Biochemical Pharmacology, Vol. 53, pp. 865–875, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00885-4

Activation of Protein Kinase C Subtypes α, γ, δ, ε, ζ, and η byTumor-Promoting and Nontumor-Promoting Agents

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ABSTRACT. Protein kinase C (PKC) subtypes α, γ, δ, ε, ζ, and η have been expressed using the baculovirus expression system. The partially purified PKC subtypes have been studied for their substrate specificities and phospholipid-independent activation by various chemically different nontumor- and tumor-promoting agents, as well as their inhibition of kinase activity by staurosporine and two related compounds. An endogenous PKC-like kinase activity of Sf9 cells was detected and analyzed for cofactor requirements and inhibition. Protamine sulfate was most efficiently phosphorylated by all of the PKC subtypes tested, although this phosphorylation was independent of phosphatidylserine (PS) and diacylglycerol (DAG) or 12-O-tetradecanoylphorbol 13-acetate (TPA). Except for PKC-ζ, all subtypes tested phosphorylated myelin basic protein (MBP), histone, or a peptide derived from the pseudosubstrate region of PKC-α in a PS/DAG-dependent manner but to varying extents. Among the various agents tested, TPA most efficiently stimulated the kinase activities of the PKC subtypes in a phospholipid-dependent manner. Phorbol 12,13-dibutyrate (PDBu) was less effective than TPA but displayed no major difference among the subtypes. Activation of PKC-α by bryostatin-1 reached only half of the TPA response whereas the other subtypes were activated more effectively. The weak tumor promoter resiniferonol 9,13,14-orthophenyl acetate (ROPA) mainly stimulated PKC-α and PKC-γ at 1 μM concentration, whereas PKC-ε and PKC-η were much less activated. Sapintoxin D, mezerein, indolactam V, and resiniferatoxin at concentrations of 1–100 nM preferentially activated PKC-α in a DAG-like manner, whereas at 1 μM other subtypes were activated as well. Preferential activation of PKC-α was also noted for tinyatoxin and thapsigargin, but their mode of activation is unclear because these two compounds did not compete for the phorbol ester binding of the PKC subtypes as the other agents did. Of the three PKC inhibitors tested, staurosporine most efficiently inhibited kinase activity of the PKC subtypes, whereas K252a and CGP 41251 were at least 10 times less effective. However, K252a showed certain specificity for inhibition of PKC-α, and CGP 41251 failed to inhibit PKC-ε and PKC-ζ. Given the different substrate specificities and modes of activation by various tumor-promoting and nontumor-promoting agents, as well as the different sensitivities towards different inhibitors, our results indicate a divergence of individual PKC subtypes in signal transduction. BIOCHEM PHARMACOL 53;6:865–875, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. protein kinase C; tumor promoters; bryostatin 1; sapintoxin D; mezerein; indolactam V

The serine/threonine-specific protein kinase C (PKC),[¶] the major receptor for a number of tumor-promoting agents, utilizes 1,2-diacylglycerol (DAG) and/or other lipids to modulate various cellular functions. Recent molecular cloning revealed that PKC consists of a family of related but distinct enzymes (for review, see [1, 2]). Today, the various PKC subtypes can be classified into three groups comprising the conventional calcium-dependent subtypes (cPKCs: α-, βI-, βII-, and γ-PKC), the nonconventional calcium-independent isoforms (nPKCs: δ-, ε-, η-, θ- and μ-PKC), and the atypical calcium-independent and DAG or phorbol ester-unresponsive subtypes (aPKCs: ζ- and ι- (λ) PKC) [1–5]. The finding that tumor-promoting phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA) are able to replace the endogenous activator DAG in the stimulation of PKC has provided insight into the role of this enzyme in the regulation of a variety of cellular processes such as exocytosis, gene expression, proliferation, differentiation, and tumor promotion [2, 6]. The various PKC

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[¶] *Abbreviations:* PKC, protein kinase C; PS, phosphatidylserine; DAG, 1,2-diacylglycerol; MBP, myelin basic protein; α-pep, α-pseudosubstrate peptide; TPA, 12-O-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; STX, sapintoxin D; RTX, resiniferatoxin; TTX, tinyatoxin; ROPA, resiniferonol 9,13,14-orthophenyl acetate; PCR, polymerase chain reaction.

Received 16 July 1996; accepted 25 October 1996.

subtypes show distinct enzymological properties, differential tissue expression with specific subcellular localization, and different modes of cellular regulation [7–9]. These features, combined with the finding that more than one subtype is usually expressed in a single cell type, have led to the notion that each member of the PKC family plays discrete roles in the processing of various physiological and pathological responses to extracellular stimuli.

The involvement of PKC in the multistage process of tumor formation and progression has emerged due to the fact that this enzyme family represents the major target for tumor-promoting phorbol esters and chemically unrelated tumor promoters [10, 11]. Although phorbol esters bind to and activate PKC in a stereoselective manner, they display a different spectrum of biological activity. For example, mezerein, a daphnane diterpene with unsaturated side chains, and sapintoxin D belong to the group of second stage tumor promoters [12]. Other daphnane analogs such as the resiniferonol esters tinyatoxin or resiniferatoxin, whose skin irritant properties are among the most potent known to humans, are not tumor promoters, although both are derived from the 2-O-deacylated parent structure of resiniferonol 9,13,14-orthophenyl acetate (ROPA), a weak tumor promoter [13, 14]. In addition, a number of structurally unrelated natural products have been found to bind to and efficiently activate PKC independent of their tumor-promoting properties. These compounds include the teleocidins and the bryostatins. (−)-Indolactam V, a semisynthetic derivative of the alkaloid-type of PKC activator with a teleocidin B-type structure, is highly inflammatory and a potent tumor promoter, whereas the macrocyclic lactone bryostatin-1, although a potent activator of PKC, is non-tumor promoting [15, 16]. Surprisingly, the latter agent inhibits some of the TPA-mediated responses in certain cell types, although it binds to the regulatory site of PKC and activates PKC activity [17]. Moreover, not all of the tumor-promoting agents act through activation of PKC, with thapsigargin being a prototype for this class of drugs [18].

The finding that cellular responses to various PKC activators are divergent in different cell types may imply that these activators bind to and selectively activate certain classes or individual members of PKC. Previous data have suggested such a selective activation of PKC subtypes [19–21] which, however, seems to be cell type specific [21].

In order to further analyze activation characteristics of individual PKC subtypes by the above-mentioned tumor-promoting and nontumor-promoting agents, we made use of the baculovirus expression system for high level expression of functional PKC subtypes [22, 23]. Partially purified PKC subtypes PKC-α, -γ, -δ, -ε, -ζ, and -η expressed in insect cells were analyzed for their activation, inhibition, and substrate specificities.

MATERIALS AND METHODS

Materials

Protamine sulfate, histone III-S and myelin basic protein were from Sigma Chemical Co. (St. Louis, MO, USA). [γ-^{32}P] ATP (1000 Ci/mmol), ^{35}S-methionine (>1000 Ci/mmol), and ^{125}I-labeled antirabbit IgG were obtained from Amersham (Arlington Heights, IL, USA). Taq polymerase and MLV-reverse transcriptase were from Pharmacia (Piscataway, NJ, USA). The baculovirus vector pAc360 and Sf9 cells were obtained from M. D. Summers (Texas A&M University, Dallas, TX, USA). The pGEM-1 was obtained from Promega. [^{3}H]Phorbol-12,13-dibutyrate ([^{3}H]PDBu) (10.2 Ci/mmol) was from NEN, Regensdorf, Switzerland. Restriction enzymes were from New England Biolabs (Boston, MA, USA) or Boehringer (Mannheim, Germany). *In vitro* transcription systems with the T7 and Sp6 polymerases were from Boehringer and the rabbit reticulocyte lysate was from Promega (Basel, Switzerland). Staurosporine, CGP 41251, and K252a were produced by Ciba-Geigy Ltd., Basel, Switzerland. The Ex-Cell 400 medium was from JR Scientific (Woodland, CA, USA). Bryostatin-1 was generously provided by Aston Molecules LTD (Birmingham, UK). All other compounds tested were obtained from LC Services (Anawa, Basel, Switzerland).

Methods

CONSTRUCTION AND EXPRESSION OF RECOMBINANT BACULOVIRUS CARRYING PKC-α, -γ, -δ, -ε, -ζ, AND -η. The construction of recombinant baculovirus for PKC-α, -γ, -δ, -ε, and -ζ is described elsewhere [22–24]. The PKC-η cDNA was obtained from mouse lung. Briefly, mouse lung polyA+ mRNA was enriched by oligo-dT cellulose (Pharmacia, Uppsala, Sweden) and used to synthesize complementary strand cDNA using MLV-reverse transcriptase in the presence of oligo-(dT) primer (Boehringer, Mannheim, Germany). Two polymerase chain reaction (PCR) fragments encompassing the entire coding region of the PKC-η were generated using the primers (5'GAG AAT TCA GAT CTC ATG TCG TCC GGC ACG ATG AAG 3') containing the start codon of PKC-η and the unique cloning sites for BglII and EcoRI as well as (5'GTC TTA GTG TCG ACC TGG AAA TG 3') carrying the Sal I site in the PKC-η coding region. These primers were used to generate the 5' half (0.97 kb) of the PKC-η coding region. The 3' half of the PKC-η coding region (1.047 kb) was PCRed using the primers (5'AGG CAC TCG AGA GAT CTG GCT ACA GTT GCA ATT CC 3') containing the stop codon of PKC-η, the XhOI, and Bgl II sites as well as the primer D 4 (5'ATT TCC AGG TCG ACA CTA AGAC 3') carrying the Sal I site in the PKC-η coding region. The 5'-half of the PKC-η coding region was digested with EcoRI and Sal I whereas the 3'-half of the PKC-η was digested by Bgl II and Sal I and inserted into the Bgl II and EcoRI cut pSP 73 vector. Restriction and sequence analysis confirmed identity to the PKC-η sequence previously published [25]. The coding region of PKC-η was then cut with Bgl II from the pSP73 and cloned in frame into the unique Bam HI site of the pAc360 shuttle vector. The recombinant transfer plasmid containing the PKC-η was cotransfected with AcNPV DNA into Sf9 insect cells using the

baculo gold system as described [26]. Standard methods of gene expression using the baculovirus vector systems were used as described elsewhere [27]. The cell line *Spodoptera frugiperda* (Sf9) was propagated as a monolayer or in suspension culture in medium containing 10% fetal calf serum.

PARTIAL PURIFICATION OF PKC SUBTYPES. Sf9 cells were infected with recombinant baculovirus at a multiplicity of infection of 10 and harvested 48 hr postinfection. Cytosolic fractions were prepared from 500 mL spinner cultures (5×10^8 cells) by disrupting infected Sf9 cells by sonication and centrifugation at 100,000 × *g* for 1 hr at 4°C exactly as described [28]. The PKC subtypes were partially purified using DEAE Fast Flow Sepharose column chromatography connected to an FPLC system (Pharmacia, Uppsala, Sweden) using buffer and elution procedure exactly as described [28]. Column fractions were analyzed for ^{3}H-PDBu binding [24] and PKC activity by measuring the incorporation of ^{32}P from γ-[^{32}P]ATP into histone III-S or myelin basic protein in the presence or absence of phosphatidylserine (PS; 0.1 mg/mL) and DAG or TPA as described [29]. The PS/TPA-dependent protein kinase activity eluting from the column as single peaks was collected, pooled, dialyzed, and stored frozen at −70°C until use. aPKC-ζ elution was monitored using the anti-PKC-ζ antiserum in conjunction with determination of activity using protamine sulfate as exogenous substrate. One unit of PKC activity is defined as 1 nmol of ^{32}P transferred from γ-[^{32}P]ATP into the respective substrate per minute at 37°C.

RESULTS

Expression of PKC Subtypes

Analysis of the phorbol ester binding of cytosolic extracts from Sf9 cells infected with the recombinant baculovirus showed different levels of phorbol ester binding (Table 1). Neither Sf9 nor Sf9 cells infected with the wild-type baculovirus (AcNPV) showed detectable levels of phorbol ester binding under our assay conditions.* Although aPKC-ζ was expressed at high levels, as we showed previously [30], it did not bind phorbol esters [31]. This is obviously related to the fact that aPKC-ζ contains only one zinc finger with divergence in sequence requirements for phorbol ester binding in contrast to the other members of the PKC family tested, which contain two zinc finger structures in their N-terminal regulatory domain [32]. These zinc-binding fingers have been reported to bind phorbol esters in a phospholipid-dependent manner [33, 34]. The expression of PKC subtypes was monitored using subtype-specific antibodies that proved to be highly specific for their respective antigens with no crossreactivities against other PKC subtypes or Sf9 proteins, as previously demonstrated [30].

TABLE 1. Phorbol ester binding capacity of PKC subtypes

PKC subtype	[^{3}H]PDBu binding activity (pmol of bound PDBu per mL of protein)
Control (Sf9-wild type)	0.8 ± 0.5
PKC-α	85.7 ± 4.9
PKC-γ	54.8 ± 6.8
PKC-δ	181.0 ± 13.9
PKC-ε	196.4 ± 8.7
PKC-ζ	2.4 ± 0.2
PKC-η	66.7 ± 6.1

PKC subtypes were expressed using the baculovirus expression system. Cytosolic extracts (10 μg) from Sf9 cells infected with wild-type or recombinant baculovirus were analyzed for their [^{3}H]PDBu binding activities as described in the Materials and Methods section. Data are expressed as pmol of bound PDBu per mg of protein and are the means ± SD (n = 3).

Substrate Specificities of Partially Purified PKC Subtypes

Partially purified extracts of insect cells expressing the various PKC subtypes were assayed for protein kinase activity under a variety of conditions. Since protamine sulfate was most efficiently phosphorylated by all PKC subtypes in a PS/DAG-independent manner, kinase activities of the subtypes towards the substrates tested were referred to their respective protamine sulfate kinase activities. Equal units of protamine sulfate kinase activities were tested. Thus, Fig. 1 allows the comparison not only of different PKC subtype activities towards a given substrate (Histone IIIS (Fig. 1A), MBP (Fig. 1B), and PKC-α-pseudosubstrate peptide (α-PEP; Fig. 1C)), but also of differential substrate phosphorylations by one PKC subtype.

Except for PKC-ζ, kinase activities of the other PKC subtypes could be highly stimulated by the addition of activators such as PS/DAG or PS/TPA. The cPKCs PKC-α and PKC-γ displayed Ca^{2+} dependence in their activation of substrate phosphorylation in the presence of PS/DAG or PS/TPA, whereas the nPKCs PKC-δ, PCK-ε, and PKC-η did not (data not shown). Substrate phosphorylations by the various PKC subtypes in the absence of activators were relatively low, especially in the case of histone phosphorylation. Thus, a comparative analysis of substrate phosphorylations by the different PKC subtypes is only possible in their activated state. Interestingly, PKC-ζ was an exception in phosphorylating the α-pseudosubstrate peptide in the absence of activators to a greater extent than did the other subtypes. This high level of α-peptide phosphorylation was reached by PKC-α, -γ, and -δ only in the presence of activators.

As published previously, histone is a poor substrate for PKC-ε [35]. In contrast to the other PKC subtypes, histone phosphorylation by PKC-ε could not be significantly further activated by the addition of PS/TPA. Phosphorylation of myelin basic protein (MBP) and histone by PKC-δ was most efficiently stimulated in the presence of activators as compared to the other subtypes tested.

* Although we detected no endogenous phorbol ester binding activity in Sf9 cells, partial purification of extracts from uninfected cells yielded a peak of low kinase activity that could be stimulated by PS/TPA. Such an endogenous PKC activity in Sf9 cells has been reported by Kazanietz et al. [20]. Because this activity eluted at lower salt concentrations than those of the transfected insect cell extracts, we could separate this endogenous activity from the recombinant PKC activity by DEAE chromatography.

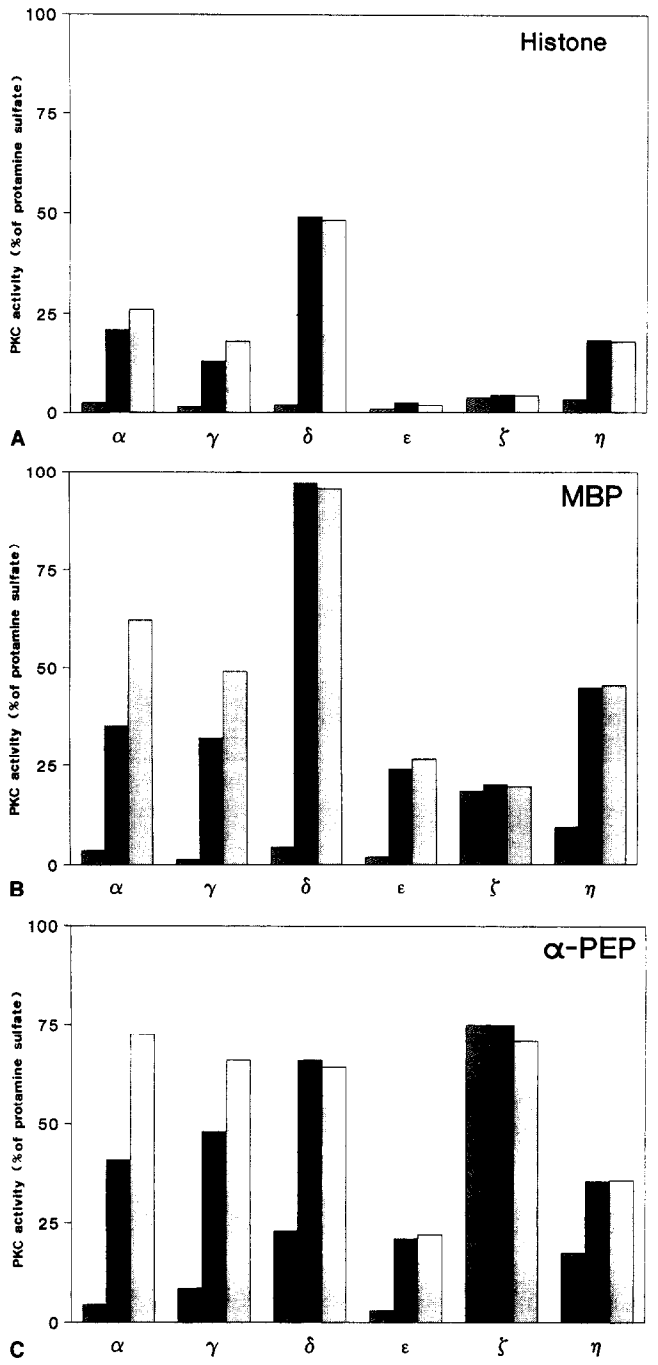

FIG. 1. Relative kinase activities of PKC-α, -γ, -δ, -ε, ζ, and -η. Protein kinase activity of partially purified cytosolic fractions (3 μg) were assayed in either the absence of cofactors (hatched bars), the presence of 100 μg/mL PS and 10 μg/mL DAG (closed bars), or in the presence of 100 μg/mL PS, 10 μg/mL DAG, 0.25 mM calcium chloride (open bars), using 0.5 mg/mL of either protamine sulfate, histone III S (A), myelin basic protein (MBP) (B), and 400 μM PKC-α pseudosubstrate peptide (α-PEP) (C) as exogenous substrates. Results were normalized to the activity with protamine sulfate as 100% (PKC-α, 41; PKC-γ, 38; PKC-δ, 15; PKC-ε, 21; PKC-ζ, 13; PKC-η, 16; values represent units of kinase activity per mg protein). Values are means of triplicates ± SD from two independent experiments.

Although being stimulated by the addition of a combination of PS/TPA, PKC-η displayed an already high level of kinase activity in the presence of phospholipid only, whereas the other PKC subtypes were significantly stimulated only in the presence of phospholipid plus DAG or TPA (data not shown). We also tested the partially purified endogenous PKC activity of Sf9 cells for phosphorylation of histone and MBP. MBP was much better phosphorylated than histone. In the presence of PS/TPA, a 20-fold stimulation of kinase activity was achieved (Fig. 2). Calcium had no effect on substrate phosphorylation. Thus, the endogenous PKC of Sf9 cells closely resembles the group of calcium-independent, PS/TPA-activated nPCKs.

In general, histone was much less phosphorylated by the PKC subtypes tested than were MBP and the α-peptide, the latter displaying the highest activator-dependent phosphorylation by PKC-α and PKC-γ, and a significant activator-independent phosphorylation by PKC-ζ.

Effects of Various Tumor-Promoting and Nontumor-Promoting Agents on PKC Activities

We tested the PKC subtypes PKC-α, -γ, -δ, -ε, and -η for their activation by various tumor-promoting and nontumor-promoting agents. PKC-ζ was omitted from this test because this subtype is unresponsive to phorbol ester activation [31, 36]. Partially purified cytosolic extracts of baculovirus-infected insect cells were assayed for MBP kinase activity after stimulation by the potential activators (Fig. 3A). Of the different compounds tested, the PDBu activation profile most closely resembled that of TPA. The macrocyclic lactone bryostatin-1 activated the PKC subtypes over a wide range of concentrations, with PKC-α being less affected. The diterpene ROPA significantly stimulated kinase activity of the cPKC subtypes PKC-α and PKC-γ at 1

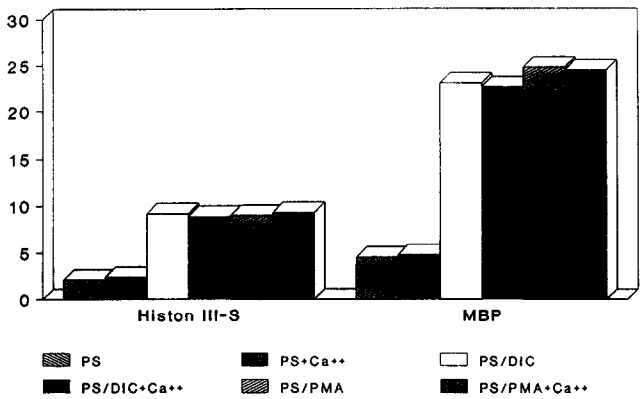

FIG. 2. Kinase activity of partially purified endogenous Sf9 PKC-like kinase. Partially purified cytosolic extract (3 μg) of uninfected Sf9 cells was assayed for kinase activity in either the absence (control) or presence of 100 μg/mL PS, 250 μM calcium chloride (Ca^{++}), 10 μg/mL DAG (DIC), 1 μM TPA (PMA), or a combination of cofactors using either 0.5 mg/mL histone III S or MBP as exogenous substrates. Values are expressed as multiplicity of activation compared to the control. A second experiment gave similar results.

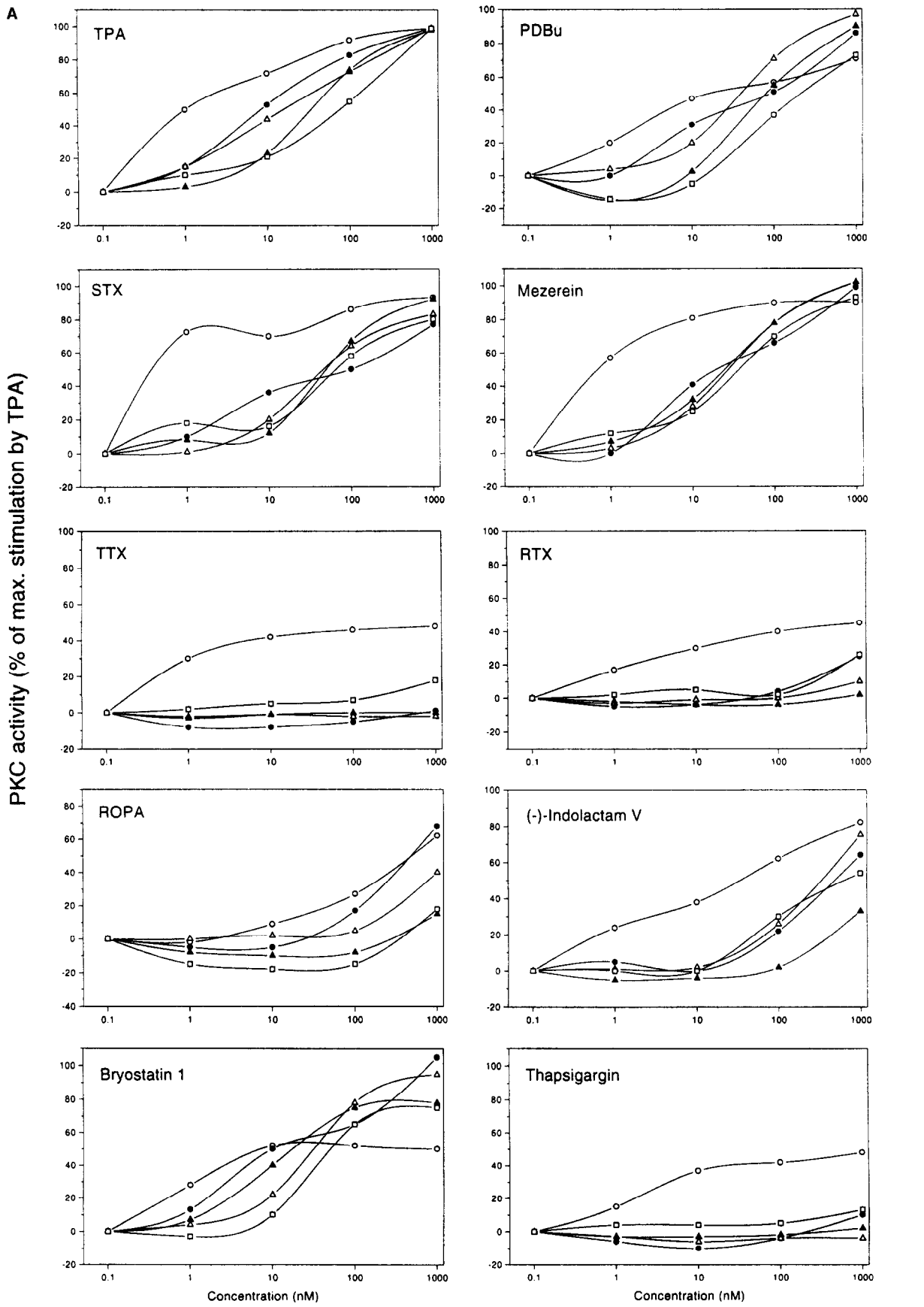
A
TPA
PDBu
STX
Mezerein
TTX
RTX
ROPA
(-)-Indolactam V
Bryostatin 1
Thapsigargin
PKC activity (% of max. stimulation by TPA)
Concentration (nM)
PKCα
PKCγ
PKCδ
PKCε
PKCη

FIG. 3

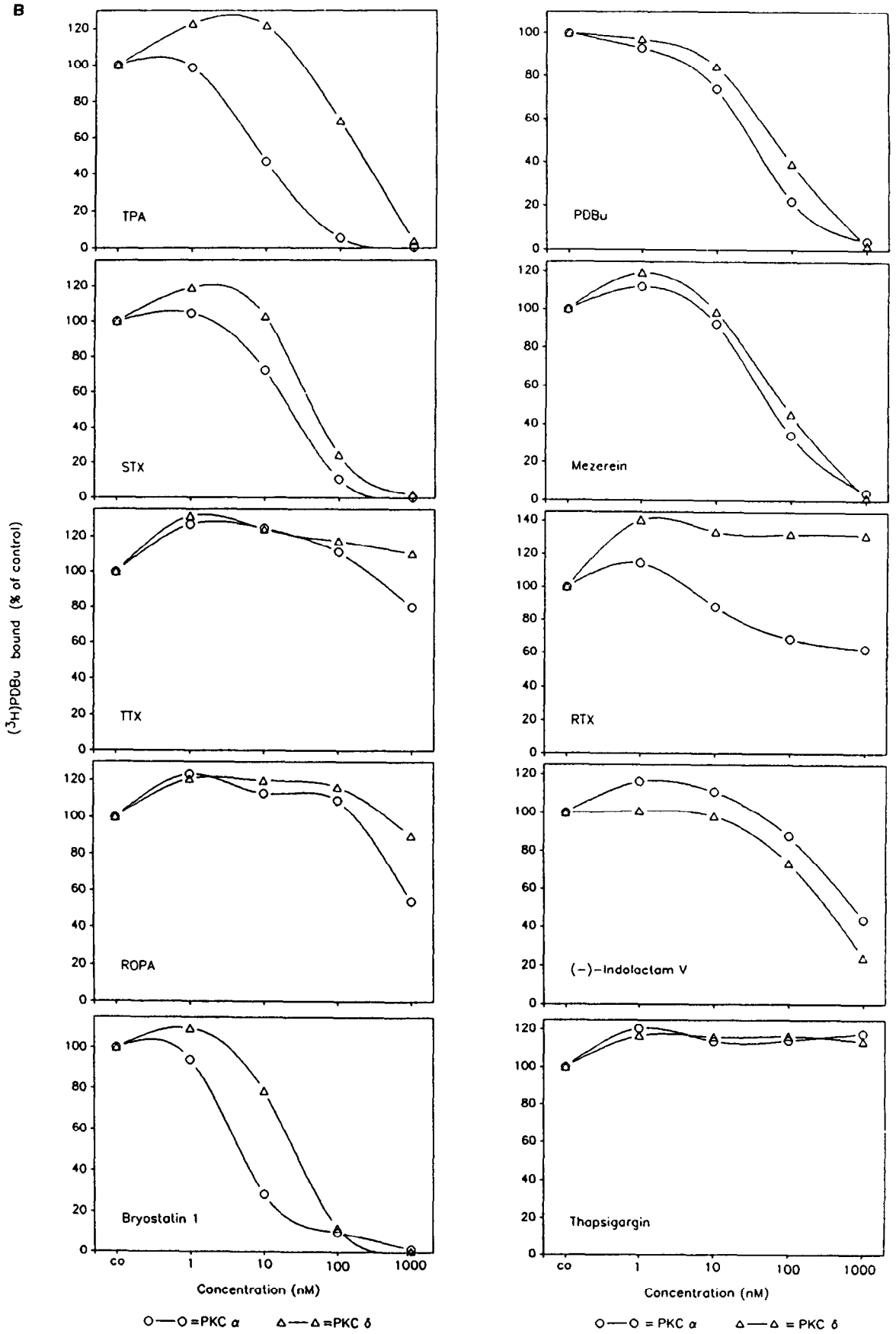

FIG. 3. Continued.

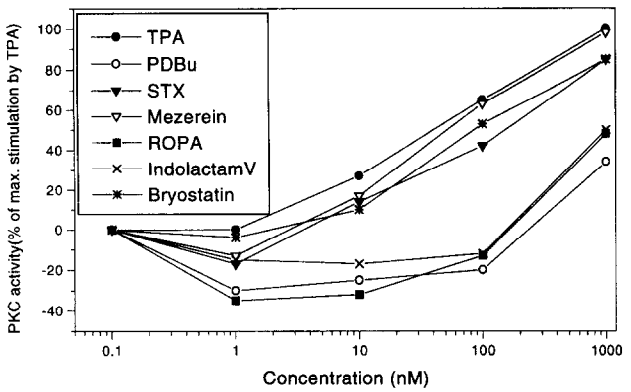

FIG. 4. Activation of endogenous Sf9 PKC-like kinase by various tumor-promoting and nontumor-promoting agents. Kinase activities were assayed as outlined in Materials and Methods using MBP as substrate. Activities are expressed as percentage of maximal activation by TPA vs. concentration of compound (nM).

μM, whereas PKC-ε and PKC-η were only marginally affected at this concentration. Interestingly, preferential activation of PKC-α could be observed for the other compounds tested. In the case of sapintoxin D (STX), mezerein, resiniferatoxin (RTX), and indolactam V, this preferential activation of PKC-α was observed at concentrations from 1–100 nM, whereas at 1 μM, all or at least some of the other subtypes were activated as well. In addition, tinyatoxin (TTX) and thapsigargin exclusively activated PKC-α over the whole range of concentrations tested. The activator profiles for the purified endogenous PKC activity of Sf9 cells is shown in Fig. 4. TPA, STX, mezerein, and bryostatin 1 are potent activators of endogenous PKC activity, whereas PDBu, ROPA and indolactam were only weakly active (Fig. 4) and TTX, RTX, and thapsigargin displayed only a marginal activity (data not shown).

To analyze if the activation by the various compounds occurred through specific binding at the phorbol ester binding site of PKC, we measured the ability of these agents to displace radioactive bound PDBu in a phorbol ester binding assay for PKC-α and PKC-δ (Fig. 3B). Except for tinyatoxin and thapsigargin, the compounds tested were able to bind to PKC at the phorbol ester binding site and displace PDBu. The higher and preferential activation of PKC-α by ROPA and RTX, respectively, could be correlated with the ability of these agents to displace more PDBu from the phorbol ester binding site of PKC-α than from PKC-δ. On the other hand, we observed no specific binding of STX, mezerein, or indolactam V to PKC-α as compared to PKC-δ, although these compounds preferentially stimulated kinase activity of PKC-α. Although tinyatoxin and thapsigargin selectively activated PKC-α as well, these two compounds were only slightly able or even unable to compete for the phorbol ester binding of PKC-α and PKC-δ. Thus, their mode of action remains unclear.

Inhibition of the PKC Subtypes by Staurosporine and Two Related Compounds

We have tested the inhibitory effect of staurosporine [37], CGP 41251 [38], and K252a [39] on kinase activities of partially purified PKC subtypes. PKC activities were assayed in the presence of the inhibitors using protamine sulfate as substrate. Similar data were obtained when inhibition of kinase activities was measured in the presence of PS/TPA using MBP as exogenous substrate (data not shown). The microbial alkaloid staurosporine, a potent but unselective inhibitor of protein kinases, most efficiently inhibited PKC activities (Fig. 5). Half-maximal inhibition of protein kinase activities of PKC-α, -γ, -δ, -ε, and -η were obtained at concentrations ranging from 2 nM for PKC-α, 4 nM for PKC-η, 5 nM for PKC-γ, 20 nM for PKC-δ to approximately 70 nM for PKC-ε. More than 1 μM of staurosporine was required to half maximally inhibit kinase activity of PKC-ζ. The natural staurosporine analog K252a is another nonselective protein kinase inhibitor [39]. Its potency to inhibit PKC subtypes is at least 10 times weaker as compared to staurosporine. However, it shows a certain specificity for inhibition of PKC-α (IC_{50} 40 nM vs. IC_{50} > 400 nM for the other PKC subtypes). CGP 41251, a chemically modified staurosporine derivative [38], is somewhat less potent than the parent compound but markedly more selective for inhibition of PKC activity. Although CGP 41251 was able to inhibit PKC-α and -γ with high potency (IC_{50} at 30 nM and 21 nM for PKC-α and -γ, respectively), it was less effective for PKC-δ (IC_{50} at 265 nM) and completely failed to inhibit PKC-ε and PKC-ζ at concentrations up to 100 μM.

Interestingly, all three inhibitors tested inhibited the partially purified endogenous PKC activity of Sf9 cells very efficiently with a potency resembling the values for PKC-α (IC_{50}: 3 nM, 17 nM, and 28 nM for staurosporine, K252a, and CGP 41251, respectively). In general, kinase activities of the cPKCs PKC-α and PKC-γ were most efficiently inhibited by all three inhibitors tested, whereas the Ca^{2+}-independent isoforms PKC-δ, PKC-ε, and PKC-ζ were much less or even not affected.

FIG. 3. Activation of PKC-α, -γ, -δ, -ε, and -η and phorbol ester binding activity of PKC-α and -δ by the various tumor-promoting and nontumor-promoting agents. Kinase activities and phorbol ester binding were assayed as outlined in Materials and Methods. (A) MBP kinase activities are expressed as percentage of maximal activation by TPA for each individual PKC subtype vs. concentration of compound (nM). (B) Phorbol ester binding activities of PKC-α and -δ. Ten micrograms of enzyme preparations were tested. Inhibition of [^{3}H]PDBu binding by increasing concentrations of the various compounds is expressed as percentage of the control.

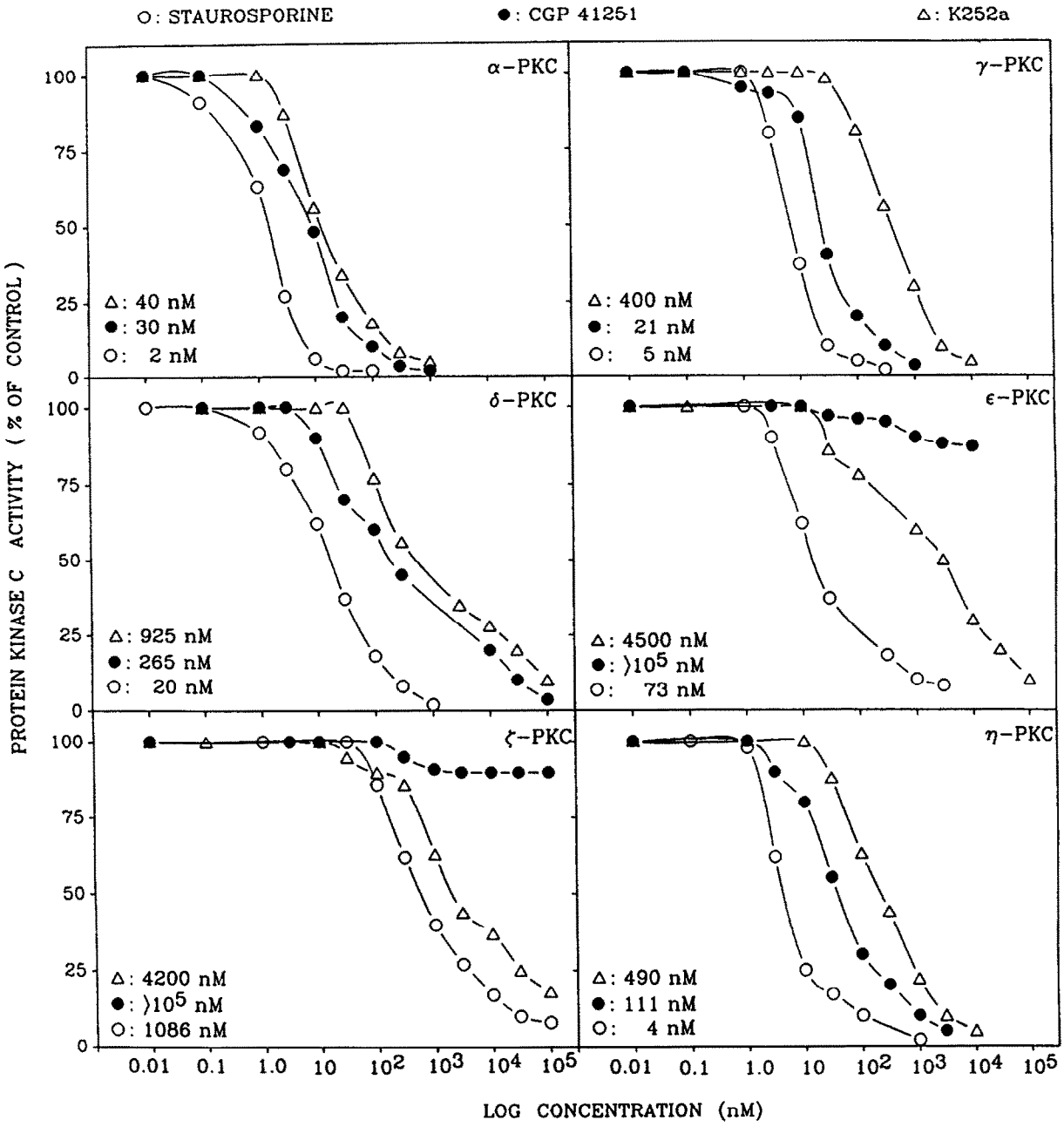

FIG. 5. Inhibition of PKC-α, -γ, -δ, -ε, -ζ, and -η by staurosporine, CGP 41251, and K 252a. Inhibition of protamine sulfate kinase activity of the various PKC subtypes is expressed as percentage of control vs. inhibitor concentration. The respective IC_{50} values are indicated. Data are means of two or three independent experiments.

DISCUSSION

The use of the baculovirus expression system is well established today for high level expression and detailed biochemical characterization of individual PKC isotypes [20, 22–24, 35, 40]. In contrast to previous assumptions, we detected and partially characterized an endogenous PKC activity in Sf9 cells, which is probably related to the nPKCs. The Sf9 PKC did not disturb our comparative analysis because we could separate this activity from that of the transfected PKC subtypes by a simple ion exchange chromatography step.

The proposed divergence of PKC subtypes in signal transduction was first monitored by comparing substrate phosphorylation. Each PKC subtype phosphorylated protamin sulfate most efficiently and independently of exogenous activators. Presumably due to negatively charged sulfate groups, this substrate interacts unspecifically with the catalytic site of PKC and is thus phosphorylated by all members of the PKC family to the greatest extent [23, 24]. In contrast to our results, Liyanage *et al.* [23] could not measure an efficient PS/TPA-stimulated phosphorylation of histone or MBP by PKC-δ and -η using crude extract preparations of recombinant subtypes in a mixed micelle

assay. Thus, partial purification of the PKC subtypes may be necessary to remove possible contamination by phosphatases or inactive, not PS/TPA-stimulated, enzyme [40].

Although the different substrate phosphorylations by the PKC subtypes varied somewhat, none of the PKC subtypes displayed exclusive specificity. Differential PKC subtype expression in cells and their differential subcellular localization as well as the access to more specific cellular substrates may confer substrate specificity and thus functional specificity on individual subtypes. The diverse biological effects of tumor-promoting and nontumor-promoting agents raises the question as to whether these substances act differentially on individual PKC subtypes. In this respect, the best-known example is bryostatin-1, which has been shown to mimic as well as to inhibit certain biological effects of TPA [17]. Recently, it has been shown in HL60 leukemia cells [41] and 3T3 fibroblasts [42] that a differentiative action of bryostatin is mediated through activation of a set of PKC subtypes different from that activated by TPA. However, such an isoenzyme-specific action of bryostatin-1 shows no selectivity for an individual PKC isoform when compared to phorbol ester [21]. In the present study bryostatin-1 was slightly better than TPA in activating PKC-γ activity, whereas it activated PKC-α only to 50% of the TPA response. The preferential *in vitro* activation of PKC-α by STX, mezerein, indolactam V, and RTX in a restricted concentration range suggests that by adjusting the concentrations appropriately, one could obtain certain selectivity in activating PKC-α in a cellular system, leaving other isotypes inactivated.

Binding of a compound to PKC and activation of the enzyme might be regarded as two separate processes. For example, 12-deoxyphorbol esters and diacylglycerol have been shown to be relatively inefficient in inducing membrane insertion and thereby activation of PKC-α, although they do bind to the enzyme [43]. Two different sites for ligand interaction on PKC-α have been suggested: a high affinity site involved in reversible binding and a low affinity site for irreversible membrane insertion and activation. We observed a contrary effect of TTX and thapsigargin on PKC-α, in that these agents were able to selectively activate PKC-α but were only marginally able to bind the enzyme or were unable to do so. TTX differs from RTX in that the carboxymethyl on the phenolic ring of the homovanillyl ester group is lacking. It remains to be determined if this slight structural difference accounts for the observed differences in phorbol ester binding activity. The tumor promoter thapsigargin is an inhibitor of microsomal Ca^{2+}-ATPase and has been reported not to bind to PKC or induce ODC [18, 44]. On the other hand, thapsigargin caused selective redistribution of PKC subtypes in GH_4C_1 cells, most probably by an indirect mechanism [45]. At present, we do not know the reason for the selective activation of PKC-α by thapsigargin.

Staurosporine is a potent, but unselective protein kinase inhibitor that presumably competes for ATP at the catalytic site of PKC [37, 38]. The potency of staurosporine to inhibit the enzyme activities of the various PKC subtypes is consistent with our previously published data concerning inhibition of PKC-α, -δ, and -ζ [24]. Interestingly, PKC-η was very efficiently inhibited by staurosporine. Although less potent than staurosporine, the more PKC-specific staurosporine derivatives K252a and CGP 41251 displayed certain selectivity for inhibition of individual PKC subtypes. Mizuno *et al.* [46] reported a much stronger inhibitory effect of K 252a on a cPKC mixture than on PKC-δ and -ε. Similarly, our data show an at least 10-fold more potent inhibition of PKC-α compared to the other isotypes. CGP 41251 has been shown to selectively inhibit PKC and to exert anti-proliferative and anti-tumor activity [38]. As this compound completely failed to inhibit PKC-ε and PKC-ζ but did inhibit the cPKCs very efficiently, it might be used as a specific probe in a cellular system to discriminate between diverse PKC subtype-mediated effects.

In the last few years, specific cellular functions of various PKC subtypes have been proposed [7, 47–53]. In renal mesangial cells, PKC-α negatively regulates phosphoinositide hydrolysis [7], whereas PKC-ε seems to be involved in phospholipase A2 regulation and prostaglandin synthesis [7], in the regulation of a phospholipase D [47], mitogen-activated protein kinase activation [48], and in the regulation of the inducible nitric oxide synthase [49]. Our data further support the divergence of individual PKC subtypes in signal transduction. In addition, we suggest that some activators as well as inhibitors act in a subtype-specific manner in cellular systems and thus may be valuable tools for the identification of specific functions of PKC isoenzymes *in vivo*.

This work was supported by grants from the Swiss National Science Foundation and the Wilhelm Sander-Stiftung.

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