Ca²⁺ modulation of *cis*-unsaturated fatty acid-induced mutant protein kinase C activity: indication of inhibitory Ca²⁺-binding site in protein kinase C- α

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The C2 domain in protein kinase C (PKC) is homologous to equivalent domains in a number of important cytoplasmic proteins. Except for its implied function in Ca²⁺ and phospholipid binding, the precise role of this domain is not well understood. We examined the role of the C2 domain of PKC- α using a mutant enzyme in which 80 % of this domain has been deleted. This mutant can be activated by phospholipid and diacylglycerol, but is independent of Ca²⁺. In this regard, its characteristics are similar to those of the novel PKCs (nPKCs), consistent with the notion that the C2 domain of PKC confers its Ca²⁺ responsiveness. However, when the C2 deletion mutant is activated by *cis*-unsaturated fatty acid, the activity is strongly inhibited by Ca²⁺ at micromolar concentrations. The Ca²⁺ inhibition is dose-dependent and is specific to *cis*-unsaturated fatty acids. The

deletion mutant can also be activated synergistically by diacylglycerol and *cis*-fatty acid, but again activation is inhibited by Ca²⁺. Our results indicate that a PKC lacking the C2 domain is Ca²⁺-responsive and there exists an additional site for Ca²⁺ that modulates the sensitivity of the enzyme to *cis*-unsaturated fatty acid but not to diacylglycerol. This modulatory Ca²⁺-binding site appears to be suppressed by the C2 domain because the presence of the domain reverses the direction of PKC activity induced by *cis*-unsaturated fatty acid. These results suggest that the modulatory Ca²⁺-binding site could act as a molecular switch selective for fatty acid activation by sensing the changes in the Ca²⁺ levels in a cell, serving a possible mechanism of differential activation of cPKC with a C2 domain and nPKC lacking this domain.

INTRODUCTION

Protein kinase C (PKC) is a family of enzymes that play an important role in transmembrane signalling pathways in cells (reviewed in refs. [1,2]). PKC activity is regulated by Ca²⁺ and lipid second messengers such as diacylglycerol and arachidonic acid, which are generated by phospholipases. A variety of hormones, neurotransmitters, cytokines and growth factors have been shown to activate phospholipases and produce these cellular messengers (reviewed in refs. [3,4]). PKC can be classified into three groups, classical (cPKC; α , β I, β II, γ subtypes), novel (nPKC; δ , ϵ , η , θ) and atypical (aPKC; ζ , λ (ι)), on the basis of distinct differences in the composition of the four conserved regions, C1–C4 [2]. The C1 and C2 regions are regulatory whereas C3 and C4 comprise the catalytic domain. cPKC contains all four conserved regions (C1–C4), whereas the other two classes, nPKC and aPKC, lack the C2 domain. aPKC differs further from nPKC by having only one cysteine-rich sequence in the C1 domain. These differences in the conserved regions result in significant differences in the enzymic properties of PKC in each group. The most dramatic example of this is the variation in responsiveness of the isotypes to tumour-promoting phorbol esters and to Ca²⁺. aPKC or recombinant PKC mutants that lack one of the two cysteine-rich zinc finger motifs in the C1 domain are insensitive to phorbol esters or diacylglycerol, indicating the importance of this motif for the interaction with these PKC activators [5–8]. Recent studies further suggest that the second cysteine-rich zinc finger motif is responsible for the phorbol ester binding [9–11]. Similarly, PKC that lacks the C2 region, such as nPKC, does not require Ca²⁺ for the activity induced by

phospholipid and diacylglycerol [1,2]. The C2 domain is therefore thought to be the region responsible for the Ca²⁺ regulation of the enzyme. It has been shown that nPKCs, such as ϵ -, and η -subtypes, have a domain with an amino acid sequence that is similar to the C2 domain at their N-termini [12,13]. Sossin et al. [14] have recently shown that the domain, called the E region, plays an important role in the regulation of phorbol ester interaction with the C1 domain of PKC. The E domain was shown to be responsible for binding to phospholipids, but the binding does not require Ca²⁺ [14]. Thus it is generally accepted that PKC can be divided into two groups with respect to Ca²⁺ sensitivity, i.e. Ca²⁺-dependent PKC with the C2 domain and Ca²⁺-independent PKC which lacks this domain.

Evidence has emerged that the individual PKC subtypes have specific cellular localizations and that they are activated in different temporal sequences by phospholipid metabolites and Ca²⁺ [15]. However, the mechanism(s) underlying isoform-specific PKC activation is still poorly understood. This is in part due to limited knowledge of the molecular interaction between the lipid/Ca²⁺ activators and specific domains of PKC. For example, the binding site of phorbol esters and diacylglycerol has been considered to be identical, yet evidence has been presented recently for two distinct sites with different affinities for these activators [16]. Likewise, the PKC domain that interacts with *cis*-unsaturated fatty acid such as arachidonic acid and oleic acid has not been identified. Understanding the role of the specific domains that are present or absent in PKC would therefore greatly facilitate the understanding of the molecular basis of such differential activation of PKC subtypes.

In this study, we have taken a deletion mutagenesis approach

Abbreviations used: aPKC, atypical protein kinase C; cPKC, classical protein kinase C; DiC₈, 1,2-dioctanoyl-*sn*-glycerol; DOPS, dioleoyl-phosphatidylserine; nPKC, novel protein kinase C; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol.

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to understand the role of the C2 domain of PKC- α . Direct comparison of the deletion mutant with its wild-type counterpart should provide insight into the differential activation between cPKC and C2-lacking PKC subtypes. PKC- α was chosen in the present study because this subtype has all four conserved regions, it is the most sensitive of the cPKC isoforms to the synergistic activation by *cis*-fatty acid and diacylglycerol, and it requires Ca^{2+} for *cis*-fatty acid-induced activation. Here we present biochemical evidence for the presence of an inhibitory Ca^{2+} -binding site in PKC- α outside the deleted region in addition to the bipartite Ca^{2+} -binding site in the C2 domain. This inhibitory Ca^{2+} -binding site may be one of the molecular mechanisms for the differential activation of PKC subtypes.

EXPERIMENTAL

Construction of PKC- α expression plasmids

A 2324 bp cDNA of bovine brain PKC- α (kindly provided by Dr. Peter Parker, Imperial Cancer Research Fund, London, U.K.) was subcloned at the *Sa*I restriction site of pBluescript II SK(–) plasmid for orientation cloning. The cDNA was then subcloned at the *Kpn*I and *Eco*RI site of the mammalian expression vector, pCDNA3 (Invitrogen). A 330 bp fragment encoding amino acids 199–308 was deleted by partial digestion with *Hind*III and self-religated. This deletion removed 10 amino acids from the V3 region and 80 % of the C2 domain leaving 24 residues at the N-terminus. Maintenance of the correct reading frame at the deleted junction was confirmed by DNA sequencing. This deletion mutant, ΔC2 -(199–308), is hereafter referred to as the C2 deletion mutant for simplicity.

Determination of PKC subtypes expressed in COS-7 cells

Before the DNA transfection experiments, COS-7 cells were examined for endogenously expressed PKC subtypes. Anti-PKC- α polyclonal antibody specific to the C-terminus (Santa Cruz Biotech) was used to detect the C2 deletion mutant and wild-type PKC- α . Other anti-PKC antibodies specific to - β , - γ , - δ , - ϵ and - ζ subtypes (polyclonal) were obtained from Life Technologies. The cell lysates were made in $2\times$ Laemmli buffer (4 % SDS, 10 % 2-mercaptoethanol, 20 % glycerol, 0.04 % Bromophenol Blue and 125 mM Tris/HCl, pH 6.8) and subjected to Western-blot analysis. Proteins were separated in an SDS/10 % polyacrylamide mini-gel and transferred on to a nitrocellulose membrane. The working concentrations of the subtype-specific anti-PKC antibodies used for immunostaining were 0.1 $\mu\text{g}/\text{ml}$ for anti PKC- α and 1 $\mu\text{g}/\text{ml}$ for anti-PKC- β , - γ , - δ , - ϵ and - ζ . Immunoreactivity was detected by the enhanced chemiluminescence method (Amersham). This revealed that α - and ζ -immunoreactivities were present, but that no others were detected in COS-7 cells. Therefore we routinely used α - and ζ -specific antibodies to distinguish the recombinant PKC from endogenous PKC in the transfected COS-7 cells (see below).

Cell culture and DNA transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % newborn calf serum in a humidified incubator (95 % air/5 % CO_2) at 37 °C. For transfection, exponentially growing cells were seeded 12–15 h before transfection at a density of 1.2×10^6 cells/100 mm plate or 4.2×10^5 cells/60 mm plate. Expression vector (3 μg) mixed with lipofectin (Life Technologies; 1:2.5, w/v) in serum-free DMEM was then overlaid on to the cells in a minimum volume of DMEM. The cells were further incubated at 37 °C, 5 % CO_2 for 5 h with occasional gentle swirling of the plates. An equal volume

of DMEM containing 10 % newborn calf serum was added and the incubation continued overnight. The DNA-lipofectin complex was removed the next day and replaced with fresh DMEM plus 10 % serum. Incubation was continued for a total time of 50 h after transfection at which time cells were washed and harvested. The cells were either immediately used for preparation of PKC or stored at -80°C until use.

Separation of deletion mutant PKC- α from endogenous PKC subtypes and catalytic fragments of PKC in COS-7 cells

Transfected COS-7 cells were harvested from two 100 mm plates, washed in PBS, pH 7.4, and homogenized with a Teflon/glass homogenizer in ice-cold buffer A [2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol (DTT), 0.2 % Triton X-100, 20 mM Tris/HCl, pH 7.5] containing protease inhibitors (20 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ chymostatin, 2 $\mu\text{g}/\text{ml}$ aprotinin). The homogenates were centrifuged for 1 h at 100 000 *g*. The supernatants were loaded on to a DEAE-cellulose column (bed volume 0.75 ml) pre-equilibrated with buffer B (2 mM EDTA, 5 mM EGTA, 2 mM DTT, 20 mM Tris/HCl, pH 7.5). After the column had been washed with 4 bed volumes of buffer C (2 mM EDTA, 1 mM EGTA, 2 mM DTT, 20 mM Tris/HCl, pH 7.5), PKC was eluted with a linear gradient (0–0.3 M) of NaCl in buffer D (1 mM EGTA, 2 mM DTT, 20 mM Tris/HCl, pH 7.5) at a flow rate of 1 ml/min. Collected samples were analysed by immunoblotting using anti-PKC- α and - ζ antibodies. All fractionation procedures were carried out at 4 °C.

Translocation and down-regulation of PKC with PMA and other agents

COS-7 cells were cultured on 60 mm plates and transfected with either the C2 deletion mutant or wild-type PKC- α -expressing plasmid. For translocation studies, the transfected cells were exposed for 30 min to 100 nM PMA, 150 $\mu\text{g}/\text{ml}$ oleic acid, 10 $\mu\text{g}/\text{ml}$ A23187 or 0.02 % DMSO (vehicle control) at 50 h after transfection. For down-regulation studies, treatment with 1 μM PMA, 10 $\mu\text{g}/\text{ml}$ A23187 or 0.02 % DMSO was initiated 14 h before harvesting of the cells. Harvested cells were homogenized in buffer E (1 mM EGTA, 0.1 mM EDTA, 2 mM DTT, protease inhibitors described above, 50 mM Tris/HCl, pH 7.5) and centrifuged at 100 000 *g*. The supernatant was saved as the soluble fraction. The pellet was resuspended in buffer E containing 0.2 % Triton X-100 and incubated for 60 min on ice. This fraction was then centrifuged for 30 min at 100 000 *g* and the supernatant was collected as the particulate fraction. Small aliquots of the soluble and particulate fractions were analysed by Western blot and subjected to densitometric analysis.

Assay of PKC

PKC activity was measured by lysine-rich histone (Type III-S; Sigma) phosphorylation in the presence of PKC activators as described previously [17]. Concentrations of the activators used in the experiments [dioleoylphosphatidylserine (DOPS), 1,2-dioctanoyl-*sn*-glycerol (DiC_8), fatty acids and Ca^{2+}] are specified in each experiment. Free Ca^{2+} concentrations were calculated by taking account of multiple equilibria of Ca^{2+} , Mg^{2+} and ATP in the presence of 1 mM EGTA [18].

Lipid preparation

DOPS and sodium salts of *cis*-unsaturated (arachidonic acid, oleic acid), *trans*-unsaturated (linoleic acid) and saturated (stearic acid) fatty acid were prepared as described previously

[17]. DiC_8 was prepared by dispersion in DOPS solution. Further dilution of all lipids was achieved by dispersion in 20 mM Tris/HCl, pH 7.5, at 4 °C.

RESULTS

Separation of the recombinant C2 deletion mutant from other endogenous PKCs in COS-7 cells

The expression of the C2 deletion mutant and wild-type PKC in COS-7 cells is shown in Figure 1(A). Both PKCs can be stained by an antibody that specifically recognizes the C-terminus of PKC- α . Wild-type PKC- α migrates at 80 kDa (upper band), whereas the C2 deletion mutant migrates at 64 kDa (lower band). To characterize the enzymic properties of the recombinant PKCs, we separated them from other PKC subtypes and proteolytic products present in the COS cells used in the expression. Since only PKC- α and - ζ immunoreactivities were detected in the COS-7 cell homogenates (results not shown), we used these subtype-specific antibodies to evaluate the separation of the deletion mutant PKC- α from endogenous PKC. These PKCs were chromatographically separated by a linear gradient of NaCl on a DEAE-cellulose column. The wild-type, C2 deletion mutant and catalytic fragment of PKC- α and PKC- ζ were consistently eluted at NaCl concentrations of 80–130, 100–170, 180–200 and 220–240 mM respectively ($n = 9$). The deletion mutant and wild-type PKC- α fractions, free from contamination by other forms, were pooled for analysis (Figure 1B). The absence of contaminating endogenous PKCs was confirmed by Western blot in each preparation.

Characteristics of the C2 deletion mutant PKC- α in the cell

Recombinant proteins with deletion mutations are often found to be unstable and non-functional because of incorrect folding. To test whether the C2 deletion mutant of PKC is functional after expression, we compared the translocation and down-

Figure 1 Expression and purification of wild-type and C2 deletion mutant PKC- α in COS-7 cells

Wild-type (wt) and deletion mutant (dm) PKC were transiently expressed in COS-7 cells and harvested 50 h after transfection. The cell homogenate was centrifuged at 100 000 g and the supernatant was applied to a DEAE-cellulose column. Fractions containing PKC were eluted with a 0–0.3 M linear NaCl gradient and detected by immunoblot using anti-PKC α antibody raised against its C-terminal residues (651–672). Wt and dm PKC were pooled separately as described in the Experimental section. (A) Immunoblot of the cell homogenate transfected with C2dm PKC or wt PKC construct. The upper (80 kDa) and lower (64 kDa) bands are wild-type and deletion mutant respectively. (B) PKC fractions separated by DEAE-cellulose column chromatography. Markers of apparent molecular mass are 116, 97, 66, 45 and 29 kDa (from top to bottom).

Figure 2 Translocation and down-regulation of PKC expressed in COS-7 cells in response to various stimuli

COS-7 cells transfected with the deletion mutant construct were treated with various agents. After stimulation, cell homogenates were centrifuged at 100 000 g to obtain cytosolic (S) and membrane-associated (P) fractions. PKC redistribution was visualized with anti-PKC- α antibody. (A) Transient stimulation for 30 min with 100 nM PMA, 150 μM oleic acid or 10 μM A23187. DMSO (0.02%) was used as a vehicle control. (B) Long-term stimulation for 14 h with PMA (1 μM) or A23187 (10 μM). The levels of PKC in each fraction were determined by densitometric analysis.

regulation of the deletion mutant and wild-type PKC- α in the cell in response to PMA and other agents. As shown in Figure 2(A), more than 90 % of the wild-type enzyme (upper band) and approx. 65 % of the C2 deletion mutant (lower band) were present in the soluble fraction in the DMSO vehicle control. Thus the deletion of amino acid residues 199–308 results in increased PKC- α association with the membrane/cytoskeletal fraction in unstimulated cells. A 30 min treatment of the cells with 100 nM PMA, however, translocated both the deletion mutant and the wild-type to the particulate fraction almost completely. Exposure to 150 $\mu\text{g}/\text{ml}$ (50 μM) oleic acid caused a minor increase in the particulate fraction for both enzymes, whereas A23187, a Ca^{2+} ionophore, had little effect on translocation. These results are consistent with the known effect of PMA on translocation of PKC from the cytosol to the membrane/cytoskeletal fraction and indicates that the deletion mutant can respond in a similar fashion to the wild-type.

Figure 3 Activation of the C2 deletion mutant by phospholipid, diacylglycerol and Ca^{2+}

(A) Phospholipid-dependent activation of the mutant PKC by synthetic phosphatidylserine (DOPS). PKC activity was measured in the presence of 10 μM DiC_8 and 1 μM free Ca^{2+} at various concentrations of DOPS. Free Ca^{2+} concentrations were controlled by EGTA- Ca^{2+} buffer as described in the Experimental section. (B) Ca^{2+} -dependence of diacylglycerol-induced activation. Activity was measured in the presence of 30 μM DOPS and 10 μM DiC_8 at the indicated free Ca^{2+} concentrations. The results presented are representative of two to four separate experiments with duplicates in each experiment and are means $\pm$ S.D.

It is also known that PMA treatment of cells for a long duration causes down-regulation of cPKC and nPKC, but no effect on aPKC which lacks one of the cysteine-rich domains in the C1 region [7]. Thus, if the C2 deletion mutant is functional after cellular expression, it should respond to long-term treatment of PMA and be down-regulated. As shown in Figure 2(B), this is the case. After 14 h of PMA treatment, both the C2 deletion mutant and wild-type PKC- α are absent from both soluble and particulate fractions. As for the DMSO control, treatment with A23187, which is not reported to down-regulate PKC, resulted in no significant changes in PKC levels in both fractions. This demonstrates that the down-regulation of the C2 deletion mutant is specific to PMA and not simply due to the instability of the enzyme.

Activation of the C2 deletion mutant by phospholipid, Ca^{2+} and diacylglycerol

To examine the role of the C2 domain in the activation of PKC, enzymic properties of the C2 deletion mutant enzyme were characterized with respect to three activation mechanisms of PKC, i.e. diacylglycerol activation, *cis*-unsaturated fatty acid

Figure 4 Activation of C2 deletion mutant by oleic acid in the presence and absence of Ca^{2+}

Top, PKC was activated by various concentrations of oleic acid in the presence ($\blacktriangle$) or absence ($\triangle$) of 0.5 mM free Ca^{2+} . Bottom, Ca^{2+} -dependence of oleic acid activation of C2 deletion mutant was examined at 100 μM oleic acid. The results presented are representative of two to four separate experiments with duplicates in each experiment and are means $\pm$ S.D.

activation and the synergistic activation by diacylglycerol and *cis*-unsaturated fatty acid [17,19]. Diacylglycerol activation requires acidic phospholipid and physiologically relevant (micromolar) concentrations of Ca^{2+} as cofactors for cPKC. Like the wild-type PKC- α , the C2 deletion mutant was activated by a synthetic phosphatidylserine, DOPS, in a dose-dependent manner in the presence of Ca^{2+} and DiC_8 , a diacylglycerol (Figure 3A). However, this activation does not require Ca^{2+} as shown in Figure 3(B). The activation is independent of Ca^{2+} when the enzyme is activated by phospholipid and diacylglycerol. In this regard, the C2 deletion mutant PKC- α is similar to the naturally occurring nPKC which lacks the C2 domain [2].

Activation of the C2 deletion mutant by *cis*-unsaturated fatty acid

cis-Unsaturated fatty acid has been shown to activate PKC subtypes independently of phospholipid and diacylglycerol, and with different Ca^{2+} requirements. Whereas activation of PKC- α by *cis*-fatty acid is Ca^{2+} -dependent, other subtypes (β and γ) do not require Ca^{2+} for activation by *cis*-fatty acid as demonstrated with purified [17] and recombinant [20] PKC preparations. As shown in Figure 4(top), the C2 deletion mutant is activated by oleic acid. Surprisingly, however, oleic acid activates the mutant

Figure 5 Conformational specificity of fatty acid-induced activation of the C2 deletion mutant at various concentrations of Ca^{2+}

Fatty acid specificity of the Ca^{2+} inhibition of the C2 deletion mutant was determined at different concentrations of free Ca^{2+} . PKC activity was measured in the presence of $50 \mu\text{M}$ of stearic acid (●), arachidonic acid (▼) or linoleic acid (△). The results presented are representative of two to four separate experiments with duplicates in each experiment and are means $\pm$ S.D.

Figure 6 Synergistic effect of *cis*-unsaturated fatty acid and diacylglycerol on the mutant PKC- α

The effect of combination of the two activators, DiC_8 and oleic acid (OA), on the C2 deletion mutant activity (■) was compared with those activated by $10 \mu\text{M}$ DiC_8 (□) or $50 \mu\text{M}$ oleic acid (▨) alone. The activity was measured at 0 to $1 \mu\text{M}$ free Ca^{2+} in the presence of $10 \mu\text{M}$ DOPS. The results presented are representative of two to four separate experiments with duplicates in each experiment and are means $\pm$ S.D.

PKC- α strongly in the absence of Ca^{2+} but not in its presence. The effect of Ca^{2+} is opposite to that on the wild-type PKC- α , the activation of which by fatty acid is dependent on Ca^{2+} [17,20] (see also Figure 7). The low K_a value ($5 \mu\text{M}$) for oleic acid in the absence of Ca^{2+} indicates that full activation of the C2 deletion mutant can be achieved by micromolar concentrations of fatty acid.

To examine further the effect of Ca^{2+} , we determined the concentration-dependence of Ca^{2+} on the fatty acid-induced activation. As shown in Figure 4(bottom) Ca^{2+} inhibits in a concentration-dependent fashion the activity of the C2 deletion mutant induced by oleic acid. This inhibitory effect is potent; $1 \mu\text{M}$ free Ca^{2+} strongly blocks the activity. This is in contrast

Figure 7 Effect of Ca^{2+} on the activation of wild-type (wt) and C2 deletion mutant (C2 dm) PKC- α by oleic acid in the presence of diacylglycerol

The oleic acid-dependence was measured to examine the effects of Ca^{2+} on PKC activity induced by both activators. The oleic acid-induced activity was measured at three different concentrations (□, $0 \mu\text{M}$; ▲, $1 \mu\text{M}$; ●, $10 \mu\text{M}$) of free Ca^{2+} in the presence of $10 \mu\text{M}$ DiC_8 and $10 \mu\text{M}$ DOPS. The results presented are representative of two to four separate experiments with duplicates in each experiment and are means $\pm$ S.D.

with the diacylglycerol activation in which Ca^{2+} has little effect on the enzyme at concentrations up to 0.5 mM . The Ca^{2+} inhibition is not limited to oleic acid. Arachidonic acid-induced activation is also inhibited by Ca^{2+} in a dose-dependent manner as shown in Figure 5. The Ca^{2+} action is stereospecific and limited to *cis*-unsaturated fatty acid. Stearic acid does not activate the enzyme whereas linoleic acid, a *trans*-unsaturated fatty acid, partially activates it but in a Ca^{2+} -independent fashion. These results indicate that the Ca^{2+} inhibition of the activity of the C2 deletion mutant is specific to *cis*-unsaturated fatty acid.

Ca^{2+} effect on the C2 deletion mutant activity induced by diacylglycerol and *cis*-fatty acid

In addition to the activation described above, wild-type PKC- α has been shown to be activated synergistically by *cis*-fatty acid and diacylglycerol *in vitro* [17,19] as well as *in situ* [21]. The synergistic activation is distinct from the other two with respect to the requirements for Ca^{2+} . Ca^{2+} is essential for PKC- α with respect to the diacylglycerol- and *cis*-fatty acid-induced activations, whereas synergistic activation can be observed even in the presence of high concentrations of Ca^{2+} chelators. Cooperativity of *cis*-fatty acid with diacylglycerol is also seen in other cPKC subtypes, although the effect is smaller than that

observed with the α -subtype [17]. Since *cis*-fatty acid greatly reduces the concentration of phospholipid and Ca^{2+} required for diacylglycerol-induced activation, and since the C2 domain contains a Ca^{2+} - and phospholipid-binding domain [22–25], we examined the effect of the deletion of this domain on the synergistic activation of PKC. As shown in Figure 6, synergy is observed at or below physiologically relevant concentrations of Ca^{2+} . This suggests that the deleted region (amino acids 199–308) is not essential for the synergistic activation. We have examined whether Ca^{2+} inhibits the deletion mutant when it is activated by both *cis*-fatty acid and diacylglycerol. In the presence of diacylglycerol, oleic acid also activates both wild-type and C2 deletion mutant PKC- α , particularly at low concentrations ($\leq 25 \mu\text{M}$) for the mutant PKC- α (Figure 7). However, the effect of Ca^{2+} is totally opposite; micromolar concentrations of Ca^{2+} (1 and $10 \mu\text{M}$) strongly enhance the wild-type PKC- α activity whereas the mutant is inhibited by these concentrations of Ca^{2+} . It is thus clear that a micromolar range of Ca^{2+} is inhibitory to the C2 deletion mutant activity induced by oleic acid in the presence of diacylglycerol as in the case of its absence (Figure 4).

DISCUSSION

The C2 domain of PKC has received considerable attention by virtue of its absence from Ca^{2+} -independent subtypes of PKC and the existence of similar sequence motifs in other important cytoplasmic proteins. A large number of C2-domain-containing proteins have been identified, many of which participate in signal transduction and membrane trafficking [22]. These include synaptotagmin [23], cytosolic phospholipase A_2 [24], rabphilin [25], GTPase-activating protein [26] and phospholipase C- γ [27]. In proteins such as synaptotagmin I and cytosolic phospholipase A_2 , the C2 sequence is essential in Ca^{2+} -dependent phospholipid binding, a required step in the association of these proteins with the synaptic vesicle membrane or the plasma membrane. In the PKC family, isotypes lacking the C2 domain do not require Ca^{2+} when they are activated by diacylglycerol and phospholipid, whereas those that contain this domain absolutely required Ca^{2+} for activation. These observations lead to the conclusion that the C2 domain is responsible for Ca^{2+} -dependent activation of cPKCs. PKC has hence been conventionally classified into two groups with respect to Ca^{2+} sensitivity; Ca^{2+} -dependent cPKCs with C2 domain, and Ca^{2+} -independent enzymes, nPKC and aPKC, without this domain.

However, the results presented in this study indicate that this view may be oversimplified. We show here that the deletion of residues 199–308, which removes 80 % of the C2 domain in PKC- α , results in Ca^{2+} -independent activation when the enzymic activity of the mutant is induced by diacylglycerol and phospholipid. In this regard, the C2 deletion mutant is similar to nPKC, consistent with the role of this domain in Ca^{2+} responsiveness. However, when the deletion mutant is activated by *cis*-fatty acid, micromolar concentrations of Ca^{2+} strongly inhibit enzymic activity. This indicates that there is an additional Ca^{2+} -binding site in PKC- α with relatively high affinity outside the deleted region, probably outside the C2 domain. The sensitivity of the mutant to micromolar concentrations of Ca^{2+} in the presence of *cis*-fatty acid but not of diacylglycerol suggests that the additional Ca^{2+} -binding site may act as a negative regulator responsive to the fatty acid signal but insensitive to the diacylglycerol signal. In contrast with the deletion mutant, wild-type PKC- α expressed in COS-7 cells requires Ca^{2+} for its activation, which is consistent with observations on purified PKC- α from rat brain [17]. These findings suggest a dual role for the C2 domain

in the regulation of PKC- α , i.e. Ca^{2+} -dependent activation of the enzyme and the suppression of the inhibitory Ca^{2+} -binding site as a negative regulator. Because of this negative regulation by the C2 domain, the inhibitory function of this Ca^{2+} -binding site is suppressed in PKC- α and it may not be involved in the regulation of the enzyme. The inhibitory site is perhaps more relevant to signalling by the nPKCs and aPKCs that lack the C2 domain.

Sutton et al. [28] have studied the structure of the C2 domain of synaptotagmin I. Their X-ray-crystallographic analysis revealed a bipartite Ca^{2+} -binding site, formed by four aspartic acid residues residing in two distant loops (Asp-172 and -178 in $\beta 2$ – $\beta 3$ loop and Asp-230 and -232 in $\beta 6$ – $\beta 7$ loop) of the C2 domain. They have further shown by site-directed mutagenesis that the contribution of aspartate residues from both loops is essential for the formation of the bipartite Ca^{2+} -binding site. On the basis of these observations, they proposed that a C2 key, a conserved amino acid sequence of 60 residues containing the Ca^{2+} -binding site formed by the four aspartates, represents an essential functional core of the C2 motif. Since the bovine brain PKC- α cDNA used in this study also contains the C2 key of synaptotagmin I (corresponding to residues 187–254 of PKC- α) and the four corresponding aspartic acid residues (Asp-187, -193, -246, -248), we examined whether these four aspartates form such a bipartite Ca^{2+} -binding site using the INSIGHT program. Molecular modelling based on the X-ray-crystallographic co-ordinates of the C2 key in synaptotagmin [28] suggests that these residues form such a cluster capable of forming a bipartite Ca^{2+} -binding site (results not shown). A recent NMR study has further shown that an additional aspartate residue participates in bipartite Ca^{2+} binding and forms an additional Ca^{2+} -binding site in synaptotagmin and PKC- β [29]. This corresponding aspartate is also present in PKC- α (Asp-254) and clusters with the other four aspartic acid residues in the model. Although it remains to be seen whether such a secondary Ca^{2+} -binding site is indeed present in PKC- α as well, our deletion study is consistent with their bipartite Ca^{2+} -binding model and the role of the Ca^{2+} -binding site in the C2 domain, since the removal of aspartate residues from PKC- α (Asp-246, -248, -254) totally abolished the Ca^{2+} -dependence of the diacylglycerol-induced activation. The possibility that the remaining two aspartate residues (Asp-187 and -193) can form a Ca^{2+} -binding site in PKC- α is unlikely because the aspartate residues from both distant loops have been shown to be critical for the formation of the Ca^{2+} -binding site [28]. Furthermore, we have recently obtained evidence that PKC- ϵ , a novel PKC, that has no key aspartic acid residues for the formation of the bipartite Ca^{2+} -binding site, can also be inhibited by micromolar concentrations of Ca^{2+} in a similar manner to that shown in this study, i.e. Ca^{2+} at micromolar concentrations is inhibitory when it is activated by arachidonic acid but it is not when PKC- ϵ is activated by diacylglycerol [30]. These observations together indicate that the Ca^{2+} inhibition shown in this study is not due to the remaining aspartate residues (Asp-187 and -193) in the deletion mutant but importantly that the fatty acid-sensitive inhibitory Ca^{2+} -binding site is also present in naturally occurring nPKC [30] (K. Murakami, L. Gannon-Murakami and F. Eshete, unpublished work).

Although the functionally defined Ca^{2+} -binding site was only found in the C2 domain, it has been suggested that cPKC has multiple Ca^{2+} -binding sites. Using paramagnetic Gd^{3+} as a probe for Ca^{2+} -binding sites on PKC- α , Maurer et al. [31,32] documented two classes of Ca^{2+} -binding sites with differing affinities. They further observed that the probe and the enzyme can effectively form a quaternary complex with peptide substrate and an ATP analogue and suggested that the high-affinity Ca^{2+} -binding site exists at or around the active site in the catalytic

domain of PKC- α [32]. Whether or not the modulatory Ca^{2+} -binding site found in our study is identical with the high-affinity binding site for Ca^{2+} reported by Maurer et al. remains to be elucidated. Nonetheless, these observations raise the possibility of multiple regulation of PKC by Ca^{2+} in both regulatory and catalytic domains of the enzyme.

The two functionally opposite Ca^{2+} -binding sites revealed in the present study may be of physiological significance with regard to signal divergence via differential regulation of PKC subtypes. Ca^{2+} binding to the bipartite site in the C2 domain in wild-type PKC- α stimulates wild-type PKC- α activity. The novel modulatory (inhibitory) site shown in this study, on the other hand, is insensitive to the diacylglycerol signal but acts as inhibitory to *cis*-fatty acid signal. It has been postulated that individual PKC subtypes transduce separate and distinct signals in the cell (for reviews, see refs. [2,33]). For this model to be operative, each PKC subtype should possess multiple domains providing the molecular machinery for sensing the second-messenger signals such as diacylglycerol, arachidonic acid and Ca^{2+} with different sensitivity. Separate domains may be required to determine the precise subcellular localization and translocation in response to the signals, and for recognition of protein substrate with specificity. The novel modulatory Ca^{2+} -binding site could play a role in the differential regulation of PKC subtypes in response to the changes in cellular Ca^{2+} levels. Functionally, cPKC responds to Ca^{2+} positively for the fatty acid signal because of the presence of the C2 region which can mask the effect of the modulatory Ca^{2+} -binding site. When this region is deleted from PKC, however, the second Ca^{2+} -binding site becomes functional and modulates the PKC activity in response to the fatty acid signal, turning on the PKC activity in the absence of Ca^{2+} and turning it off in its presence.

In summary, our study suggests that another functional Ca^{2+} -binding site is present in PKC- α in addition to the bipartite Ca^{2+} -binding site in the C2 domain. Our results suggest that this modulatory Ca^{2+} -binding site could act as a molecular switch selective for the fatty acid activation of PKC by sensing the changes in the Ca^{2+} levels in a cell. This notion is further supported by our recent observation that micromolar concentrations of Ca^{2+} can inhibit *cis*-fatty acid-induced activity of nPKC- ϵ [30]. We propose that the modulatory Ca^{2+} -binding site serves a mechanism of differential activation of cPKC with a C2 domain and nPKC lacking this domain.

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