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Expression and Partial Characterization of Rat Protein Kinase C-δ and Protein Kinase C-ζ in Insect Cells Using Recombinant Baculovirus

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Abstract Expression of rat protein kinase C-δ (PKC-δ) and PKC-ζ in insect cells using recombinant baculovirus resulted in the production of proteins with a molecular size of approximately 76 kD and 78 kD, respectively, as determined by immunoblotting with subtype-specific antisera. Although the PKC-ζ cDNA encoded for 592 amino acids, a 76 kD protein was also generated by in vitro transcription/translation. Extracts of cells expressing PKC-δ were able to bind phorbol ester to levels comparable to extracts of cells expressing PKC-α. No phorbol ester binding was, however, detected in insect cell extracts expressing PKC-ζ. However, similar levels of protein kinase activity were detected in lysates of cells expressing PKC-δ or PKC-ζ when protamine sulfate was used as exogenous substrate. Compared to protamine sulfate, both, myelin basic protein (MBP) or histone, were poor substrates for PKC-δ and PKC-ζ. In contrast to PKC-ζ, the PKC-δ enzyme activity phosphorylated MBP or histone in a phosphatidylserine-(PS)/diacylglycerol(DG)-dependent manner, albeit not to the same extent as PKC-α. Lack of stimulation of the enzyme activity of PKC-ζ by PS/DG, was confirmed by endogenous phosphorylation of insect cell proteins by PKC-ζ, whereas several insect cell proteins were phosphorylated by PKC-δ in a PS/DG-dependent manner, including a protein of 78 kD.

Our data demonstrate that the 76 kD PKC-ζ, in contrast to PKC-δ, is unable to bind phorbol esters and displays a protein kinase activity that is independent of PS or PS/DG. In addition, staurosporine was about 2–4 order of magnitudes less effective in inhibiting the protein kinase activities of PKC-δ and PKC-ζ when compared to PKC-α.

Key words: rat protein kinase C, recombinant baculovirus, antisera, phorbol ester, isoenzymes

Protein kinase C (PKC) has been originally identified as a phospholipid-sensitive, calcium-dependent protein kinase which can be fully activated either by diacylglycerol (DG) or by tumor promoting phorbol esters such as 12-O-tetradecanoyl-phorbol 13-acetate (TPA) (Castagna et al., 1982; Bell and Burns, 1991; Nishizuka, 1988; Huang, 1989; Jaken, 1990). These type of tumor promoters are able to replace DG in the activation of PKC and have provided insights into the role of this enzyme in the regulation of a variety of cellular processes including exocytosis, gene expression, proliferation, differentiation, and tumor promotion (Castagna et al., 1982; Nishizuka, 1986; Weinstein, 1990; Jaken, 1990; Houslay, 1991; O'Brian and Ward, 1989). Subsequent molecular cloning of PKC revealed the existence of a family of related but distinct enzymes (Parker et al., 1989; Huang, 1989; Bell and Burns, 1990; Ono et al., 1988; Ono et al., 1989b; Nishizuka, 1988; Osada et al., 1990; Bacher et al., 1991). According to structural features and their dependence on calcium for activity, the members of the PKC family have been classified into a calcium-dependent group of enzymes (cPKCs: PKC-α, PKC-β1, PKC-β2, and PKC-γ) and a calcium-independent group of non-conventional PKCs (nPKCs: PKC-δ, PKC-ε, PKC-ζ, and PKC-η) (Parker et al., 1989; Huang, 1989; Bell and Burns, 1990; Ono et al., 1988; Nishizuka, 1988; Osada et al., 1990; Bacher et al., 1991; Schaap and Parker, 1990). Comparison of their amino acid se-

Abbreviations used: PS, phosphatidylserine; DG, diacylglycerol; TPA, 12-O-tetradecanoyl-phorbol 13-acetate.

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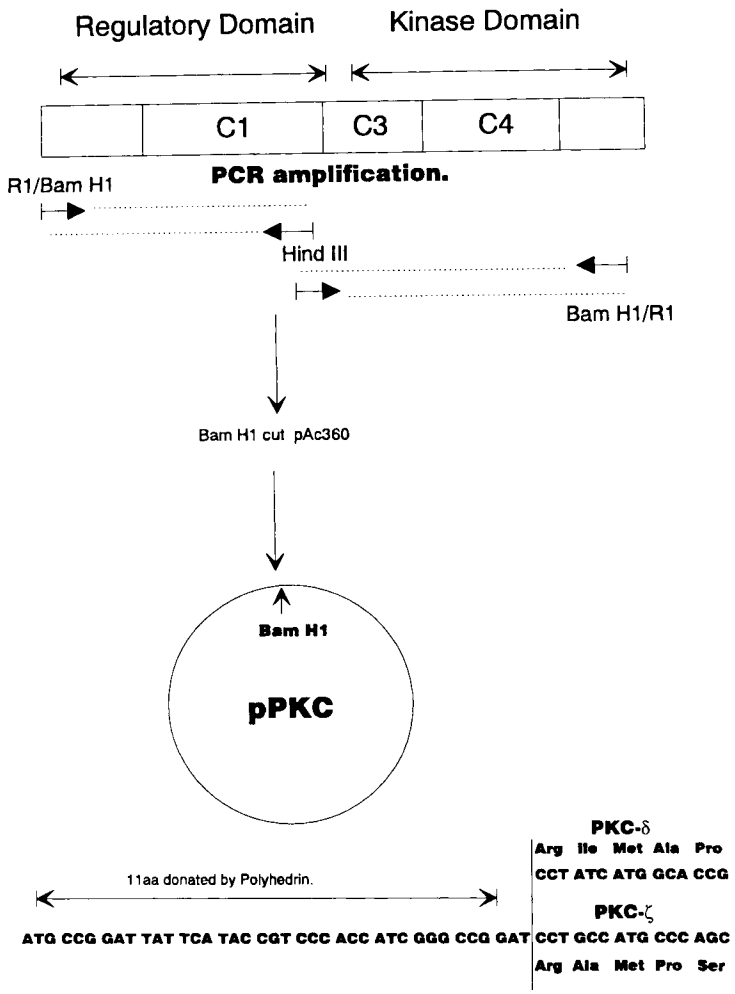

Fig. 1. Cloning strategy for recombinant baculovirus bvPKC-δ and bvPKC-ζ. Rat brain PolyA$^+$ RNA was used to synthesize first strand cDNA which was amplified as two PCR fragments encompassing the entire coding region of the PKC-δ and PKC-ζ as described in Material and Methods. The 3′-half and the 5′-half of PKC-δ and PKC-ζ coding regions were joined by digestion with EcoR I and Hind III and ligation into EcoR I cut pGEM-1 (pPKC). The full length cDNAs of PKC-δ and PKC-ζ were cloned into the unique BamH I of the transfer vector pAc360 and used to produce recombinant baculovirus.

quences revealed that the calcium-dependent group of cPKCs contains four large conserved regions, three of which are present in the calcium-independent group of nPKCs (C1–C3, Fig. 1) (Huang, 1989; Parker et al., 1989; Bell and Burns, 1991; Nishizuka, 1988).

The protein kinase domain of PKC is confined to the C-terminus and is separated by a protease-sensitive hinge region from the regulatory N-terminal region (Nishizuka, 1988; Huang, 1989; Parker et al., 1989; Bell and Burns, 1991; Schaap et al., 1990a; Huang et al., 1988; Kishimoto et al., 1989). The latter domain displays two invariant cystein-rich zinc fingers (Bell and Burns, 1991; Nishizuka, 1988; Parker et al., 1989), with the exception of the PKC-ζ which carries only one of these finger structures that is unable to bind phorbol esters (Ono et al., 1989b). These cystein-rich zinc fingers have been reported to function in the phospholipid-dependent binding of tumor promoting phorbol esters or DG (Nishizuka, 1988; Bell and Burns, 1991; Jaken, 1990; Huang, 1989; Ono et al., 1989a; Kaibuchi et al., 1989; Burns and Bell, 1991; Cazaubon et al., 1989, 1990). Recent reports suggest that each of the cystein-rich zinc finger alone is sufficient to bind phorbol esters albeit with reduced affinity compared to the holoenzyme (Ono et al., 1989a; Cazaubon et al., 1990; Burns and Bell, 1991).

With the exception of the calcium-dependent cPKCs and of the PKC-ε, that have been characterized to a certain extent in vitro (Akita et al.,

1990; Burns et al., 1990; Huang et al., 1988; Marais and Parker, 1989; Schaap and Parker, 1990; Akita et al., 1990; Ohno et al., 1988), only limited information is available about the biochemical properties of the other members of the calcium-independent nPKCs (Bacher et al., 1991; Osada et al., 1990; Ono et al., 1988, 1989b). Since specific function(s) for each PKC subtype in the regulation of discrete cellular responses have been proposed (Nishizuka, 1988; Kikkawa et al., 1989; Weinstein, 1990; O'Brian and Ward, 1989; Jaken, 1990; Bell and Burns, 1991), the analysis of the biochemical properties of individual PKC subtypes should provide some understanding for the diversity of his enzyme family at the cellular level.

Expression of proteins in insect cells using recombinant baculovirus has emerged as the method of choice for high level expression of protein kinases especially for PKC (Patel and Stabel, 1988; Schaap and Parker, 1990; Fiebich et al., 1990; Burns et al., 1990; Burns and Bell, 1991). Here we report cloning and expression as well as partial biochemical characterization of rat PKC-δ and PKC-ζ using the baculovirus expression system. Our results show that in contrast to PKC-δ, the 76 kD PKC-ζ expressed in insect cells is unable to bind phorbol esters and displayed a protein kinase activity that is independent of PS or PS/DG. In addition, the PKC-ζ subtype, in contrast to PKC-α and PKC-δ was almost insensitive towards the potent protein kinase inhibitor staurosporine.

MATERIALS AND METHODS

Materials

Protamine sulfate, Histone III-S, and myelin basic protein were from Sigma Chemical Co. (St. Louis). [γ-^{32}P]ATP (1,000 Ci/mmol), ^{35}S-methionine (>1000 Ci/mmol), and ^{125}I-labeled anti-rabbit IgG were obtained from Amersham (Arlington Heights, IL). Taq polymerase and MLV-reverse transcriptase were from Pharmacia (Piscataway, NJ). The baculovirus vector pAc360 and Sf9 cells were obtained from M.D. Summers (Texas A&M University). The pGEM-1 was obtained from Promega. [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) (10.2 Ci/mmol) was from NEN. Restriction enzymes were from New England Biolabs or Boehringer (Mannheim). In vitro transcription system with the T7 and Sp6 polymerases were from Boehringer (Mannheim) and the rabbit reticulocyte lysate was from Promega. Cell culture materials and foetal calf serum were from Gibco/BRL. The Ex-Cell 400 medium was from JR Scientific (Woodland, CA).

Methods

Isolation of PKC-δ and PKC-ζ cDNA clones. Molecular cloning techniques used are essentially described in Sambrook et al. (1989). Wistar rat brain total RNA was isolated according to Chomczynski and Sacchi (1987). PolyA^{+} RNA was affinity purified by oligo-dT cellulose (Pharmacia, Piscataway, NJ) and used to synthesize single stranded cDNA using MLV-reverse transcriptase in the presence of oligo-(dT) primer (Boehringer, Mannheim). Two Polymerase Chain Reaction (PCR) fragments encompassing the entire coding region of the PKC-δ and PKC-ζ were generated using primers (Applied Biosystems) synthesized according to published sequences (Ono et al., 1988, 1989b). D1(5′CGGAATTCGGATCCTATCATGGCACCGTTCCTG3′) containing the start codon of PKC-δ and the unique cloning sites for EcoR I and BamH I as well as D2(5′CGCTTCCGGGAAGCTTTCTGG3′) carrying the unique Hind III site in the PKC-δ coding region were used to generate the 5′ half (0.95 kb) of the PKC-δ coding region, whereas the 3′ half of PKC-δ (1.15 kb) coding region was PCRed using the primer D3(5′CCGAATTCGGATCCCAGGTCTGGGAGCTCA3′) containing the stop codon of PKC-δ and EcoR I and BamH I cloning sites as well as the primer D4(5′GTGACCCAGAAAGCTTCCCGG3′) carrying the unique Hind III site (Fig. 1). Similarly, primer Z1(5′CTGAATTCGGATCCTGCCATGCCCAGCAGG3′) containing the start codon and the EcoR I and BamH I cloning sites and primer Z2(5′CGTGTTCCTCTGGAAGCTTC3′) carrying the unique Hind III site in the PKC-ζ coding region were used to generate the 5′ half (1.0 kb) of PKC-ζ. The 3′-half of the PKC-ζ coding region (0.75 kb) was generated using the primers Z3(5′CGATCGAATTCGGATCCTCACACGGACTCC3′) containing the stop codon and EcoR I and BamH I sites as well as Z2('GCAGAGGCAGAGGAAGCTTC3′) carrying the unique Hind III site. The 3′- and 5′-halves of PKC-δ and PKC-ζ coding regions were joined by digestion with EcoR I and Hind III and ligated into EcoR I cut pGEM-1. Restriction and sequence analysis confirmed that these two cDNAs inserted into the pGEM-1 (pGEM-δ and pGEM-ζ) were identical to PKC-δ and PKC-ζ sequences previously published (Ono et al., 1988, 1989b).

Cells and virus. Standard methods of gene expression using the baculovirus vector systems were used as outlined in Summers and Smith (1987). The cell line Spodoptera frugiperda (Sf9) was propagated as a monolayer or in suspension culture in medium containing 10% foetal calf serum. Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant viruses were propagated using spinner cultures of Sf9 cells.

Construction of recombinant baculovirus. The baculovirus transfer vector pAc360 (Luckow and Summers, 1988) was used to express PKC-δ and PKC-ζ. The coding region of PKC-δ and PKC-ζ were cloned into the unique Bam HI site of the pAc360. The recombinant transfer plasmids pPKC-ζ and pPKC-δ (Fig. 1) were then co-transfected with AcNPV DNA into Sf9 insect cells as described by Smith et al. (1983). Recombinant virus bvPKC-δ and bvPKC-ζ, carrying a single copy of the respective PKC coding regions, were isolated by a modification of the limiting dilution procedure of Fung et al. (1988) and amplified in spinner cultures of virus-infected Sf9 cells. Titers ranged between 1 and 3×10^8 plaque forming units per ml. The generation of the pig kidney cell line LLC-PK1 overexpressing the bovine brain PKC-α has been described elsewhere (Wartmann et al., 1991).

In vitro transcription and translation of PKC-ζ and PKC-δ cDNAs. pGEM-δ plasmids carrying the coding region of PKC-δ were either linearized with Bgl I or Sal I and transcribed in vitro using either the Sp6 or the T7 polymerases, respectively. pGEM-ζ carrying the full length PKC-ζ cDNA was linearized with Sal I and transcribed in vitro using the T7 polymerase. Approximately 5 μg of in vitro synthesized RNAs were translated in vitro in the rabbit reticulocyte lysate in the presence of 1 μCi of ^{35}S-methionine for 90 min at 37°C. The reactions were then stopped by boiling in SDS and the radiolabeled proteins resolved on a 10% SDS gel were visualized by fluorography as described previously (Borner et al., 1989). Controls for the in vitro transcription/translation were Brome Mosaic Virus (BMV) RNA (Promega) and appropriately linearized plasmid constructs FA and Sp6-neo (Boehringer, Mannheim) whose translation products are 40 kD and 30 kD, respectively.

Determination of PKC activity and phorbol ester binding. Sf9 cells were infected with recombinant baculovirus at a multiplicity of infection of 10 and harvested 48 h after postinfection. Cytosol and membrane fractions were prepared using the sequential extraction with NP-40 and SDS exactly as described by Borner et al. (1989). For ^{3}H-PDBu binding, subcellular fractions were prepared in the absence of detergent and analyzed as described previously (Fabbro et al., 1988). PKC activity was assayed by measuring the incorporation of ^{32}P from γ-[^{32}P]ATP into histone III-S, protamine sulfate, or myelin basic protein in the presence or absence of phosphatidylserine and DG or TPA (Novabiochem) as described (Fabbro et al., 1988). One unit of PKC activity is defined as 1 nmol of ^{32}P transferred from γ-[^{32}P]ATP into the respective substrates per minute at 37°C. Endogenous phosphorylation of cytosolic fractions were performed as described above in the absence of added exogenous protein substrate.

Western blot analysis of PKC-δ and PKC-ζ. Peptides (Applied Biosystems), based on the C-terminal sequences of rat PKC-δ (KGFSFVNPKYEQFLE, Ono et al., 1988) and of PKC-ζ (GFEYINPLLLSAEESV, Ono et al., 1989b), were coupled to Keyhole limpet hemocyanine by glutaraldehyde as described by Harlow and Lane (1988) and used to immunize rabbits as previously described (Borner et al., 1989). Aliquots of subcellular fractions were resolved on 8% SDS gels, transferred on nitrocellulose (Bio-Rad), and analyzed for immunoreactivity (dilutions 1:1,000) using labeled ^{125}I-anti-rabbit IgGs as described earlier (Borner et al., 1989). Specificities of anti-PKC-δ and anti-PKC-ζ antisera towards their respective antigens as well as towards other PKC subtypes were tested using insect cell extracts expressing recombinant PKC-α, PKC-β1, PKC-β2, PKC-γ, PKC-ε, and PKC-η kindly provided by Dr. S. Stabel. The anti-PKC-δ and anti-PKC-ζ antisera proved to be highly specific for their respective antigens as no cross-reactivities against the others PKC subtypes were detected (data not shown).

Other analytical methods. Protein concentrations were determined as described by Bradford (1976) using bovine serum albumin as standard (Bio-Rad). Concentration of nucleic acids was determined by OD_{260} absorption (Sambrook et al., 1989).

RESULTS

Construction of Recombinant Baculovirus Carrying PKC-δ and PKC-ζ

The full length rat PKC-δ and PKC-ζ cDNAs obtained by PCR revealed open reading frames for PKC-δ of 2.07 kb and for PKC-ζ of 1.78 kb encoding for 673 and 592 amino acids, respectively, which were identical to the previously published sequences for rat PKC-δ and PKC-ζ (Ono et al., 1988, 1989b). Recombinant bvPKC-δ and bvPKC-ζ baculovirus were constructed using the transfer vector pAc360 (Luckow and Summers, 1988) which allows the expression of the recombinant proteins fused to the first 11 amino acids of the baculovirus polyhedra protein (Fig. 1). Using this transfer vector the expression of these two nPKC subtypes is under the transcriptional and translational control of the polyhedrin protein regulatory sequences (Luckow and Summers, 1988). Although by this cloning strategy 13 amino acids were added N-terminal of both nPKC subtypes (Fig. 1), this approach resulted in high level expression of these two nPKCs.

Expression of PKC-δ and PKC- ζ in Insect Cells

Sf9 cells infected with either bvPKC-δ or bvPKC-ζ expressed novel proteins of about 78 kD and 76 kD, respectively, as determined by Coomassie blue staining of SDS gels (data not shown) which were absent in wild-type baculovirus-infected insect cells. These two novel proteins of 78 kD and of 76 kD immunoreacted specifically with their corresponding anti-PKC antisera and were absent from cells infected with the wild-type baculovirus or uninfected control cells (Fig. 2). Subcellular fractionation of insect revealed that significant amounts of the total cellular PKC-δ and PKC-ζ proteins were located in membrane fractions (Fig. 2). About 20–30% of the total cellular 76 kD PKC-ζ and 78 kD PKC-δ immunoreactive proteins could only be solubilized by SDS (Fig. 2). The antisera also occasionally reacted with proteins around 50 to 60 kD, suggesting a possible proteolytic fragmentation of PKC-δ and PKC-ζ proteins in insect cells (data not shown). The apparent molecular size of the protein generated by in vitro transcription/translation of the PKC-δ cDNA was consistent with the 78 kD PKC-δ protein expressed in insect cells, suggesting that the 13 amino acids at the N-terminus of PKC-δ did not

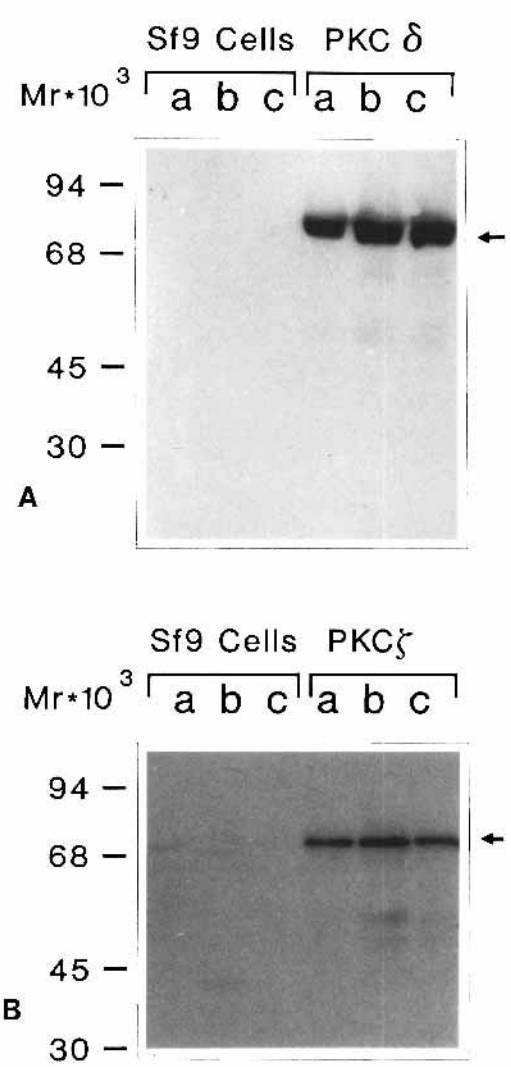

Fig. 2. Western blot analysis of PKC-δ and PKC-ζ expression. Equal amounts of protein (10 μg) from subcellular fractions (a–c) obtained from insect cells infected with either the wild-type baculovirus (Sf9), bvPKC-δ (PKC-δ), or bvPKC-ζ (PKC-ζ) were resolved on a 8% SDS gel and immunoblotted using the specific anti-PKC-δ **(A)** and anti-PKC-ζ **(B)** antisera as described in Methods. (a) cytosol; (b) NP-40 membrane extract; (c) final membrane pellet (SDS-solubilized). The final membrane pellet obtained after extraction of the membrane fractions with NP-40 was completely solubilized in hot SDS.

affect its mobility in SDS gels (Figs. 2, 3). Although the full length cDNA for PKC-ζ encoding for 592 amino acids predicts a calculated molecular weight of 68 kD, its major in vitro translation product was a protein with an apparent molecular size of 76 kD (Fig. 3). The finding that the PKC-ζ cDNA generated in vitro a protein similar in size (i.e., 76 kD) to that expressed from insect cells (which carries 13 additional amino acids at its N-terminus) indicates that

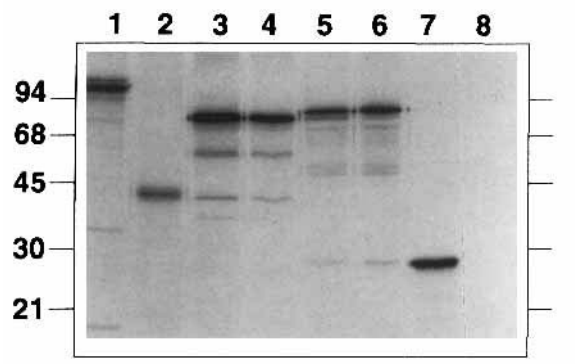

Fig. 3. In vitro transcription/translation of PKC-δ and PKC-ζ. The pGEM-1 carrying the full length cDNAs of either PKC-δ or PKC-ζ were linearized and transcribed in vitro using either the Sp6 or the T7 polymerases (Sp6, T7). The synthesized RNAs were translated using the rabbit reticulocyte lysate in the presence of ^{35}S-methionine using the appropriate controls as described in Methods. Lanes 1, BMV RNA; 2, FA; 3, PKC-z (T7); 4, PKC-z (T7); 5, PKC-d (Sp6); 6, PKC-d (T7); 7, Sp6-neo, and 8, translation in the absence of added RNA.

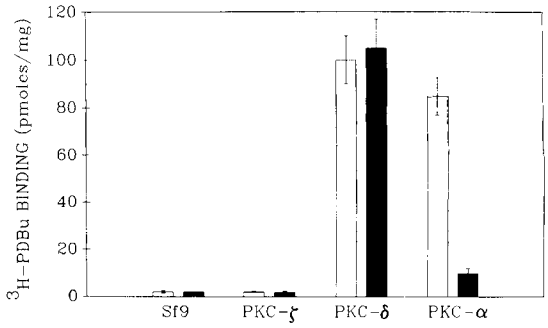

Fig. 4. Phorbol ester binding of PKC-α, PKC-δ, and PKC-ζ. Subcellular fractions (5 μg) obtained from insect cells expressing either the wild-type baculovirus (Sf9), bvPKC-δ (PKC-δ), or bvPKC-ζ (PKC-ζ) as well as LLC-PK1 cells overexpressing PKC-α (PKC-α) were analyzed for ^{3}H-PDBu binding as described in Methods. Values are means of triplicates ± SD from two independent experiments. Cytosolic (□) and membrane (■) fractions.

the PKC-ζ subtype has an anomalous migration behavior in SDS gels (Figs. 2, 3).

Phorbol Ester Binding of PKC-δ and PKC-ζ

We have previously shown that the overexpression of bovine PKC-α in the pig kidney cell line, LLC-PK1, results in a 15–20-fold increase in phorbol ester binding (Wartmann et al., 1991). Similarly, cytosolic and membrane fractions from bvPKC-δ-infected cells were found to specifically bind significant amounts of phorbol ester compared to uninfected or wild-type baculovirus infected cells (Fig. 4). Levels of phorbol ester binding in cytosolic fractions of cells expressing the PKC-δ were comparable to the levels found in cells overexpressing the PKC-α (Fig. 4). Equal amounts of phorbol ester binding were found in the cytosolic and membrane fractions of cells expressing the PKC-δ, whereas in cells overexpressing the PKC-α the phorbol ester binding was predominantly localized in the cytosolic fraction (Fig. 4). Subcellular distribution of phorbol ester binding in cells expressing the PKC-δ and PKC-α well agreed with the distribution of these two PKC subtypes as determined by immunoblotting (Figs. 2 and 4, see also Wartmann et al., 1991). In contrast, no significant levels of phorbol ester binding were detected in subcellular fractions of uninfected Sf9 cells or insect cells expressing the PKC-ζ (Fig. 4), although expression levels of PKC-ζ were comparable to PKC-δ as determined by immunoblotting and/or Coomassie blue staining (data not shown). These results suggest that the 76 kD PKC-ζ expressed in insect cells is unable to bind phorbol esters.

Protein Kinase Activity of PKC-α, PKC-δ, and PKC-ζ

Cytosolic fractions of cells overexpressing PKC-α as well as insect cells expressing PKC-δ and PKC-ζ were assayed for protein kinase activity under a variety of conditions (Fig. 5). In order to equalize the amounts of the respective PKC subtypes for the determination of protein kinase activity, the levels of expression of each PKC subtype was monitored by phorbol ester binding and/or immunoblotting. Cytosolic fractions of Sf9 cells expressing PKC-δ and PKC-ζ were able to phosphorylate protamine sulfate in a PS/DG-independent manner to levels comparable to PKC-α (Fig. 5C). Whereas, PKC-α also efficiently phosphorylated histone and myelin basic protein (MBP) in a PS/DG-dependent manner, both histone and MBP were, however, poor substrates for PKC-δ and PKC-ζ (Fig. 5). In addition, stimulation of PKC-δ protein kinase activity by PS/DG or PS/TPA using histone or MBP as substrate was marginal (2–3-fold) compared to PKC-α (10-fold) (Fig. 5). No further stimulation of PKC-δ protein kinase activity on these two standard substrates was observed when calcium was added in combination with PS/DG (data not shown).

No stimulation of PKC-ζ protein kinase activity was detected in the presence of PS/TPA, PS/DG, or PS using either of the exogenous substrates (Fig. 5). These data indicate that the enzyme activity of PKC-ζ is independent of cofac-

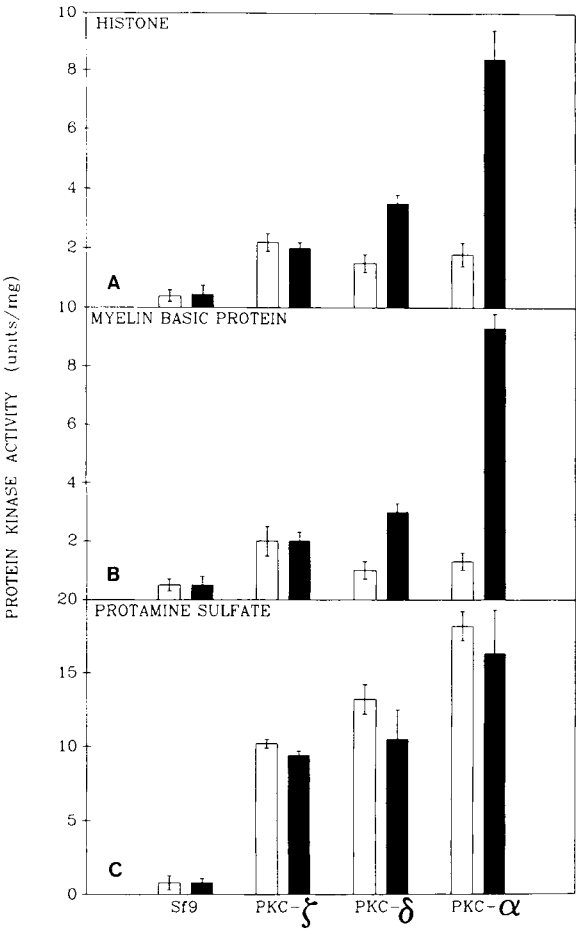

Fig. 5. Protein kinase activity of PKC-α, PKC-δ, and PKC-ζ. Cytosolic fractions (1–5 μg) obtained from insect cells infected with wild-type baculovirus (Sf9), bvPKC-δ (PKC-δ), or bvPKC-ζ (PKC-ζ) as well as of cells overexpressing PKC-α were analyzed for protein kinase activity as described in Methods. Protein kinase activity of cytosolic fractions in either absence (□) or presence (■) of 20 μg PS and 300 nM TPA using 0.5 mg/ml of either histone (**A**), myelin basic protein (**B**), or protaminesulfate (**C**) as exogenous substrates. Values are means of triplicates ± SD from two independent experiments.

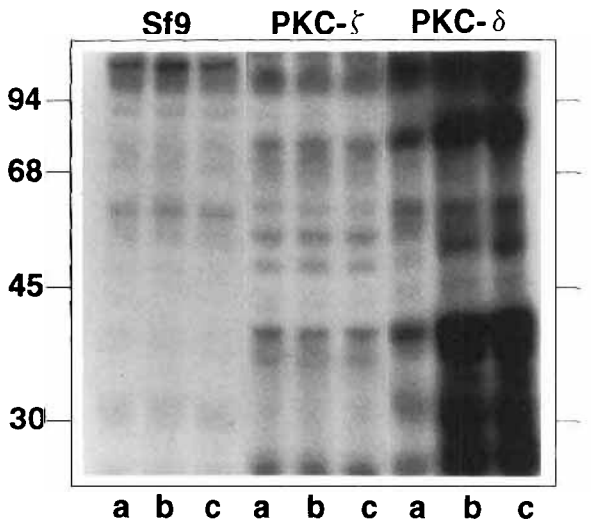

Fig. 6. Endogenous phosphorylation in cell extracts expressing PKC-δ and bvPKC-ζ. About 30 μg of cytosolic fractions of Sf9 cells infected with wild-type baculovirus (Sf9), bvPKC-δ (PKC-δ) or bvPKC-ζ (PKC-ζ) were incubated with 1 μCi γ[^{32}P]ATP in the absence (a) or presence of 20 μg PS and 2 μg DG (b) or 20 μg PS and 300 nM TPA (c) for 10 min at 37°C. The reactions were stopped with hot SDS, resolved on a 10% SDS-gels and autoradiographed as described in Methods.

tors. Lack of stimulation of PKC-ζ protein kinase activity by cofactors was also observed when the phosphoryation of cytosolic proteins of insect cells were analyzed in the absence of exogenous substrates (Fig. 6). In the absence of cofactors, the phosphoprotein pattern of cytosolic fractions of insect cells expressing the PKC-ζ was qualitatively and quantitatively different from the pattern obtained from control insect cells or cells expressing recombinant PKC-δ (Fig. 6, lane a). Furthermore, addition of PS/DG or PS/TPA did not enhance the phosphorylation of any of the cytosolic proteins from cells that had been infected with either wild-type or recombinant bvPKC-ζ (Fig. 6, lanes a–c). In contrast, addition of cofactors significantly stimulated the phosphorylation of a variety of cytosolic protein from insect cells infected with bvPKC-δ including a 78 kD protein (Fig. 6, lanes a–c). This protein was similar in size to the PKC-δ, suggesting that PKC-δ may undergo a cofactor-dependent autophosphorylation. It should be noted that no quantitative difference in the phosphoprotein pattern was obtained when the stimulations was carried out with either PS/DG or PS/TPA (Fig. 6, lanes b and c). Taken together these data tend to indicate that in contrast to PKC-δ, the PKC-ζ expressed from insect cells is unable to phosphorylate exogenous and endogenous protein substrates in a PS/TPA-dependent manner.

Effects of Staurosporine on PKC-α, PKC-δ, and PKC-ζ

We have tested the effects of the microbial alkaloid staurosporine, a potent but unselective inhibitor of protein kinases (Tamaoki et al., 1986; Tamaoki and Nakano, 1990; Meyer et al., 1988) on cytosolic fractions of cells expressing PKC-α, PKC-δ, or PKC-ζ. A concentration of about 5 nM of staurosporine was required to half-maximally inhibit the protein kinase activity of PKC-α when protamine sulfate was used

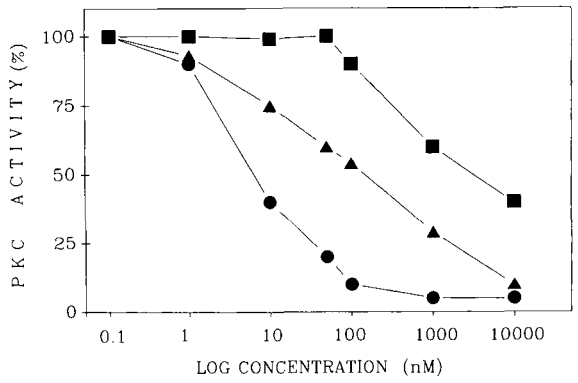

Fig. 7. Effects of staurosporine on PKC-α, PKC-δ, and PKC-ζ. Protein kinase activity of aliquots of cytosolic fractions (5 μg) obtained from cells expressing either PKC-α, PKC-δ, or PKC-ζ were analyzed in the absence of added cofactors using protaminesulfate as exogeneous substrate in the presence of increasing concentrations of staurosporine as described in Methods. Protein kinase activity is expressed as % of protein kinase activity in the absence of staurosporine. PKC-α (●), PKC-δ (▲), and PKC-ζ (■).

as exogenous substrate (Fig. 7). A similar IC_{50} for staurosporine was obtained when PKC-α was assayed in the presence of cofactors using either MBP or histone as exogenous substrates (data not shown). This concentration of staurosporine required for the half-maximal inhibition of the PKC-α protein kinase activity is comparable to previously published data (Tamaoki et al., 1986; Meyer et al., 1988) (Fig. 7). In contrast, the protein kinase activity of cytosolic fractions of cells expressing either PKC-δ or PKC-ζ were less effectively inhibited by staurosporine (Fig. 7). Concentrations of approximately 500 nM and 5,000 nM of staurosporine were required to half-maximally inhibit the protein kinase activity of PKC-δ and PKC-ζ, respectively, using protamine sulfate as exogenous substrate (Fig. 7). Similar results were obtained when PKC-δ and PKC-ζ where assayed using either histone or MBP in the presence of cofactors (data not shown). These data indicate that staurosporine selectively inhibits the protein kinase activity of PKC-α, PKC-δ, and PKC-ζ, the PKC-ζ being the *least* sensitive towards this potent protein kinase inhibitor.

DISCUSSION

The use of the baculovirus expression system has allowed the purification and biochemical characterization of all of the conventional calcium-dependent cPKC and of one the calcium-independent nPKC-ε (Schaap and Parker, 1990; Burns et al., 1991; Patel and Stabel, 1989; Burns and Bell, 1991; Fiebich et al., 1990). The biochemical properties of the other members of the calcium-independent nPKCs only been studied following expression of these subtypes in COS cells (Ono et al., 1988, 1989b; Bacher et al., 1991; Osada et al., 1990; Ohno et al., 1989). However, purification PKC subtypes obtained from COS cells for biochemical analysis is hampered by the fact that these cells contain significant levels of PKC-α as well as of other yet unidentified PKC activities (Ono et al., 1988, 1989b; Schaap et al., 1989). Since insect cells do not express detectable endogenous cofactor-dependent PKC activity, the baculovirus expression system appears to be the method of choice to express, purify, and characterize individual PKC subtypes. Therefore, the cDNAs of the rat PKC-δ and PKC-ζ isolated by PCR were used to construct recombinant baculovirus using the transfer vector pAC360. Although by this strategy 13 additional amino acids are added N-terminal to both nPKC subtypes high level expression (about 2–4% of the total insect cell protein) of functional nPKC-δ and nPKC-ζ subtypes were obtained.

The apparent molecular size of 78 kD for the PKC-δ protein produced from insect cells or in vitro is consistent with the molecular size of PKC-δ purified from porcine spleen published earlier (Leibersperger et al., 1990). However, the apparent molecular size of 76 kD of the PKC-ζ produced in vitro or insect cells is in contrast to the molecular size of 64 kD reported for the PKC-ζ partially purified from COS cells (Ono et al., 1989b). Since in this study, however, extracts of COS cells have not been analyzed for immunoreactive PKC-ζ protein before purification, proteolytic degradation of PKC-ζ cannot be ruled out (Ono et al., 1989b). For example, proteolytic degradation during partial purification of the nPKC-ε subtype expressed from COS cells has been reported (Schaap et al., 1988). Although on occasion small amounts of immunoreactive proteins of 60 and 50 kD were observed in insect cells expressing PKC-δ and PKC-ζ we have not been able to immunodetect a major 64 kD PKC-ζ. Furthermore in a variety of cells tested, only proteins with apparent molecular sizes of 72–76 kD immunoreacted specifically with our anti-PKC-ζ antisera (Crabos et al., 1991 and unpublished observations). Taken together these data suggest that the molecular size of 64 kD reported for PKC-ζ may be due to a

proteolytic fragmentation of the PKC-ζ (Ono et al., 1989b). Since the open reading frame of the PKC-ζ cDNA encodes for a protein with 592 amino acids with a calculated molecular weight of 68 kD, the apparent molecular size of 76 kD for the PKC-ζ produced in insect cells or in vitro can only be explained by an anomalous migration of this particular PKC subtype in SDS gels. According to our data the 13 additional amino acids at the N-terminus of either PKC-δ or PKC-ζ do not affect their mobilities in SDS gels.

Equal amounts of PKC-δ and PKC-ζ were found in the soluble and membrane fractions of insect cells. This subcellular distribution was confirmed by phorbol ester binding for PKC-δ and is in contrast to the subcellular distribution of the bovine PKC-α overexpressed in the LLC-PK1 or other cells (Huwiler et al., 1991; Kiley et al., 1990; Leibersperger et al., 1990; Wartmann et al., 1991). Whether the subcellular distribution of the PKC-δ and PKC-ζ in insect cells reflects an intrinsic property of these two nPKC subtypes and/or may be related to the fact that compared to the PKC-α these two nPKC subtypes lack the conserved C2 region remains to be studied (Nishizuka, 1988; Bell and Burns, 1990; Huang, 1989; Jaken, 1990). Recently, the subcellular distribution of the various PKC isoforms have been analyzed by immunoblotting in a variety of cell types (Huwiler et al., 1991; Kiley et al., 1990, 1991; Strulovici et al., 1991; Heidenreich et al., 1990; Crabos et al., 1991; Leibersperger et al., 1990). Although these results are far from being conclusive, they indicate that differences in the subcellular localization exist between the members of calcium-independent nPKCs and calcium-dependent cPKCs with respect to membrane association induced by calcium, TPA, or hormones.

In contrast to PKC-δ, PKC-ζ was unable to bind to and to be activated by phorbol esters, confirming the results obtained previously by Ono et al. (1989b). However, we have been unable to confirm the observation that histone phosphorylation by PKC-ζ is dependent on PS but independent of calcium and DG or TPA (Ono et al., 1989b). Neither PS nor PS/TPA had any effect on the enzyme activity of PKC-ζ irrespective of the substrates used. The reasons for this divergent results are not clear but may be due to the finding that the PKC-ζ analyzed by Ono et al. (1989b) underwent proteolysis during purification. Except for the PKC-ζ, all members of the PKC family contain two zinc finger structures and are capable to bind to and to be activated by phorbol ester in a phospholipid-dependent manner (Ono et al., 1988; Nishizuka, 1988; Bell and Burns, 1990; Osada et al., 1990; Bacher et al., 1991, our results). At present, there is no evidence that the zinc fingers in the PKC molecule serve functions other than to bind phorbol esters in phospholipid-dependent manner (Burns and Bell, 1991; Cazaubon et al., 1990; Kaibuchi et al., 1989; Ono et al., 1989a). The phospholipid-dependent phorbol ester binding sites have not yet been precisely identified in the PKC molecule and it is unclear whether one or both of the zinc fingers are required for high affinity phorbol ester binding and phorbol ester- or DG-dependent protein kinase activity (Kaibuchi et al., 1989; Cazaubon et al., 1989; Cazaubon et al., 1990; Burns and Bell, 1991; Ono et al., 1989a,b). Recent data, however, support the idea that each of the two zinc finger structures of the PKC-γ is sufficient to bind phorbol esters, albeit with a lower affinity as in the holoenzyme (Burns and Bell, 1991). It is, intriguing to note that the PKC-ζ, which contain only one, albeit well conserved "PKC-like zinc finger," is unable to bind to and to be activated by phorbol esters. These data may suggest that this subtype has a lipid requirement different from the other PKC subtypes. In this respect, the mode of activation of PKC-ζ might resemble that of the raf protein kinase whose activation has also been proposed to occur by a yet unidentified lipid mediator (Li et al., 1991). Alternatively, not all of the proteins containing only one zinc finger structure like the PKC-ζ, such as the diacylglycerol kinase (Sakane et al., 1990; Schaap et al., 1990b), the raf family of protein kinases (Li et al., 1991), or the n-chimaerin (Ahmed et al., 1990), are able to bind phorbol esters in a phospholipid-dependent manner. Among these proteins only the n-chimaerin has been reported to be capable of binding phorbol esters (Ahmed et al., 1990).

Nevertheless, the PKC-ζ expressed from insect cells was able to phosphorylate protamine sulfate to similar extents as PKC-δ. Presumably due to negatively charged sulfate groups, this substrate is efficiently used by all members of PKC family independent of the addition of cofactors (Bazzi and Nelsestuen, 1987; Souvignet and Chambaz, 1990). However, compared to PKC-α, the standard protein substrates histone and MBP were poor substrates for the enzyme activity of PKC-δ and PKC-ζ. Interestingly, purified PKC-ε expressed from insect cells or PKC-δ

isolated from porcine spleen also poorly utilizes histone and MBP as substrates compared to pseudosubstrate-based peptides or protamine sulfate (Schaap and Parker, 1990; Leibersperger et al., 1990). Although the PKC-δ expressed in insect cells was able to phosphorylate MBP or histone in a PS/TPA-dependent manner, stimulation by cofactors was modest when compared to the effector-dependent stimulation of the PKC-α. In contrast, the cofactor-dependent phosphorylation of endogenous substrate in insect cells by PKC-δ was more pronounced than the PS/TPA-dependent phosphorylation of MBP and histone. Preliminary data suggest that the 78 kD protein band phosphorylated in a PS/TPA-dependent manner in the insect cell free system was identified by immunoprecipitation as the autophosphorylated form of PKC-δ. With the exception of PKC-ζ, cofactor-dependent autophosphorylation of PKC is a characteristic feature for all members of the PKC family (Bacher et al., 1991; Osada et al., 1990; Ohno et al., 1988; Borner et al., 1989; Huang et al., 1986; Mochly-Rosen and Koshland, 1987). The finding that the addition of calcium to PS/DG did neither enhance exogenous nor the endogenous substrate phosphorylation is consistent with reports showing that the enzyme activity of the members of the nPKC group is independent of calcium (Schaap et al., 1990; Bacher et al., 1991; Leibersperger et al., 1990).

Compared to PKC-α, the kinase activity of PKC-δ and PKC-ζ were less effectively inhibited by staurosporine. Staurosporine is a potent, but unselective protein kinase inhibitor which presumably competes for ATP at the catalytic site of PKC without affecting its phorbol ester binding (Tamaoki et al., 1986; Meyer et al., 1988; Tamaoki and Nakano, 1990). The potency of staurosporine to inhibit the enzyme activity of PKC-α (IC_{50}: 5 nM) is consistent with the results reported previously (Tamaoki et al., 1986; Meyer et al., 1988; Tamaoki and Nakano, 1990). In contrast the IC_{50} values for PKC-δ and PKC-ζ were about 2 to 3 orders of magnitude higher than for PKC-α. Similarly, the inhibitory potency of the staurosporine-like, K252a, has also been reported to differ by two order of magnitude between calcium-dependent and calcium-independent PKC activities (Gschwendt et al., 1989). In another study, however, no difference in the IC_{50} values for staurosporine between the calcium-dependent cPKC subtypes and the calcium-independent nPKC-ε were detected, although the potency of staurosporine was about 50-fold lower than previously reported (Schaap and Parker, 1990). Taken together, these data suggest that the calcium-independent nPKCs may be less effectively inhibited by staurosporine than the calcium-dependent cPKCs. Whether the differential sensitivity of the various PKC subtypes may be related to differences in the primary structure of their respective catalytic domains remains to be established. It is noteworthy, that PKC-ζ, which has the least conserved catalytic domain of all the PKC subtypes (Bell and Burns, 1990; Ono et al., 1988; Nishizuka et al., 1988) is only inhibited at submicromolar concentrations of staurosporine.

Using the baculovirus expression system we have provided evidence for the effector dependence, substrate preference, and sensitivities to staurosporine of PKC-δ and PKC-ζ. The results presented indicate that the 76 kD PKC-ζ expressed from insect cells is unable to bind to and to be activated by phorbol esters and is almost insensistive to the potent protein kinase inhibitor staurosporine.

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