Protein kinase C group B members PKC- δ , - ϵ , - ζ and PKC-L(η)

Comparison of properties of recombinant proteins *in vitro* and *in vivo*

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Of the recently identified protein kinase C (PKC) types of group B (δ , ϵ , ζ , η , PKC-L), only PKC- ϵ has been characterized in great detail. In order to compare the regulatory and catalytic properties of these new kinases, we have expressed PKC- δ , - ϵ , - ζ and PKC-L as recombinant proteins from their cDNAs in insect cells via baculovirus vectors and in mammalian COS-1 cells. After expression in insect cells, phorbol ester binding and kinase activities of the group B enzymes were compared with the respective activities of a member of group A, PKC- γ . Although PKC- δ and PKC-L(η) bind phorbol ester to a similar or the same extent as PKC- γ , they show a distinctively different behaviour towards conventional PKC substrates such as histone, myelin basic protein, protamine and protamine sulphate, suggesting either that phorbol esters are not able to fully activate these enzymes or that their substrate specificities are very different from those of the group A enzymes. PKC- ζ , a polypeptide of 80 kDa, does not bind phorbol ester and does not phosphorylate these substrates to a significant extent. Consistent with their ability to bind phorbol ester, recombinant PKC- δ and PKC- ϵ are down-regulated in COS cells by prolonged treatment with phorbol ester, whereas PKC- ζ protein levels remain unaltered.

INTRODUCTION

Protein kinase C (PKC), originally defined as a phospholipid- and Ca^{2+} -dependent protein kinase with a key role in intracellular signal transduction (reviewed in [1–3]), has expanded into a multi-gene family of protein kinases characterized by conserved structural features. These include a highly conserved kinase domain and a regulatory domain with one or two zinc-finger-like structures and a basic pseudosubstrate prototope (reviewed in [4–6]). Based on structural similarities between individual members, the family has recently been divided into two groups, tentatively named groups A and B. Group A enzymes possess a conserved domain (C2 domain) in the regulatory region which is lacking in group B members. This block of amino acids has been proposed to be responsible for the dependence of group A enzymes on Ca^{2+} in activity assay *in vitro* [2,7].

Whereas group A enzymes (PKC- α , - β 1, - β 2, - γ) have been studied rather extensively, group B enzymes (PKC- δ , - ϵ , - ζ , - η , PKC-L) are much less well characterized due to their more recent discovery. PKC- δ , - ϵ and - ζ have been isolated from brain cDNA libraries [7–10], whereas PKC- η and PKC-L have been cloned from a mouse epidermis and a human keratinocyte library respectively [11,12]. In contrast to all other PKC types identified so far, expression of PKC- η and PKC-L is predominantly observed in skin and lung tissue, supporting the concept that different members of the PKC family might play different roles in different cellular contexts. PKC-L appears to be the human homologue of the mouse PKC- η [6,11,12].

The ability to bind and to respond to phorbol ester tumour promoters is one of the features of PKC that has been used to characterize this class of proteins ([3,13,14] and references therein). Through mutagenesis and antibody inhibition studies the zinc finger structures in the N-terminal part of PKC molecules have been recognized as the sites of action of phospholipid and phorbol ester activators [15,16]. PKC- ζ is so far unique among the PKC enzymes in that it contains a single zinc-finger-like structure, and has been claimed not to bind phorbol ester tumour promoters

but to phosphorylate histone in a phospholipid-dependent manner after transient expression of the cDNA in COS cells [7]. Therefore PKC- ζ appears not to fulfil this original criterion of a phorbol ester-binding protein, although it can clearly be considered as a member of the PKC family based on sequence similarity [8]. In order to rigorously establish common and differing properties of the group B enzymes, we have expressed PKC- δ , - ζ and PKC-L(η) in insect cells, which have proved to be an extremely suitable system in which to study recombinant PKC proteins since these cells do not show endogenous PKC kinase activity or phorbol ester binding [17–20]. Here we show that, in contrast to the previous publication [7] which determined an apparent molecular mass for PKC- ζ of 64 000 Da on SDS/polyacrylamide gels, the PKC- ζ cDNA (which would give a predicted molecular mass of 67 740 Da) actually gives rise to an 80 000 Da product which does not bind phorbol ester and is not significantly active with activator-dependent or activator-independent conventional PKC substrates.

Long-term treatment of cells with phorbol ester leads to down-regulation of measurable PKC activity and phorbol ester binding sites [21,22]. It has been shown that this is a consequence of the disappearance of the PKC polypeptide due to enhanced proteolytic degradation [23]. It is not clear if the phenomenon of down-regulation will provide some insight into the physiological mechanism of PKC activation, or whether binding of phorbol ester to the PKC molecule is sufficient to trigger a proteolytic attack. It is clear, however, that the group A PKC polypeptides α , β and γ are all down-regulated by extended exposure to phorbol ester, although with different sensitivities. This has been shown for endogenous PKC proteins as well as for group A PKC types expressed from vectors in mammalian cells [24–27]. We show here that the group B enzymes PKC- δ and PKC- ϵ are also down-regulated by phorbol ester in COS cells, whereas PKC- ζ protein levels are not affected, suggesting that phorbol ester binding by the polypeptide itself is a prerequisite for down-regulation and that down-regulation is not mediated by some indirect mechanism intrinsic to all PKC types.

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MATERIALS AND METHODS

cDNA cloning

For cDNA cloning, probes were generated using oligonucleotides from published sequences [7,8] and amplification of DNA fragments directly on a λ phage library by the PCR [28]. The following oligonucleotides were chosen from the published cDNA sequences of rat PKC- δ , - ϵ and - ζ [7,8] as PCR primers and synthesized: δ -5', ⁵⁰²AGAAGCCCACCATGTACCCTG⁵²²; δ -3', ¹²⁹⁰TTCCGACAGTCTCTGGTGTC¹²⁷¹; ϵ -5', ¹⁴⁴TGCTCTTCAATCCTGCCCTC¹⁶³; ϵ -3', ⁹⁷⁰ACAGTGGTCACAGAACGTGG⁹⁵¹; ζ -5', ³⁵⁵GTGTCCTCACAGATGGAGCTG³⁷⁵; and ζ -3', ⁹²⁷ACGCCCGATGACTCTGATGAG⁹⁰⁷ (numbers give nucleotide positions of respective sequence in published cDNAs [7,8]). Amplification using the respective pair of primers resulted in the generation of fragments of 787 bp for PKC- δ , 827 bp for PKC- ϵ and 573 bp for PKC- ζ , and was carried out as follows. 10⁷ phages in solution were disrupted by incubation for 5 min at 70 °C and the released DNA was subjected to 30 rounds of PCR amplification in a total volume of 100 μ l containing 20 mM-Tris/HCl, pH 8.3, 2.5 mM-MgCl₂, 50 mM-KCl, 100 μ M-deoxynucleotides, 200 nM 5'- and 3'-primer pairs and 1 unit of Taq polymerase (Beckman). Denaturation was for 1 min at 95 °C, annealing for 3 min at 55 °C, and extension for 2 min at 72 °C in a water-cooled thermocycler (Bio-Med). Amplified DNA was separated on an agarose gel, and fragments were eluted, labelled with ³²P by random priming [29] and used to screen an oligo(dT)-primed rat brain cDNA library from 6-week-old-male Sprague-Dawley rats in λ ZAP II (Stratagene, La Jolla, CA, U.S.A.). Hybridizations were carried out in 50% formamide at 42 °C and filters washed at 65 °C with 0.1 $\times$ SSC/0.1% SDS [30]. Positive signals were selected and phages were purified by three rounds of plaque assays. The pBluescript SK plasmid vector was rescued from the λ ZAPII vector by *in vivo* excision according to the manufacturer's protocol (Stratagene). Plasmid clones were analysed by restriction enzyme analysis and DNA sequencing with Sequenase (USB, Cleveland, OH, U.S.A.) using T7 and T3 17-mer primers (Stratagene).

Generation of antibodies

Peptides corresponding to the published C-terminal amino acid sequences of rat PKC- δ (K-FVNPKYEQFLE) and rat PKC- ζ (K-INPLLLSAEESV) [8] were purchased from Neosystems (Strasbourg, France) and were coupled to keyhole-limpet haemocyanin (Calbiochem) using glutaraldehyde. They were then injected subcutaneously into laboratory rabbits and serum was collected according to published protocols [31]. Anti-peptide antisera were used at 1:1000 dilutions, without further purification, in Western blots as described [32].

Cloning of cDNAs in a mammalian expression vector and transfection of COS-1 cells

PKC- δ cDNA was transferred as a 2.8 kb *Eco*RI fragment from the pBluescript plasmid (Stratagene) into the *Eco*RI site of the expression vector pMT-2 [33]. PKC- ϵ cDNA was excised from the pBluescript plasmid as a 3 kb *Nco*I/*Bam*HI fragment which was filled in with Klenow polymerase to produce blunt ends and then ligated to the *Eco*RI-cut pMT-2 plasmid, which had also been filled in. In order to generate a full-length PKC- ζ clone, clones PKC- ζ_4 and PKC- ζ_{10} (see Fig. 1) were joint at a common *Pst*I site (position 688) and the complete PKC- ζ cDNA was then transferred as a 2.1 kb *Eco*RI fragment into the *Eco*RI site of pMT-2.

Transfection of COS-1 cells on 60 mm or 100 mm dishes was carried out with DEAE-dextran as described by Cullen [34]. Cells were harvested after 46–48 h by lysis in boiling hot SDS sample

buffer [35] with or without prior exposure to phorbol 12-myristate 13-acetate (PMA) for 16 h at a final concentration of 400 nM.

Baculovirus expression of PKC- δ , - ζ and PKC-L

cDNAs coding for PKCs were transferred from the pBluescript plasmid vector (Stratagene) into baculovirus expression vectors pAcC5 (kindly provided by Robin Clark, Cetus Corp., Emeryville, CA, U.S.A.) and pAcYM1 [36] (kindly provided by Dr D. Bishop, NERC Institute of Virology, Oxford, U.K.) by standard cloning procedures [37]. Properties of these vectors are described by Luckow & Summers [38].

PKC- δ cDNA was excised from the pBluescript plasmid with *Apa*I and *Bam*HI and cloned into the *Bam*HI site of pAcYM1 after addition of *Bam*HI linkers. PKC- ζ cDNA was excised as an *Eco*RI fragment from pBluescript-PKC- ζ , and the ends were filled in and ligated to blunt-ended *Bam*HI-cut pAcYM1 vector. PKC-L cDNA [12] in pBluescript was linearized at the 5'-end with *Sma*I, *Nco*I linkers were added, and the cDNA was excised at the 3'-end with *Kpn*I and ligated into the *Nco*I/*Kpn*I sites of pAcC5. In order to destroy the ATG codon upstream of the PKC-L start codon generated by this ligation, the DNA was linearized again with *Nco*I, blunt-ended with Mung bean nuclease and re-ligated.

Isolation of recombinant baculoviruses expressing PKC polypeptides was carried out according to the protocols of Summers & Smith [39] and as described in [17,40].

Phorbol ester binding on intact insect cells

Insect cells (Sf9) (2.5×10^6) were seeded on 60 mm dishes and infected with recombinant baculoviruses. At 48 h post-infection, cells were washed twice with phosphate-buffered saline (PBS) and incubated with 11 nM-[³H]phorbol dibutyrate (PDBu) (0.5 μ Ci/ml) (Amersham) in PBS for 30 min at room temperature. After incubation, cells were washed twice with PBS and scraped in PBS, and cell-associated radioactivity was counted in a liquid scintillation counter.

PKC activity assay

Insect cells (3×10^6) were infected with recombinant baculovirus, harvested after 2 days, lysed in 50 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 10 mM-EGTA, 2 mM-dithiothreitol (DTT) and 0.1% Triton X-100, and centrifuged at 10000 *g* for 20 min at 4 °C.

Aliquots of the supernatant were used undiluted or diluted in 50 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 2 mM-DTT and 0.1% Triton X-100 in PKC activity assays using detergent-mixed micelles [41], and histone H1S, myelin basic protein, protamine or protamine sulphate (all from Sigma) or histone H1 (Boehringer) as substrate. PKC activity assays were performed for 5 min at 30 °C as described [17] and contained in a volume of 40 μ l of 50 mM-Tris/HCl, pH 7.4, 1.25 mg of protein substrate/ml, 12.5 mM-MgCl₂, 750 μ M-CaCl₂, 0.25% Triton X-100 and 125 μ M- γ -³²P-labelled ATP, with or without 625 μ g of phosphatidylserine/ml (Lipid Products, Surrey, U.K.)/2 μ M-PMA (Sigma). After the reaction, 30 μ l aliquots were spotted on to 1 cm $\times$ 1 cm P-81 phosphocellulose paper squares (Whatman). These were washed for 3 $\times$ 10 min in 30% acetic acid and bound radioactivity was counted.

RESULTS

Isolation of cDNA clones for rat PKC- δ , - ϵ and - ζ

For the isolation of PKC- δ , - ϵ and - ζ cDNAs, type-specific hybridization probes were generated by the PCR using oligonucleotides derived from the 5' regions of published PKC- δ , - ϵ and - ζ sequences [7,8]. Amplified DNA fragments were labelled

Fig. 1. Schematic representation of rat PKC- δ , - ϵ and - ζ cDNA clones

Open reading frames are shown by stippled boxes. Start (ATG) and stop (TGA or TAG) codons are indicated and the locations of PCR products used for isolation of respective clones are shown by solid bars below clones.

Table 1. PDBu-binding activities of insect cells expressing recombinant proteins

Insect cells (2.5×10^6) were infected with recombinant baculoviruses expressing PKC- γ , - δ , - ζ , PKC-L(η) or the unrelated adenovirus E1A protein as a control. PDBu binding was measured as cell-associated radioactivity. The values represent means of triplicates from two separate experiments.

Expressed protein	[3 H]PDBu bound (pmol/ 10^6 cells)
E1A	0.28 ± 0.01
PKC- γ	2.95 ± 0.19
PKC- δ	1.78 ± 0.09
PKC- ζ	0.26 ± 0.02
PKC-L	3.13 ± 0.16

with 32 P and used to screen a rat brain cDNA library. Screening of 10^6 λ plaques with the PCR products shown in Fig. 1 yielded five PKC- δ clones, nine PKC- ϵ clones and nine PKC- ζ clones. After plasmid rescue from the phages, one PKC- δ clone, two PKC- ϵ clones and five PKC- ζ clones were analysed further by restriction enzyme digestion and sequencing. The PKC- δ clone carried a 2.8 kb full-length insert which was confirmed by partial DNA sequence analysis; the PKC- ϵ clones (ϵ_5 and ϵ_1) carried 3 kb and 4.6 kb full-length inserts respectively (Fig. 1). The 4.6 kb PKC- ϵ cDNA had an extended 3' untranslated region compared with the shorter clone (Fig. 1). The three longest PKC- ζ clones carried inserts of 2.3 kb (ζ_8), 2.5 kb (ζ_3) and 1.9 kb (ζ_4). Restriction enzyme analysis and DNA sequencing showed that all three PKC- ζ clones were identical to the one initially identified by Ono *et al.* [8] as an incomplete PKC- ζ cDNA. Ono *et al.* [7] later interpreted this cDNA as representing a possibly incomplete splicing intermediate, since sequence divergence occurs at a specific point upstream of the one zinc finger of PKC- ζ . In order to isolate the full-length PKC- ζ cDNA as described by Ono *et al.* [7] a hybridization probe was generated which only contained sequences unique to the 'properly spliced' PKC- ζ product (cf. Fig. 1) and used to rescreen the library. The specific probe was generated by digesting the original ζ -PCR probe with *Sph*I, which generates a 100 bp 5' fragment specific for the sequence described by Ono *et al.* [7] (cf. Fig. 1). Rescreening of the library with this fragment yielded one clone (ζ_{10}) which carried a 1.5 kb insert generated by internal priming on the PKC- ζ mRNA and extended to position -2 relative to the ATG start codon (Fig. 1).

In order to obtain a full-length PKC- ζ cDNA, the inserts of clones ζ_4 and ζ_{10} were jointed at a common *Pst*I site (position 688).

Subsequently, all full-length cDNAs were cloned into the mammalian expression vector pMT-2 [42] and PKC- δ and PKC- ζ were cloned into the baculovirus expression vector pAcYM1 [36], and these were used to express recombinant proteins in COS-1 cells and insect cells respectively.

Expression of PKC group B proteins from baculovirus expression vectors, phorbol ester binding and kinase activities

PKC- δ , - ζ and PKC-L(η) cDNAs [12] were cloned into baculovirus expression vectors, recombinant viruses were isolated using standard protocols [39] and expression of PKC proteins

Table 2. Kinase activities of recombinant proteins towards different substrates

Insect cells (2.5×10^6) were infected with recombinant baculoviruses expressing PKC- γ , - δ , - ζ , PKC-L(η) and the unrelated adenovirus E1A protein as a control. At 2 days post infection cells were harvested and lysed. Soluble fractions were diluted 1:10 and 5 μ l was used in kinase assays in the absence (-) or presence (+) of the activators phosphatidylserine/PMA (PS/PMA). Shown are results (units/ml) determined in triplicate in one set of experiments with all substrates. The experiment was repeated several times with essentially identical results. Abbreviation: MBP, myelin basic protein.

Substrate ...	Activity (units/ml)							
	Histone		MBP		Protamine		Protamine sulphate	
	-	+	-	+	-	+	-	+
PKC- γ	12.4 ± 0.4	83.2 ± 5.0	16.2 ± 1.1	173.1 ± 8.8	163.5 ± 11.7	322.8 ± 6.5	532.7 ± 14.1	588.7 ± 19.9
PKC- δ	32.1 ± 1.7	34.2 ± 1.2	37.7 ± 1.7	57.6 ± 0.3	75.3 ± 4.6	129.1 ± 4.5	131.3 ± 5.0	139.7 ± 3.8
PKC- ζ	7.1 ± 0.2	6.4 ± 0.3	12.8 ± 0.2	8.9 ± 0.4	10.7 ± 0.5	23.2 ± 1.2	45.8 ± 2.8	43.2 ± 2.7
PKC-L	7.7 ± 0.3	10.0 ± 0.6	10.3 ± 0.6	13.9 ± 1.4	29.5 ± 1.9	71.6 ± 1.5	72.5 ± 3.2	97.3 ± 4.5
E1A	3.1 ± 0.4	3.3 ± 0.1	4.1 ± 0.4	3.7 ± 0.4	3.5 ± 0.3	4.6 ± 0.7	6.3 ± 0.6	7.0 ± 0.8

Fig. 2. Expression of PKC- δ (a) and - ζ (b) polypeptides in insect cells

Insect cells were infected with recombinant viruses carrying cDNAs for PKC- δ and PKC- ζ . At 2 days post infection, cells were harvested, lysed in SDS sample buffer and analysed by immunoblotting with anti-peptide antisera as indicated. Control infections were carried out with a recombinant virus expressing the adenovirus E1A gene [43].

was analysed by immunoblotting of whole cell lysates from infected insect cells. Fig. 2 shows detection of PKC- δ and PKC- ζ polypeptides in insect cells using anti-peptide antisera raised against synthetic peptides derived from the C-terminus of rat PKC- δ and - ζ . For control of antibody specificity, cells infected with a control virus expressing the unrelated adenovirus E1A protein [43] were also analysed. Infection of insect cells with baculoviruses expressing PKC- δ or PKC- ζ led in both cases to the production of 80 kDa proteins (Fig. 2).

Phorbol ester binding properties of recombinant PKC proteins PKC- δ , - ζ and PKC-L(η) were assayed on whole live insect cells infected with the respective recombinant baculoviruses and were compared with phorbol ester binding by group A member, PKC- γ [17]. To this purpose, insect cells were infected with baculoviruses expressing PKC polypeptides or the unrelated adenovirus E1A protein as a control [43], the cells were incubated for 48 h to express the recombinant proteins and were then labelled with [3 H]PDBu. Cell-associated radioactivity was determined in triplicate in several independent experiments (Table 1). Cells expressing PKC- γ bound between 2.8 and 3 pmol of PDBu/ 10^6 cells, consistent with previously published values [27]. Phorbol ester binding by PKC-L(η) was in the same range, whereas binding by PKC- δ of (1.7–2 pmol/ 10^6 cells) was consistently lower. PDBu binding by PKC- ζ -expressing cells was never higher than in cells expressing the control protein E1A (Table 1).

In order to analyse the catalytic properties of the group B enzymes, infected insect cells were used to compare the kinase activities of PKC- δ , - ζ and PKC-L(η) with that of a representative from the PKC group A, PKC- γ . Insect cells were infected with control virus E1A or with recombinant baculoviruses expressing PKC- γ [17], PKC- δ , PKC- ζ or PKC-L(η). After 48 h, cells were harvested and recombinant proteins were extracted and used in standard PKC kinase assays with different substrate proteins in a mixed micelle assay in the presence and absence of the activators phosphatidylserine and PMA. The results of one of several experiments are shown in Table 2. As reported before, the protein kinase activity of recombinant PKC- γ produced in insect cells was highly dependent on phospholipid and PMA using the conventional substrate histone H1 [17]. The same is true for the other group A members of the PKC family ([19]; S. Stabel and P. J. Parker, unpublished work).

Phosphorylation of myelin basic protein by PKC- γ was also greatly stimulated by the presence of the activators phospholipid and PMA. With protamine as a substrate the requirement for activators was less, and even less if protamine sulphate was

Fig. 3. Expression of PKC- δ , - ϵ , - ζ in COS-1 cells, antibody specificity and size comparison

(a) COS-1 cells were transfected with expression constructs of PKC- γ [27], - δ , - ϵ and - ζ in pMT-2 as indicated. After 48 h, cells were lysed in boiling SDS sample buffer [35] and lysates were electrophoresed through SDS/8% polyacrylamide gels, blotted on to nitrocellulose and reacted with anti-peptide antibodies as indicated below the autoradiographs. (b) For size comparison, samples as in (a) were co-electrophoresed through an SDS/8% polyacrylamide gel as indicated. The gel was blotted and incubated with a mixture of antibodies (δ -, ϵ - and ζ -specific, and antibody 0442 for the detection of PKC- γ).

offered as a substrate (Table 2). This feature of differential requirements for activators depending on the substrate used has been recognized as a characteristic for natural (group A) enzyme preparations from brain [44–47].

In contrast with the group A enzyme, none of the PKC group B enzymes showed highly efficient phosphorylation of either substrate, although the amount of recombinant protein present in the assay, determined as extracted soluble protein of all PKC types, was comparable (results not shown). Of the group B enzymes analysed here, PKC- δ showed the highest phosphotransferase activity under these assay conditions, followed by PKC-L(η). PKC- ζ had a very low but measurable activity towards protamine sulphate only, and showed in no case enhancement of

Fig. 4. Down-regulation of rat PKC- δ , - ϵ and - ζ in COS-1 cells

(a) COS-1 cells were transfected with expression constructs of PKC- δ , - ϵ and - ζ cDNAs in the vector pMT-2 or with the vector alone and grown for 48 h with (+) or without (-) addition of 400 nM-PMA for the last 16 h. After this time cells were harvested in boiling hot SDS sample buffer [35] and analysed by electrophoresis through SDS/polyacrylamide gels and immunoblotting with anti-peptide antibodies as indicated. (b) Western blots of samples as above were cut into bottom and top halves. The top halves were developed with type-specific anti-peptide antibodies and the bottom halves were developed with the antibody 0442 [48], which detects the cellular protein tubulin as an internal gel loading control.

activity by phospholipid/PMA (Table 2). Kinase assays with extracts from control infections (E1A) under the same conditions did not show any activity (Table 2). Essentially the same results for group B enzymes were obtained when Ca^{2+} in the assay was replaced by 0.5 or 5 mM-EGTA (results not shown).

Expression of PKC- δ , - ϵ and - ζ in COS-1 cells and down-regulation

For expression and analysis in mammalian cells, the PKC cDNAs were cloned into the expression vector pMT-2 [33] and introduced into COS-1 cells by DNA transfection. Transient expression of the respective proteins was monitored on Western blots of whole cell lysates at 48 h post-transfection.

Fig. 3(a) shows Western blots of COS cells transfected with expression constructs of PKC- γ [27], PKC- δ , PKC- ϵ or PKC- ζ and probed with different anti-peptide antisera. Identical Western blots were incubated with an anti-peptide serum against group A PKCs (0442) [48] or type-specific group B antisera. In each case, specific polypeptides corresponding to the input DNA construct were detected, demonstrating that there was no or very little cross-reactivity between these anti-peptide antibodies. In addition to PKC group A members, antibody 0442 reacted with tubulin (55 kDa) present in all samples as described before [32]. Sometimes, but not always, the appearance of an additional C-terminal polypeptide of about 44–47 kDa was observed with all transfected cDNAs, and this may be due to the translational start occurring at an internal methionine, or to proteolytic degradation *in vivo* upon high-level expression.

The published size of 64 kDa for PKC- ζ after transient expression in COS-7 cells and partial purification [7] differs from the apparent molecular mass of 78–80 kDa observed here in insect

cells as well as in COS-1 cells. For a direct size comparison, whole-cell lysates from COS cells transfected with cDNAs of PKC- γ , - δ , - ϵ and - ζ were separated through electrophoresis in the same gel and immunoblotted with a mixture of all antibodies needed for specific detection of individual PKC types (Fig. 3b). The molecular masses of PKC proteins deduced from such an immunoblot were 77–79 kDa for PKC- δ , 90–91 kDa for PKC- ϵ and 78–80 kDa for PKC- ζ .

In order to investigate the behaviour of group B enzymes upon exposure to phorbol ester *in vivo*, transfected cells were treated with the phorbol ester PMA (400 nM) for 16 h. Protein levels were analysed by immunoblotting of whole-cell lysates with PKC type-specific antibodies (Fig. 4). Exposure of transfected COS cells to the phorbol ester PMA for 16 h led to a noticeable decrease of PKC- δ and PKC- ϵ protein levels, whereas the level of PKC- ζ protein remained virtually or completely unaltered (Fig. 4).

An additional C-terminal polypeptide of about 44–47 kDa (mentioned above) was sometimes seen in transfected COS cells (Figs. 3a and 3b). This distinct fragment was also down-regulated in the case of PKC- δ and - ϵ , whereas exposure of PKC- ζ to PMA affected the expression of neither the full-length product nor the (possibly) catalytic fragment (Fig. 4).

Fig. 4(b) shows a parallel control experiment in which Western blots were cut in half and the top halves were probed with the type-specific antibodies, and the bottom halves were probed with the antibody 0442 for the detection of tubulin [32] to act as a control for equal protein loading of the lanes. Unaltered levels of tubulin in treated and untreated samples confirmed that PMA treatment specifically affected the PKC polypeptides and not unrelated cellular proteins.

DISCUSSION

Full-length cDNA clones encoding rat PKC- δ , - ϵ and - ζ were isolated from a rat brain cDNA library by screening with probes generated by PCR using oligonucleotides derived from the published sequences of PKC- δ , - ϵ and - ζ [7,8]. Whereas the longest cDNA clones for PKC- δ and - ϵ carried the expected open reading frames, the majority of PKC- ζ clones of ≥ 2 kb length were identical to the incomplete cDNA clone λ CKR ζ 3 initially described by Nishizuka & coworkers [8]. In a subsequent publication, Ono *et al.* [7] postulated that this originally isolated cDNA clone probably represented an incompletely spliced intermediate of PKC- ζ mRNA, since translation of its sequence would predict a PKC- ζ protein which would initiate from an internal methionine (position 184) just downstream of the last conserved cysteine residue in the PKC- ζ zinc finger. Only by using a probe which contained DNA sequences unique to the full-length PKC- ζ clone described by Ono *et al.* [7] were we able to identify an overlapping clone which carried the postulated PKC- ζ N-terminus (cf. Fig. 1). The failure to identify full-length PKC- ζ clones in the first round of hybridization may indicate that this mRNA is rare compared with the incompletely spliced product or that it is under-represented in the library due to differential amplification of λ clones containing different inserts. It is striking that identical apparently incompletely spliced mRNA products should be re-isolated with such high frequency by different laboratories. It is, however, difficult to imagine a potential role for an abundant mRNA with this structure. The cDNA clones isolated were analysed by restriction mapping and DNA sequencing to confirm their identity with PKC subtypes and to determine the extent of their 5' and 3' untranslated sequences.

Polyclonal antibodies against the C-terminus of PKC- δ and PKC- ζ were raised and used to detect expression of recombinant proteins in COS cells and in insect cells. Expression of recombinant PKC- ϵ in COS cells has been reported by several groups [7,10,49,50], and PKC- ϵ protein with an apparent size of 90 kDa has been expressed and purified from a baculovirus expression vector [18,51]. PKC- δ and - ζ have also been expressed from mammalian expression vectors in COS-7 cells by Nishizuka and coworkers [7,8]; recombinant polypeptides were, however, identified by kinase assays after separation of COS cell extracts on hydroxyapatite columns. It was not possible in all cases to obtain a clear distinction between endogenous and recombinant PKC polypeptides or their proteolytic fragments.

Through the use of highly specific antibodies we show here expression and detection of recombinant PKC- δ and - ζ polypeptides by type-specific antibodies after blotting whole cell lysates, which decreases the possibility of generating proteolytic fragments. PKC- δ , with a predicted molecular mass of 77.5 kDa, shows an apparent molecular mass of 77–79 kDa in SDS/polyacrylamide gels. PKC- ζ , with a predicted molecular mass of 67.7 kDa, also migrates as a polypeptide of 78–80 kDa after expression in insect cells or COS cells, in contrast with the previously published apparent molecular mass of 64 kDa [7]. It seems conceivable that in the latter case a proteolytic degradation product has been erroneously identified as the full-length product of PKC- ζ .

By expression in insect cells which do not show endogenous phorbol ester binding, we could show that PKC- ζ with its unique structure of a single zinc finger in its regulatory domain indeed does not bind phorbol ester and, consistent with this, is not down-regulated by PMA after transient expression in mammalian COS cells. As has been discussed previously, this finding supports the notion that down-regulation is a direct consequence of phorbol ester binding to the PKC molecule in question, and is

not mediated via a trans-acting mechanism, e.g. involving activated endogenous PKC- α of COS cells which could lead to down-regulation of co-expressed subtypes [27,52].

Comparison of substrate and activator requirements for the group B enzymes with a representative of group A, PKC- γ , which has been characterized as a recombinant protein by several groups [17,19,20], revealed that all conventional PKC substrates, such as histone, myelin basic protein, protamine free base and protamine sulphate, are poor substrates for this class of isoenzymes and that their activity is stimulated poorly or not at all by phospholipid/PMA. Although crude extracts of infected insect cells were used in our analyses, the fact that crude preparations of PKC- γ from insect cells showed identical properties to the purified recombinant protein (S. Stabel, unpublished work; [17,19,20]) supports this approach. Furthermore, using the same crude preparations of infected insect cells for all enzymes under identical conditions may even minimize variables introduced through different purification schemes employed for individual proteins, leaving as the only variable the amount of recombinant protein expressed, which can be controlled by immunoblotting. We feel that the analysis of crude insect cell preparations, together with appropriate negative controls, is a simple and powerful approach to establish the general characteristics of individual isoenzymes. Using the standard substrate for group A PKCs, histone III-S (Sigma), in a mixed micelle assay none of the group B enzymes showed comparable activities to PKC- γ under identical conditions. In the absence of activators, histone phosphorylation by PKC- δ was consistently 3-fold higher than with PKC- γ . In contrast to PKC- γ , however, this activity was not stimulated by phospholipid/PMA. PKC-L(η) and PKC- ζ showed detectable, albeit low, phosphorylation of histone which also could not be stimulated by phosphatidylserine/PMA. Using myelin basic protein as a substrate a very similar pattern emerged. PKC- δ kinase activity was slightly stimulated in the presence of phosphatidylserine/PMA, while PKC- ζ and PKC-L(η) only showed basal levels of phosphorylation on this substrate. The phosphorylation of protamine free base and protamine sulphate by PKC purified from rat brain has recently been analysed in greater detail by Souvignat & Chambaz [47]. In agreement with their findings on natural PKC from rat brain, we observed that phosphorylation of protamine free base by all recombinant PKC types was consistently stimulated up to 2-fold by phosphatidylserine/PMA. Phosphorylation of the polyanionic substrate protamine sulphate, on the other hand, was not enhanced by the presence of activators. In contrast to PKC- γ , however, group B enzymes only produced low phosphorylation of this substrate. Taken together, the results of kinase activity assays using substrates with known effects on group A PKCs demonstrate that members of group B show a very distinct behaviour, which may be due to differences in their substrate preference or in their mode of activation. The only group B enzyme that has been studied so far in this respect is PKC- ϵ . Recombinant PKC- ϵ has been expressed in mammalian COS cells [10,49,50] and in insect cells from a baculovirus vector [18,51]. PKC- ϵ also binds phorbol ester and displays phospholipid- and PMA-dependent kinase activity on substrate peptides, but less on histone. Removal of the regulatory domain of PKC- ϵ by trypsinolysis drastically enhanced phosphorylation of histone, suggesting either that substrate specificity is influenced by the regulatory domain [51,53] or that conventional *in vitro* assay conditions do not suffice to fully activate PKC- ϵ kinase activity on this substrate. Through our analyses it also appears that the other group B members, PKC- δ and PKC-L(η), resemble PKC- ϵ in this respect. PKC- ζ with its single zinc finger does not bind phorbol ester and is the least active kinase on all substrates tested. It will be interesting to reveal potential substrates for this

enzyme and the mechanism of regulation of its kinase activity *in vivo*. The kinase activity of purified PKC- ϵ has been shown to be independent of Ca^{2+} [18]. All kinase assays with PKC- δ , - ζ and -L(η) gave the same results in the presence or absence of Ca^{2+} , suggesting that these enzymes are also independent of Ca^{2+} in their activities and that the low phosphotransferase activity of these enzymes is not due to inhibitory effects of Ca^{2+} .

In conclusion, the lack of conserved region C2, which characterizes PKC- δ , - ϵ , - ζ and PKC-L(η), appears not to be the only characteristic shared by this group. Although others have reported phospholipid- and phorbol ester-stimulated kinase activities of group B members on histone, through a direct parallel comparison of several enzymes it rather appears that histone and other conventional PKC substrates are poor substrates for group B PKCs, supporting the concept of distinct substrate specificities and thus probably distinct roles for PKCs in different cellular contexts. Although differences in catalytic properties among individual group B group members are apparent, they are all characterized by a poor response to phosphatidylserine/phorbol ester. Considering the efficient binding of phorbol ester by PKC- δ and PKC-L(η), a challenging task will be to identify the events triggered by binding of this class of substances to these enzymes.

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