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Unique substrate specificity and regulatory properties of PKC-ε: a rationale for diversity

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PKC-ε was isolated from a murine brain cDNA library. The clone, λ61PKC-ε, encoded a polypeptide of 737 amino acids that is homologous to other PKCs. Northern analysis showed that the 7 kb mRNA for this cDNA is widely expressed. The protein when expressed in COS-1 cells displayed phorbol ester-binding activity. However in order to detect the kinase activity of PKC-ε, it was necessary to employ a synthetic peptide substrate based upon the pseudosubstrate site. Subsequent analysis demonstrated that PKC-ε, while showing certain properties characteristic of the PKC family, has a quite distinct substrate specificity and is independent of Ca^{2+}.

Protein kinase C; Substrate specificity; Ca^{2+}; Phosphorylation; Gene family; Protein kinase C-ε

1. INTRODUCTION

Protein kinase C (PKC), as first described by Nishizuka and colleagues, has been implicated in the control of many cellular processes (reviewed in [1,2]). It is now clear that the general properties used to define the enzyme are shared by a group of related proteins first identified through structural analysis [3–9]. Among these reports, Housey et al. [8] described a partial cDNA clone, RP-16, which was only distantly related to the others. In order to define the relationship of this gene product to the PKC family of proteins and determine its biochemical properties, we have isolated a cDNA of this clone. The sequence of this clone is highly homologous to two recently published PKC-ε cDNA clones [10,11], and is therefore also named PKC-ε. By using a synthetic peptide as a substrate, based upon the putative pseudosubstrate site of PKC-ε, we have been able to partially purify PKC-ε. The initial characteristics of this kinase are shown to be different from PKC-α, -β1, -β2, and -γ with respect to Ca^{2+} dependence and substrate specificity.

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2. MATERIALS AND METHODS

2.1. *Isolation of PKC-ε from a mouse brain cDNA library*

The synthesis of oligodeoxyribonucleotides, the conditions for the screening of cDNA libraries and the subsequent analysis of positive clones were as described previously [12,13].

2.2. *Northern blot analysis*

Total RNA was isolated from rat tissues, using the guanidinium/cesium chloride method [14]. Total RNA was separated on a 1% agarose-formaldehyde gel (20 μg/lane) and transferred to a nitrocellulose filter. The filter was hybridised with a ^{32}P-labeled random primed PKC-ε cDNA probe (2×10^9 cpm/μg DNA) in 50% formamide, 2 × SSC (15 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 0.1% SDS, 10 × Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA, at 42°C for 20 h [14]. Three moderate 'stringency washings were done for 15 min at 50°C in 2 × SSC, 0.1% SDS and then one wash for 15 min at 55°C in 0.5 × SSC, 0.1% SDS. The filter was autoradiographed with 2 intensifying screens.

2.3. *Expression in COS-1 cells and anion-exchange chromatography*

COS-1 cells (5×10^5 cells/10 cm dish) were transfected with 20 μg of plasmid DNA, using the calcium phosphate precipitation technique [15]. Cells were harvested after 48 h in 1 ml of lysis buffer at 4°C (20 mM Tris-HCl, pH 7.5, 5 mM EDTA,

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1% Triton X-100, 0.3% β-mercaptoethanol, 250 μg/ml leupeptin, 50 μg/ml phenylmethylsulphonyl fluoride, and 10 mM benzamidine) and centrifuged at 13000 rpm for 20 min in a refrigerated microfuge. Supernatants (1 dish of cells per column) were directly applied to a TSK-DEAE-5PW HPLC column (LKB, Sweden) at a flow rate of 0.75 ml/min, equilibrated in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.3% β-mercaptoethanol. Proteins were eluted in 0.75 ml fractions with a 10 min isocratic wash followed by a linear gradient of NaCl in the same buffer (0–0.6 M NaCl in 40 min).

2.4. *Kinase assay of PKC-ε and phorbol ester binding*

Kinase activity was assayed by using a modified procedure [16] of the mixed micelle assay [17]. A sample of each fraction (5 μl) was assayed in a total assay volume of 40 μl, containing 1.25 mg/ml histone III-S (Sigma) or 0.1 mM pep-ε. The pseudosubstrate site peptide pep-ε (see text) was synthesized on an Applied Biosystems 430A peptide synthesizer. The phorbol 12,13-dibutyrate (PDB)-binding assays were carried out as previously described [18] except that 20–40 μg/ml phosphatidylserine was used.

2.5. *Generation of polyclonal antibodies and immunoblot analysis*

A synthetic peptide (NQEEFKGFSYFGEDLMP) based on the C-terminal sequence of PKC-ε was synthesized, coupled to Keyhole limpet hemocyanin (KLH) and used to immunize rabbits as described previously [19]. Protein samples were separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose and blotted [20]. Antiserum was used at a 1/3000 dilution.

3. RESULTS AND DISCUSSION

3.1. *cDNA cloning of PKC-ε and alignment with other PKC members*

An oligodeoxyribonucleotide probe was designed, based upon the published sequence of a partial cDNA clone, RP-16 [8]. The oligodeoxyribonucleotide, 5′-CCTTCACGATGACGTTCTCTACCCTGTCTGGCTTAGCAA-3′, is contained in the C_4 region of PKC-ε (fig.1b), and was used to screen a murine brain cDNA library to obtain the complete sequence of this protein. From a number of positive plaques, restriction analysis showed that four (λ61-, λ70-, λ88-, λ97-PKC-ε) contained apparently identical full-length coding regions and all of these have been shown to encode Ca^{2+}-independent phorbol ester-binding proteins (not shown). The complete sequence of λ61PKC-ε was obtained according to Sanger et al. [13]. The analyses presented below were carried out using λ61PKC-ε, which is referred to in the text as λPKC-ε.

The predicted amino acid sequence of λPKC-ε is 737 amino acids long with a molecular mass of 83570 Da. Alignment of this sequence with PKC-ε from rat and with nPKC from rabbit shows a 99% homology between these three sequences (fig.1a). 11 amino acid changes are present in the V_2/V_3 region, which also contains a deletion of Gly-390 in the rabbit sequence. 8 amino acid changes are present in the C_4 region. The putative regulatory domain of PKC-ε, V_1/C_1, is completely conserved between these three species.

The homology of PKC-ε with PKC-α, -β and -γ sequences shows that there is considerable conservation, with 48% identity between all four subtypes in the kinase domain and 44% identity between all four in the C_1 region (within the regulatory domain) [4]. However, the homology of PKC-ε with -α, -β and -γ is clearly lower than those between -α, -β and -γ themselves, which show an overall homology of more than 70%. The major areas of divergence with PKC-α, -β1, -β2 and -γ (as defined in [4]) are the regions V_1 and C_2 that are extended and deleted, respectively (fig.1b). Based on the homology to other PKC subtypes and from functional considerations, this protein is considered to be a member of the PKC superfamily and is referred to as PKC-ε (see below and [10]).

3.2. *Tissue distribution of PKC-ε*

Northern blot analysis of rat tissues with a PKC-ε probe reveals a single mRNA of 7 kb that is abundant in brain and also expressed to some degree in all other tissues studied with heart and lung showing quite high levels of expression (fig.2). This mRNA species corresponds to that identified with an RP16 probe [8] and more recently with rat [10] and rabbit [11] PKC-ε cDNAs (see below).

3.3. *Expression of PKC-ε in COS-1 cells, and characterisation of the partially purified enzyme*

In order to define the properties of the protein encoded by λPKC-ε, the cDNA was subcloned into the expression vector pmt-2 (pmt-PKC-ε). Transient expression in COS-1 cells led to a 5-fold increase in cellular phorbol ester binding (not shown), as has been observed with the expression of other PKC subtypes [5]. This increased binding activity was associated with the selective expression of a polypeptide of apparent molecular mass 89 kDa on SDS-gel electrophoresis (not shown).

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M  MVVFNGLLKIKICEAVSLKPTANSLRHAVGPRPQTFLLDPYIALNVDDSRIGQTATKQKTNSPAWHDEFV  70
Ra ------------------------------------------------------------------------
Rb ------------------------------------------------------------------------

   TDVCNGRKIELAVFHDAPIGYDDFVANCTIQFEELLQNGSRHFEDWIDLEPEGKVYVIIDLSGSSGEAPK 140
   ------------------------------------------------------------------------
   ------------------------------------------------------------------------

   DNEERVFREMMRPRKRQGAVRRRVHQVNGHKFMATYLRQPTYCSHCRDFIWGVIGKQGYQCQVCTCVVHK 210
   ------------------------------------------------------------------------
   ------------------------------------------------------------------------

   RCHELIITKCAGLKKQETPDEVGSQRFSVNMPHKFGIHNYKVPTFCDHCGSLLWGLLRQGLQCKVCKMNV 280
   ------------------------------------------------------------------------
   ------------------------------------------------------------------------

   HRRCETNVAPNCGVDARGIAKVLADLGVTPDKITNSGQRRKKLAAGAESPQPASGNSPSEDDRSKSAPTS 350
   ------------------------------------------------------------------------
   ----------------------------------------IG-------T--S----E---------

   PCDQELKELENNIRKALSFDNRGEEHRASSATDGQLASPGENGEVRPGQAKRLGLDEFNFIKVLGKGSFG 420
   ----------------------------S---------------Q-----------------------
   ----------------------------A-S-----G--  ------Q-----------------------

   KVMLAELKGKDEVYAVKVLKKDVILQDDDVDCTMTEKRILALARKHPYLTQLYCCFQTKDRLFFVMEYVN 490
   ------------------------------------------------------------------------
   ------------------------------------------------------------------------

   GGDLMFQIQRSRKFDEPRSRFYAAEVTSALMFLHQHGVIYRDLKLDNILLDAEGHCKLADFGMCKEGIMN 560
   ------------------G-----------------------------------S-----------L-
   ------------------------------------------------------------------L-

   GVTTTTFCGTPDYIAPEILQELEYGPSVDWWALGVLMYEMMAGQPPFEADNEDDLFESILHDDVLYPVWL 630
   ------------------------------------------------------------------------
   ------------------------------------------------------------------------

   SKEAVSILKAFMTKNPHKRLGCVAAQNGEDAIKQHPFFKEIDWVLLEQKKIKPPFKPRIKTKRDVSNFDQ 700
   ------------------------------------------------M-----------------------
   ------------------------------------------------------------------------

   DFTREEPILTLVDEAIIKQINQEEFKGFSYFGEDLMP 737
   ----------------V-------------------
   -------V--------V-------------------
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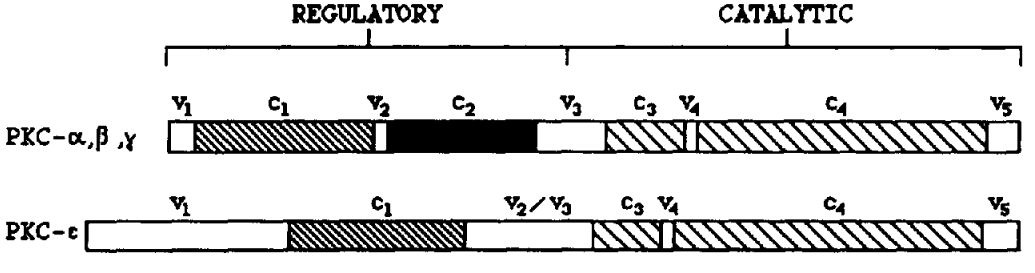

Fig.1. Protein sequence of mouse PKC-ϵ (as predicted from the cDNA sequence), and alignment with PKC-ϵ from rat (Ra) and rabbit (Rb). Only divergent amino acids are shown for rat and rabbit. With the exception of a deleted glycine at position 390, no other amino acid deletions or insertions are present. Below is shown a schematic diagram of the overall domain structure of PKC-ϵ relative to that of PKC-α,β,γ. This indicates the long extension of PKC-ϵ at the amino-terminus and the deletion of the C_2 domain.

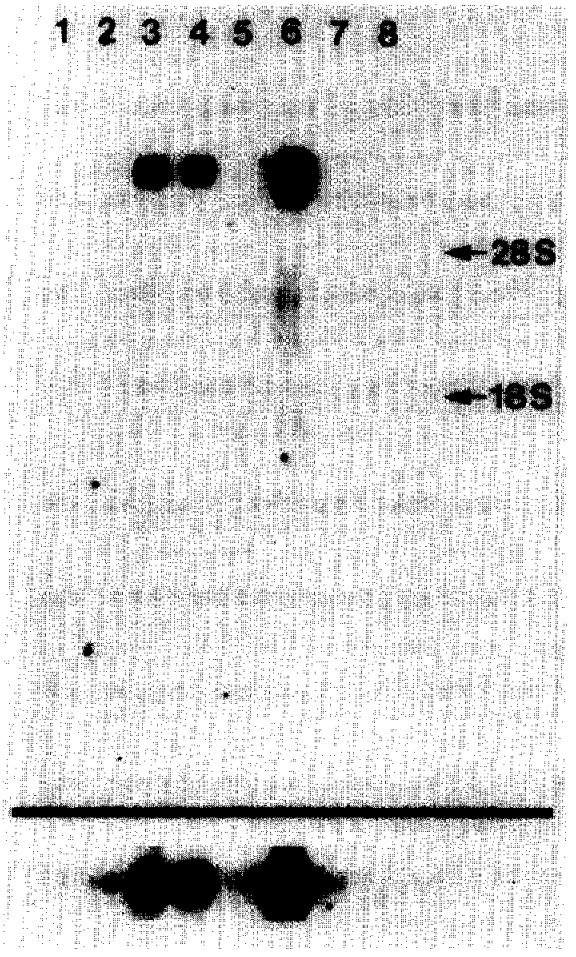

Fig.2. RNA distribution in rat tissues. A 739 bp *Xho*I fragment, containing 345 nucleotides of a 5'-untranslated sequence and 294 nucleotides of a 5'-translated sequence, was used as a PKC-ϵ specific probe which recognized a single transcript of 7 kb. The filter was autoradiographed at −80°C for 5 days (top panel) and for 4 weeks (lower panel). Lanes: 1, liver; 2, kidney; 3, lung; 4, heart; 5, gut; 6, brain; 7, thymus; 8, spleen. Although not clear from the reproduction, in the original autoradiograph, lanes 1 and 8, like lanes 2, 5 and 7, do show a positive signal, albeit somewhat weaker.

The majority of the induced binding activity was found in the cytosolic fraction and was Ca^{2+} independent, in contrast to the binding activities of PKC-α, -β and -γ. However using a conventional assay, no significant change in Ca^{2+}/phospholipid/TPA-dependent histone (III-S) kinase activity was observed in the pmt-PKC-ϵ transfected cells.

In order to investigate further the potential kinase activity of PKC-ϵ, use was made of the observation by Kemp and colleagues [21] that a modified (serine for alanine substitution) 'pseudosubstrate' site peptide from PKC-α serves as a good substrate for purified PKC (probably a mixture of subtypes α, β and γ). In PKC-ϵ there is a sequence immediately amino-terminal to the cysteine repeat region that resembles the pseudosubstrate sites of other PKC subtypes: this sequence (ERMRPRKRQGAVRRRV) was synthesized (residues 149–164) with the alanine (underlined above) replaced by a serine residue (referred to as pep-ϵ).

When pep-ϵ was employed as a substrate to screen transfected COS-1 cells, it was apparent that PKC-ϵ efficiently phosphorylated this substrate (PKC-α could also phosphorylate pep-ϵ at a rate equivalent to histone). Fractionation of cell extracts showed that multiple peaks of kinase activity were obtained following transfection with pmt-PKC-ϵ (peaks I–III; fig.3). Peak I is retarded and elutes in the isocratic wash on this column: this peak contains some of the PKC-ϵ activity and has on occasions been found to co-elute with peak II, suggesting that these activities may separate for incidental reasons, e.g. interaction with other cellular components. Peak II represents the majority of PKC-ϵ activity but also contains the endogenous PKC-α activity. Peak III was consistently found to elute 8 min after peak II on this column: the activity in this peak is characteristic of neither PKC-ϵ nor the endogenous PKC-α (see below).

A polyclonal antibody directed against the C-terminus of PKC-ϵ (see section 2.5) was used to characterize further the three protein kinase peaks obtained from pmt-PKC-ϵ transfected cells. As shown in fig.4a, this antiserum specifically recognizes a polypeptide of ~90 kDa in peaks I and II from pmt-PKC-ϵ transfected cells (lanes 2 and 4) and showed no immunoreactivity with equivalent fractions from control transfections (lanes 1 and 3). No immunoreactive 90 kDa PKC-ϵ was detected in peak III (lane 6) although on longer exposure (fig.4b) an immunoreactive polypeptide of ~50 kDa is apparent; it is possible that this represents a catalytic fragment of PKC-ϵ (see below).

The properties of the individual peaks obtained are summarised in table 1. It is clear from these

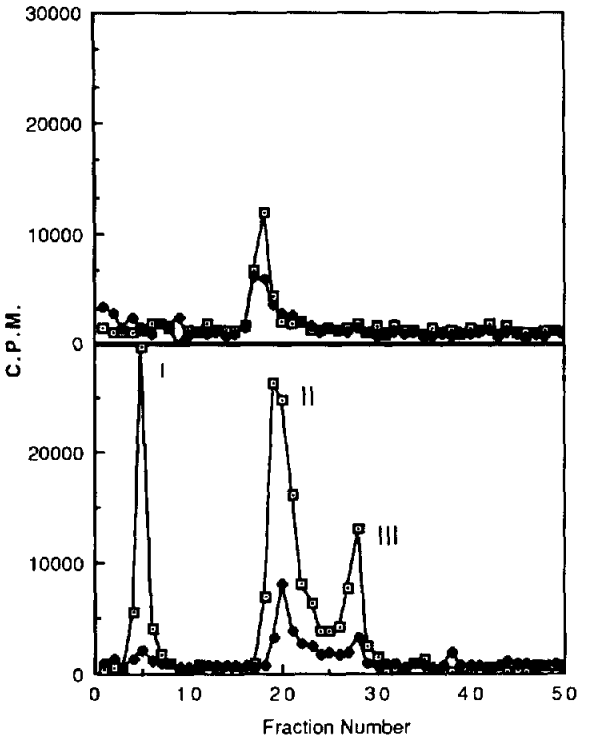

Fig.3. Separation of protein kinase activities by anion exchange chromatography. Extracts from COS-1 cells transfected with the pmt-2 vector alone (top panel) or pmt-PKC-ϵ (lower panel) were subjected to anion-exchange chromatography on a TSK DEAE-5PW column. Fractions were assayed for kinase activity with histone (closed symbols) or with pep-ϵ (open symbols) as substrate (see text).

data that peaks I and II display a Ca^{2+}-independent phorbol ester-binding activity and a phospholipid/phorbol ester-dependent pep-ϵ kinase activity which is also Ca^{2+}-independent. This contrasts strongly with the activities of the endogenous PKC-α, which shows Ca^{2+} dependence for both phorbol ester binding and kinase activity (both pep-ϵ and histone kinase activities). There is barely detectable histone kinase activity associated with peak I, while that of peak II corresponds to the endogenous PKC-α and is characterized by Ca^{2+} dependence (table 1; see also fig.3 and text). This analysis indicates that peak I is PKC-ϵ and peak II is a mixture of the endogenous PKC-α and PKC-ϵ. The PKC-ϵ kinase activity in peak II is evident when one compares the Ca^{2+}-independent pep-ϵ kinase activity in the control, 0.03 U/ml, with the activity in peak II, 0.25 U/ml (table 1).

In contrast to peaks I and II, peak III shows

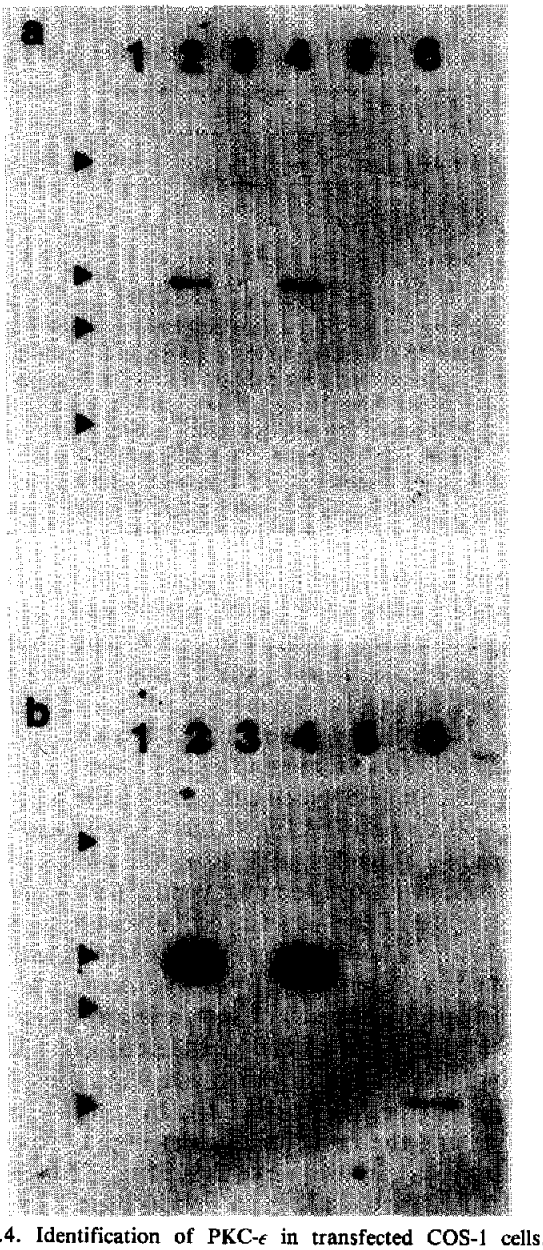

Fig.4. Identification of PKC-ϵ in transfected COS-1 cells. Fractions containing the three peaks of pep-ϵ kinase activity from pmt-PKC-ϵ transfected cells were immunoblotted using an antiserum directed against the C-terminus of the predicted PKC-ϵ sequence. The equivalent fractions from a control transfection were analysed in parallel. For both panels, lanes 2, 4 and 6 contain peaks I, II and III, respectively, and lanes 1, 3 and 5 contain the equivalent fractions from a control transfection with pmt-2. The fraction in lane 3 contains the endogenous PKC-α (see top panel of fig.3): this is not recognised by the PKC-ϵ specific antiserum. The markers shown are in descending order: 200, 92.5, 69, and 46 kDa. (a) A blot exposed for 20 h at room temperature; (b) a longer exposure of the same blot (48 h at $-80°C$).

Table 1

Characteristics of protein kinase activities in transfected COS-1 cells

	pep-ϵ kinase activity					Histone-IIIS kinase activity					PDB binding	
Addition:	None	PS	PS/C	PS/T	PS/T/C	None	PS	PS/C	PS/T	PS/T/C	− C	+ C
Control	<1	<1	<1	3	15	2	<1	<1	8	20	1	4.4
Peak I	<1	1	1	25	24	<1	<1	<1	<1	4	24	27
Peak II	<1	<1	4	25	61	<1	<1	<1	2	35	27	25
Peak III	29	53	40	51	43	13	13	16	16	13	<0.1	<0.1

Characteristics of fractionated kinase activities from control and PKC-ϵ expressing COS-1 cells. Activities separated as described in the legend to fig.3 were assayed for kinase activities, which are expressed as nmol/min per ml × 10^2. The control activity is the endogenous PKC-α peak observed in the pmt-2 transfected cells (see upper panel of fig.3). The standard kinase assay [18] was carried out with various combinations of PKC effectors present as indicated: PS, phosphatidylserine; C, calcium; T, tetradecanoylphorbol acetate; where calcium is omitted, it is replaced by 5 mM EGTA. The phorbol 12,13-dibutyrate (PDB)-binding activity was carried out in the presence of 5 mM EGTA (− C) or in the presence of 0.75 mM total calcium (+ C); units are expressed as pmol PDB bound/ml. All assays are means of duplicate determinations. The data shown are from one of several independent experiments

both pep-ϵ and histone kinase activities and both are independent of the effectors tested (table 1). Furthermore this fraction does not bind phorbol esters (table 1). It seems likely that this fraction contains a catalytic fragment of PKC-ϵ that is no longer subject to regulation and shows an altered specificity. This interpretation would be consistent with the immunological data showing the presence of a ~50 kDa protein (fig.4b) that may represent a C-terminal fragment of PKC-ϵ (the antiserum is directed against a C-terminal peptide). However in the absence of further evidence it cannot be ruled out that the activity present in peak III is due to an unrelated induced or activated protein kinase.

Further investigation of substrate specificity has shown that none of the commercially available histones (Sigma) nor α-casein will act as substrates for PKC-ϵ (peak I or peak II). In contrast, protamine and the random copolymers poly-Arg,Ser and poly-Lys,Ser will serve as substrates for PKC-ϵ, but they are phosphorylated in a phospholipid/phorbol ester-independent manner. Thus pep-ϵ is the only phospholipid/phorbol ester-dependent substrate for PKC-ϵ that has been identified to date.

Recently Ono and colleagues have described the cDNA cloning of PKC-ϵ from a rat cDNA library [10]. Furthermore they also show a brief characterization of their expressed rat PKC-ϵ enzyme. However, they detect only one kinase activity peak, using histone as a substrate, which would appear to correspond to peak III (fig.3). The authors conclude from these studies that PKC-ϵ shows completely different regulatory features compared to other PKC subtypes; this is clearly at variance with the data reported here for the murine clone. Also recently Ohno et al. [11] reported the cDNA cloning of PKC-ϵ from rabbit. These authors also find a PKC-ϵ protein that essentially co-elutes with the endogenous PKC-α on anion-exchange chromatography. However it was found necessary to immunodeplete the PKC-α in order to detect histone kinase activity associated with the expressed PKC-ϵ. As discussed [11], this could have been due to the conditions of the assay or to the poor use of histone as a substrate. The results here resolve this important point by showing that it is the specificity of PKC-ϵ that is different.

In addition to the apparent Ca^{2+} independence, a unique property of PKC-ϵ within the context of the PKC family is its distinct substrate specificity which precludes its ready detection with a histone (III-S) substrate. The use of the pseudosubstrate site as a predictive tool for the generation of a synthetic substrate has been important in overcoming the problem of specificity for PKC-ϵ, although it is clear from the results that this peptide substrate is not exclusively phosphorylated by PKC-ϵ, since it is also used as a substrate for PKC-α. Nevertheless the use of pseudosubstrate site peptides may well be of value in other situations where novel kinases are defined through molecular biological approaches.

The implication of these results is that PKC-ϵ

will selectively phosphorylate a particular set of intracellular proteins in response to diacylglycerol production and that this can occur independent of Ca^{2+} release. Thus in the context of the phosphatidylinositol pathway, PKC-ϵ and PKC-α (for example) will not simply function interchangeably, but will act in parallel to target different sets of intracellular proteins and so diversify the cellular response. Furthermore agonist-induced degradation of other lipids that produce diacylglycerol independent of a Ca^{2+} mobilising signal, may selectively lead to the activation of PKC-ϵ. Thus cellular responses to agonists could be modified through the expression of combinations of PKC subtypes that differ in their intracellular targets and in their Ca^{2+} requirement.

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