase(s) involved will resolve this issue.

Although the possible action of a common PKC kinase or PKC chaperone would account for the dominant negative activity of these PKC mutants, competition for lipid effector should be

considered, since there is evidence that some part of these priming phosphorylations may be due to autophosphorylation [8]. In principle, competition for the allosteric activator DAG would block activation-dependent phosphorylation. However, while this might account for the cPKC and nPKC effects, there is much evidence that aPKC ζ is not regulated by DAG (see, for example, [26]). It is therefore very unlikely that competition for DAG accounts for the effects of these proteins. This conclusion is substantiated by the finding that the PKC α regulatory domain does not affect wtPKC α accumulation as a fully phosphorylated form. The observation that a catalytic domain (T/A)₃ mutant, soluble or membrane-targeted, also fails to behave as PKC α (T/A)₃ indicates that the effective inhibitory properties of these mutants requires an intact protein. This is likely to reflect structural (e.g. the operation of two binding sites) and/or localization constraints on the action of these dominant negative constructs.

Irrespective of the precise competent step in the processing of these PKC isoforms, the evidence here demonstrates that there is an inhibition at the level of the priming phosphorylations. This highlights the requirement for these phosphorylations in the normal operation of PKC. In particular, it is concluded that the conserved threonine within the sequence Thr-Phe-Cys-Gly-Thr present in the activation loops of PKC α , - δ , - ϵ and - ζ plays an essential role in maintaining the proteins in their fully primed, active (if latent) conformations. The inability to complete the phosphorylation of one of these proteins and produce the active (latent) form is what is responsible for conferring dominant negative properties on the overexpressed mutants.

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REFERENCES

- Nishizuka, Y. (1995) *FASEB J.* **9**, 484–496
- Dekker, L. V. and Parker, P. J. (1994) *Trends Biochem. Sci.* **19**, 73–77
- Hug, H. and Sarre, T. F. (1993) *Biochem. J.* **291**, 329–343
- Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993) *J. Biol. Chem.* **266**, 13–16
- Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M. and Cantley, L. C. (1994) *J. Biol. Chem.* **269**, 32358–32367
- Palmer, R. H., Dekker, L. V., Woscholski, R., Le Good, A., Gigg, R. and Parker, P. J. (1995) *J. Biol. Chem.* **270**, 22412–22416
- Singh, S. S., Chauhan, A., Brockerhoff, H. and Chauhan, V. P. S. (1993) *Biochem. Biophys. Res. Commun.* **195**, 104–112
- Flint, A. J., Paladini, R. D. and Koshland, Jr., D. E. (1990) *Science* **249**, 408–411
- Pears, C., Stabel, S., Cazaubon, S. and Parker, P. J. (1992) *Biochem. J.* **283**, 515–518
- Dutil, E. M., Keranen, L. M., DePaoli-Roach, A. A. and Newton, A. C. (1994) *J. Biol. Chem.* **269**, 29359–29362
- Hansra, G., Bornancin, F., Whelan, R., Hemmings, B. A. and Parker, P. J. (1996) *J. Biol. Chem.* **271**, 32785–32788
- Lee, H. W., Smith, L., Pettit, G. R. and Smith, J. B. (1996) *Am. J. Physiol.* **271**, C304–C311
- Filipuzzi, I., Fabbro, D. and Imber, R. (1993) *J. Cell. Biochem.* **52**, 78–83
- Bornancin, F. and Parker, P. J. (1997) *J. Biol. Chem.* **272**, 3544–3549
- Cazaubon, S. M. and Parker, P. J. (1993) *J. Biol. Chem.* **268**, 17559–17563
- Cazaubon, S., Bornancin, F. and Parker, P. J. (1994) *Biochem. J.* **301**, 443–448
- Orr, J. W. and Newton, A. C. (1994) *J. Biol. Chem.* **269**, 27715–27718
- Bornancin, F. and Parker, P. (1996) *Curr. Biol.* **6**, 1114–1123
- Keranen, L. M., Dutil, E. M. and Newton, A. C. (1995) *Curr. Biol.* **5**, 1394–1403
- Zhang, J., Wang, L., Schwartz, J., Bond, R. W. and Bishop, W. R. (1994) *J. Biol. Chem.* **269**, 19578–19584
- Tsutakawa, S. E., Medzihradsky, K. F., Flint, A. J., Burlingame, A. L. and Koshland, D. E. (1995) *J. Biol. Chem.* **270**, 26807–26812
- Edwards, A. S. and Newton, A. C. (1997) *J. Biol. Chem.* **272**, 18382–18390
- Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. and Ullrich, A. (1986) *Science* **233**, 853–859
- Olivier, A. R. and Parker, P. J. (1991) *Eur. J. Biochem.* **200**, 805–810
- Schaap, D., Parker, P. J., Bristol, A., Kriz, R. and Knopf, J. (1989) *FEBS Lett.* **243**, 351–357
- Ways, D. K., Cook, P. P., Webster, C. and Parker, P. J. (1992) *J. Biol. Chem.* **267**, 4799–4805
- Pears, C. J., Kour, G., House, C., Kemp, B. E. and Parker, P. J. (1990) *Eur. J. Biochem.* **194**, 89–94
- Wooton, D., Ways, D. K., Parker, P. J. and Owen, M. J. (1993) *J. Biol. Chem.* **268**, 17975–17982
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Danielian, P. S., White, R., Lees, J. A. and Parker, M. G. (1992) *EMBO J.* **11**, 1025–1033
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Schaap, D. and Parker, P. J. (1990) *J. Biol. Chem.* **265**, 7301–7307
- Marais, R. M. and Parker, P. J. (1991) *Methods Enzymol.* 234–241
- Genot, E. M., Parker, P. J. and Cantrell, D. A. (1995) *J. Biol. Chem.* **270**, 9833–9839
- Diaz-Guerra, M. J., Bodelon, O. G., Velasco, M., Whelan, R., Parker, P. J. and Bosca, L. (1996) *J. Biol. Chem.* **271**, 32028–32033
- Johnson, L. N., Noble, M. E. and Owen, D. J. (1996) *Cell* **85**, 149–158
- Stempka, L., Girod, A., Muller, H. J., Rincke, G., Marks, F., Gschwendt, M. and Bossemeyer, D. (1997) *J. Biol. Chem.* **272**, 6805–6811