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Characterization of Ligand and Substrate Specificity for the Calcium-Dependent and Calcium-Independent Protein Kinase C Isozymes[1]

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SUMMARY

Analysis of [^{3}H]phorbol-12,13-dibutyrate (PDBu) binding was performed with protein kinase C (PKC)-α, -β_1, -γ, -δ, -ϵ, -η, and -ζ produced in Sf9 insect cells using the baculovirus expression system. With the exception of PKC-ζ, all of the PKC isozymes bound [^{3}H]PDBu with high affinity (K_d < 1 nM), either in the presence or in the absence of calcium. Scatchard analysis using 100% phosphatidylserine vesicles revealed slightly lower affinity for the calcium-independent isozymes (PKC-δ, -ϵ, and -η) than for the calcium-dependent isozymes (PKC-α, -β, and -γ). Competition for [^{3}H]PDBu binding by different classes of PKC activators showed that 12-deoxyphorbol esters, mezerein, and octahydromezerein likewise possessed lower affinity for the calcium-independent isozymes. The mezerein analog thymeleatoxin was the most marked example, being almost 20-fold less potent for binding to PKC-ϵ and -η than to PKC-β_1. In contrast, the indole alkaloids (−)-indolactam V and (−)-octylindolactam V and the postulated endogenous activator 1,2-diacylglycerol bound with similar affinities to all of the PKC isoforms, suggesting that different residues/configurations in the binding sites of the different PKC isozymes might be involved in interaction with the pharmacophore of the activators. The seven PKC isozymes also showed clearly different substrate specificities with exogenous peptide and protein substrates. The heterogeneous behavior of the different members of the PKC family with ligands and substrates may contribute to the heterogeneity of PKC-mediated pathways at the cellular level.

Activation of PKC plays a key regulatory role in a variety of cellular functions, such as gene expression, cellular growth and differentiation, and exocytosis (1). A central factor contributing to the complexity of the PKC pathway is that PKC represents a family of at least nine isozymes with different patterns of tissue expression. Thus far, cDNAs for the PKC isoforms α, β_1, β_2, γ, δ, ϵ, η, ζ, and θ have been cloned (1, 2). The diversity in behavior of the PKC isozymes is reflected in different mechanisms controlling their enzymatic activities, including different cofactor requirements, susceptibilities to proteolysis, insertion into membranes, subcellular localization, and/or selectivity for activators and substrates (3). The availability of cloned PKC isozymes now permits the detailed assessment of these differences.

Two functional domains are found in all PKC isozymes, a carboxyl-terminal catalytic domain involved in substrate phosphorylation and an amino-terminal regulatory region involved in inhibition of the catalytic domain and subject to binding of activators and regulation by cofactors. The phorbol ester tumor promoters (and probably the postulated endogenous activators, the diacylglycerols) bind to the cysteine-rich zinc fingers present in the C1 domain of the regulatory region. The C2 domain, which is in the regulatory region of PKC isozymes α, β, and γ, confers calcium dependency and is absent in the more recently described, calcium-independent isozymes (δ, ϵ, η, ζ, and θ).

Although there is general agreement that PKC is the major receptor for phorbol esters and related compounds, there is marked heterogeneity in the pharmacological and biological responses induced by different PKC activators, from biphasic dose-response curves to selective and specific effects for certain compounds (3). For example, 12-deoxyphorbol-13-monoesters not only lack the typical inflammatory, hyperplastic, and tumor-promoting effects of PMA in mouse skin but also antagonize those functional responses when they are elicited by PMA (4, 5). Mezerein was described as a second-stage tumor pro-

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[1] M.G.K. wants to dedicate this paper in memory of Dr. Maria Amelia Enero.

ABBREVIATIONS: PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate; PS, phosphatidylserine; PMA, phorbol-12-myristate-13-acetate; PC, phosphatidylcholine; MBP, myelin basic protein; DPP, 12-deoxyphorbol-13-phenylacetate; OAG, 1-oleoyl-2-acetylglycerol; kb, kilobase(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ac, acetylated.

moter in mouse skin, because it was only effective if the animals were first treated briefly with a complete tumor promoter such as PMA (6). The common assumption used to explain the heterogeneity of the functional responses to phorbol esters and related activators is that the compounds differ in their recognition and activation of the different PKC isozymes. Because individual isozymes might have specific patterns of protein phosphorylation, selective activation of one of them might "turn on" a particular signaling pathway in the cell, leading to a selective pattern of functional responses. To begin to evaluate this possible mechanism, we decided to perform an analysis of the pattern of recognition of phorbol esters and related compounds by the different PKC isozymes.

In this report we examined the binding of a series of PKC activators to PKC-α, -β_1, -γ, -δ, -ϵ, -η, and -ζ expressed in the baculovirus-insect cell expression system. We also performed a comparative analysis of phosphorylation by the PKC isozymes of a series of peptides and proteins. Our results indicate substantial differences between PKC isozymes, and we believe that these results could help explain the biological heterogeneity of the PKC pathway.

Experimental Procedures

Materials. [^{3}H]PDBu (18.6 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). PDBu, PMA, mezerein, thymeleatoxin, (−)-indolactam V, and (−)-octylindolactam V were obtained from LC Services Corp. (Woburn, MA). OAG was purchased from Avanti Polar Lipids (Pelham, AL). PS, PC, benzamidine, histone H1, MBP, protamine sulfate, and interleukin-2 receptor carboxyl-terminal peptide were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents and media, PKC-α pseudosubstrate peptide, glycogen synthase-(1–12) peptide, AcMBP-(4–14) peptide, Syntide 2, and Kemptide were purchased from GIBCO (Gaithersburg, MD). Peptides KRTLRR and VRKRTLRRL and peptides corresponding to the histone H1 phosphorylation site, PKC-ϵ pseudosubstrate, insulin receptor kinase, S6 acceptor site, and myosin light chain kinase substrate were obtained from Bachem (Torrance, CA). Peptides for the pseudosubstrate region of PKC-η and -ζ were synthesized by Peninsula Laboratories (Belmont, CA). All other reagents and chemicals were of high quality.

Cell culture. *Spodoptera frugiperda* (Sf9) insect cells were cultured at 27° without CO_2 in spinner flasks, using Grace's insect medium containing 10% fetal bovine serum, 3.3 g/liter lactalbumin hydrolysate, 3.3 g/liter yeastolate, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone.

Construction of recombinant baculovirus transfer vectors. The full length cDNA clones for bovine PKC-α and mouse PKC-β to -η were inserted into the pVL1393 expression vector (Invitrogen, La Jolla, CA) with the shortest possible 5′ untranslated sequences, as recommended (7). The 2.1-kb insert encoding PKC-α was isolated from the parent vector, pUCbα306 (kindly provided by Dr. Axel Ullrich, Max-Plank Institute for Biochemistry Martinsried, Germany), by digestion with *Nco*I (partial digestion) and *Eco*RI. A *Nco*I linker (CATGCCATGGCATG) was inserted in the *Sma*I site of the baculovirus vector pVL1393, so that the PKC-α insert could be ligated unidirectionally into the vector. PKC-β_1 and PKC-γ baculoviruses were kindly provided by Dr. Hubert Hug (University of Freiburg, Freiburg, Germany) (8). To construct the pVL1393-PKC-δ transfer vector, plasmid ELneo-PKC-δ (a kind gift of Dr. Walter Kolch, Institute for Clinical Molecular Biology and Tumor Genetics, Munich, Germany) containing the full length cDNA coding for PKC-δ (9) was digested with *Nar*I, generating a 3.5-kb fragment, the restriction sites were blunted with Klenow enzyme, and, after digestion with *Eco*RI, the resulting 2.3-kb fragment that coded for PKC-δ was isolated and subcloned into the *Sma*I-*Eco*RI sites of pVL1393. To generate the PKC-ϵ baculovirus vector, a 2.3-kb fragment was excised from the parent vector pUC19-PKC-ϵ (10) with *Nco*I and *Eco*RI and ligated into the *Nco*I-*Eco*RI-digested pVL1393 vector described for PKC-α. For construction of the PKC-η baculovirus vector, the full length cDNA clone was isolated from a mouse lymph node cDNA library (a kind gift of Dr. Wendy Davidson, National Cancer Institute, Bethesda, MD) and subcloned into the *Hinc*II site of pUC19. To shorten the 5′ untranslated region and create restriction sites for subcloning, the cDNA was modified by the polymerase chain reaction. The 5′ oligonucleotide GGGTCCGGCAGCAGCGAATTCATGTCGTCCGGCACGATG and the 3′oligonucleotide TTTGGTTCTGACTCCGAATTCGGCTACAGTTGCAATTCC (containing the underlined *Eco*RI sites) were used as primers, with the pUC19-PKC-η plasmid as a template. The resulting 2.1-kb fragment was isolated, digested with *Eco*RI, and subcloned into the *Eco*RI site of pVL1393 to generate pVL1393-PKC-η. Construction of the pVL1393-PKC-ζ plasmid has been described elsewhere (11). cDNA clones for the recently isolated PKC-θ (12) were not available.

Expression of recombinant PKC isozymes. After purification of the transfer vectors by two rounds of CsCl density gradient centrifugation, Sf9 insect cells were co-transfected with wild-type viral Autographa California nuclear polyhedrosis visus DNA and the individual PKC transfer vectors using the transfection kit from Invitrogen. After 3–4 days, a plaque assay was performed, and recombinant viral plaques were identified under an inverted microscope by the lack of polyhedrin. Their identity was confirmed by hybridization with specific ^{32}P-labeled probes for each of the PKC isozymes. Purified viruses were usually obtained after two or three rounds of plaque purification; a high titer viral stock was generated, and the titer was determined (7).

For recombinant protein production, 1 liter of approximately 2 × 10^6 Sf9 cells/ml in spinner flasks was infected with the recombinant viruses at a multiplicity of infection of 10. After 60–72 hr, the cells were centrifuged (1000 rpm, 10 min) and washed twice with phosphate-buffered saline, and the pelleted cells were kept at −70° until used.

For purification of the individual PKC isozymes, the cell pellet was resuspended in 50 ml of a homogenization buffer of the following composition: 20 mM Tris·HCl, pH 8.0, 5 mM EDTA, 5 mM EGTA, 0.3% (v/v) 2-mercaptoethanol, 10 mM benzamidine, 50 μg/ml phenylmethylsulfonyl fluoride, and 250 μg/ml leupeptin. The cells were disrupted in a Potter-Elvehjem homogenizer at 4°, and the homogenate was centrifuged at 100,000 × *g* for 60 min. The supernatant was adjusted to pH 8.0 and loaded in a TSK-GEL DEAE-5PW column (15 cm × 2 cm; Tosohaas, Philadelphia, PA) that had been previously equilibrated with 20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 0.3% (v/v) 2-mercaptoethanol, 10 mM benzamidine. The column was eluted with a 375-ml linear gradient of NaCl (0–400 mM) in the equilibration buffer, at a flow rate of 2.5 ml/min. Fractions containing high PKC activity (measured as phosphorylation of PKC-α pseudosubstrate peptide) were pooled and dialyzed overnight against 20 mM Tris·HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 50% glycerol, before storage at −70°. For most of the recombinant isozymes (PKC-α to -ϵ), the peak of kinase activity eluted at approximately 100–200 mM NaCl. PKC-ζ eluted at a slightly higher salt concentration. A low level of endogenous kinase activity eluted before the recombinant peak (at <100 mM NaCl). Identities of the isozymes were confirmed by Western blots of total cell lysates and of the active fractions using isozyme-specific antibodies (data not shown; see also Results). All the purification steps were performed at 4°.

PKC assay. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into substrates, as described previously (13). Phospholipid vesicles (20% PS/80% PC) were prepared by sonication (14), and PMA was used as a PKC activator at a final concentration of 1 μM. Incubation was at 30° for 10 min. The kinase assay was linear with time over this incubation period, as determined for phosphorylation of the PKC-α pseudosubstrate peptide. The following substrates were used: histone H1 (from calf thymus), MBP (from bovine brain), protamine sulfate (from salmon), PKC-α pseudosubstrate peptide (RFARKGSLRQKNV), PKC-ϵ pseudosubstrate pep-

tide (ERMRPRKRQGSVRRRV), PKC-η pseudosubstrate peptide (FTRKRQRSMRRRVHQ), PKC-ζ pseudosubstrate peptide (EDKSIYRRGSRRWRKL), glycogen synthase-(1–12) peptide (PLSRTLSVAAKK), AcMBP-(4–14) peptide (Ac-QKRPSQRSKYL), histone H1 phosphorylation site (Hi-7) peptide (RRKASGP), insulin receptor kinase substrate (RRLIEDAEYAARG), S6 phosphate acceptor peptide (RRLSSLRA), myosin light chain kinase (smooth muscle) substrate (KKRAARATSNVFA), Ca^{2+}/calmodulin-dependent kinase II substrate (Syntide 2, PLARTLSVAGLPGKK), cAMP-dependent protein kinase substrate (Kemptide, LRRASLG), interleukin-2 receptor (carboxyl-terminal) peptide (QRRQRKSRRTI), and the peptides KRTLRR and VRKRTLRRL. Phosphorylation of the PKC-α pseudosubstrate peptide (50 μM) was linear with protein over the concentrations of PKC isozymes used in the assays.

Binding of [^{3}H]PDBu to recombinant PKC isozymes. [^{3}H]PDBu binding to PKC was measured using the polyethylene glycol precipitation assay developed in our laboratory (15), with minor modifications. For determination of the dissociation constants (K_d) and the number of sites (B_{max}) for the different PKC isozyme preparations, typical saturation curves with increasing concentrations of the radioactive ligand (generally between 0.125 and 8 nM) were obtained in triplicate. The assay mixture (250 μl) contained 50 mM Tris·HCl, pH 7.4, 100 μg/ml phospholipid (100% PS, unless otherwise indicated), 4 mg/ml bovine IgG, variable concentrations of [^{3}H]PDBu, and 100 μM calcium chloride or 1 mM EGTA. Incubation was carried out at 37° for 5 min, unless otherwise indicated. Samples were chilled to 0° for 5 min, 200 μl of 35% polyethylene glycol in 50 mM Tris·HCl, pH 7.4, were added, and the samples were incubated at 0° for an additional 15 min to induce precipitation of the protein. The tubes were then centrifuged in a Beckman 12 microcentrifuge at 4° (12,000 rpm, 15 min). A 100-μl aliquot of the supernatant was removed for determination of the free concentration of [^{3}H]PDBu, the remaining supernatant was aspirated, and the pellet was carefully dried with disposable wipes. The tip of the centrifuge tube containing the pellet was cut off and transferred to a scintillation vial for determination of total bound [^{3}H]PDBu. Aquasol (3 ml) was added both to aliquots of the supernatants and to the pellets, and radioactivity was determined in a LKB 1218 scintillation counter. Nonspecific binding was measured using an excess of nonradioactive PDBu (30 μM), as described (15). Specific binding was calculated as the difference between total and nonspecific binding.

To measure competition for [^{3}H]PDBu binding by different compounds, reactions were performed under similar conditions but using a fixed concentration of [^{3}H]PDBu (3 nM) and increasing concentrations of the nonradioactive ligand. In a typical competition assay, six to eight different concentrations (in triplicate) of the competing ligand were used. ID_{50} values were determined from the competition curve, and the K_i for the competing ligand was calculated from the ID_{50} by using the relationship $K_i = ID_{50}/(1 + L/K_d)$, where L is the concentration of free [^{3}H]PDBu at the ED_{50} and K_d is the dissociation constant for [^{3}H]PDBu under the specific assay conditions for the corresponding PKC isozyme. When diacylglycerols were tested for competition of [^{3}H]PDBu binding, they were dried and resuspended together with the phospholipids, as described (14). In general, triplicate determinations differed by <10%.

Immunological detection of PKC isozymes. Proteins were resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gels, electrophoretically transferred to nitrocellulose, and probed with specific antibodies using standard techniques. For PKC-α, -β, and -γ, monoclonal antibodies developed against the catalytic (α) or regulatory (β and γ) domains for each specific isozyme were used (Upstate Biotechnology, Inc., Lake Placid, NY). For immunological detection of PKC-δ and -ζ, commercial polyclonal antibodies developed against peptides corresponding to the published amino acid sequences of rat PKC-δ (residues 662–673) and rat PKC-ζ (residues 480–492) were used (Research & Diagnostic Antibodies, Berkeley, CA). PKC-ϵ was detected with a polyclonal antibody generated in rabbits using a peptide from the carboxyl terminus (amino acids 316–326) of rat PKC-ϵ (GIBCO, Gaithersburg, MD). Anti-PKC-η antibody was generated in rabbits in our laboratory. For this purpose a peptide corresponding to the carboxyl-terminal region of rat PKC-η (CINQDEFRNFSYVSPELQL) was synthesized, coupled to keyhole limpet hemocyanin, and injected subcutaneously into rabbits. Antibodies purchased from commercial sources were used at the dilutions suggested by the manufacturers. PKC-η antiserum was used at a 1/1000 dilution. Goat anti-rabbit or anti-mouse antiserum coupled to alkaline phosphatase (1/1000) was used as secondary antibody, and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad, Richmond, CA) were used as color development reagents. The antibodies used in this study were selected as the most specific and selective for the individual PKC isozymes among the many different commercial antibodies or antisera generated in our laboratory that we tested.

Other methods. Protein concentration was determined using a Bio-Rad protein assay kit, with bovine serum albumin as the standard.

Results

Expression of recombinant PKC isozymes in Sf9 cells. In this study we took advantage of the baculovirus expression system to obtain high levels of expression of the individual PKC isozymes. Because each expressed isozyme came from a defined cDNA, no problems of contamination between PKC species were expected, as may occur when PKC isozymes are isolated from tissues or cells. The yield of purified recombinant protein differed between preparations but ranged between 100 and 800 μg of recombinant protein/liter of cells. The time course of expression of the recombinant proteins showed the highest levels of intact enzymes after 60–72 hr of infection (data not shown). Longer periods of infection led to the appearance of degradation products, as detected by immunostaining, and, although high kinase levels were found in cell lysates, they were independent of PS and phorbol esters, suggesting that they were mainly due to catalytic fragments. Total homogenates of Sf9 cells infected for 60 hr with the individual recombinant baculoviruses showed high levels of kinase activity (as measured by phosphorylation of the PKC-α pseudosubstrate peptide) and [^{3}H]PDBu binding, in comparison with noninfected cells.[2] No detectable increase in binding was found in Sf9 cells infected with the PKC-ζ baculovirus, even if 50 nM [^{3}H]PDBu was used (data not shown). Immunological detection of PKC isozymes α, β_1, γ, δ, ϵ, η, and ζ is shown in Fig. 1. Noninfected Sf9 cells or cells infected with the recombinant viruses for 60 hr were lysed, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Western blot analysis using antibodies specific for each PKC isozyme revealed bands of approximately 80 kDa for PKC-α, -β, -γ, -δ, and -η, 85–90 kDa for PKC-ϵ, and 75 kDa for PKC-ζ. Noninfected cells did not show any detectable signal. Similarly, Sf9 cells infected with wild-type virus did not reveal any band when probed with the anti-PKC antibodies, and the levels of phosphorylation and [^{3}H]PDBu binding were similar to those found in noninfected cells (data not shown). In conclusion, the baculovirus-Sf9 insect

[2] Although other authors report no endogenous PKC activity in Sf9 cells (16, 17), in most cases we were able to detect a low but significant level of PKC activity and [^{3}H]PDBu binding. The estimated amount was approximately 10^{-7} pmol/cell. Similar endogenous levels in Sf9 cells were reported by Liyanage *et al.* (19). This amount of endogenous activity generally represented 0.1–5% of the recombinant protein, depending on the specific levels of expression obtained for different isozymes and different preparations. In any case, this low level of endogenous activity was separated from the recombinant PKC by chromatography on TSK-GEL DEAE-5PW, as described in Experimental Procedures.

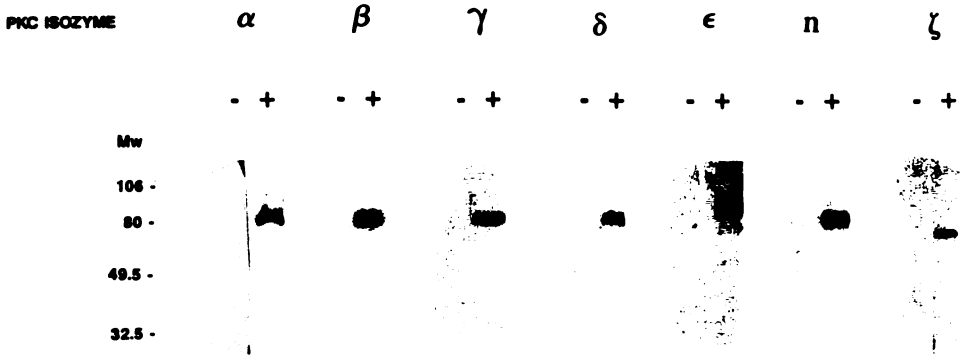

Fig. 1. Expression of PKC isozymes in Sf9 insect cells. Cells (2×10^6) in 60-mm dishes were infected with the recombinant PKC baculoviruses at a multiplicity of infection of 10 and were lysed after 60 hr. Aliquots of the samples were subjected to Western blot analysis with the specific antibodies for the different PKC isozymes, as described in Experimental Procedures. −, Noninfected; +, infected.

cell system was highly efficient for producing fully active PKC isozymes, as has already been described by several laboratories (16–19).

Characterization of [^{3}H]PDBu binding to the PKC isoforms. After partial purification of the isozymes as described in Experimental Procedures, [^{3}H]PDBu binding to the individual PKC preparations was measured. As described in earlier studies on the mixture of PKC isozymes in the mouse brain particulate fraction (20), the kinetics of [^{3}H]PDBu (0.5 nM) binding to PKC-α, -β_1, -γ, -δ, -ϵ, and -η was very fast, and maximum binding levels were attained by 2.5–5 min. Longer times of incubation resulted in variable slow loss of [^{3}H]PDBu binding. [^{3}H]PDBu binding to PKC-α was somewhat more stable than that to the other PKC isozymes, and even after 20 min of incubation 90% of the binding to PKC-α still remained (data not shown). PKC-ζ was unique among PKC isoforms, in that no specific binding was found even at a concentration of 50 nM [^{3}H]PDBu (data not shown). These results are similar to our results obtained when [^{3}H]PDBu was bound to unfractionated lysates of Sf9 cells that overexpress PKC-ζ (11).

Scatchard analysis for the different PKC isozymes revealed that [^{3}H]PDBu bound with high affinity in the presence of 100% PS vesicles (Fig. 2). Dissociation constants (K_d) for [^{3}H]PDBu with the different isozymes in the absence of calcium (1 mM EGTA) ranged between 0.14 nM (PKC-β_1) and 0.71 nM (PKC-δ) and were not substantially changed when determined in the presence of 0.1 mM calcium (Table 1). A preparation of PKC-α purified to homogeneity from Sf9-infected cells bound [^{3}H]PDBu in the presence of calcium with affinity similar to that of the partially purified enzyme ($K_d = 0.31$ nM), suggesting that binding properties were not modified by further purification. For all isozymes, the number of binding sites (B_{max}) in the presence of 100% PS vesicles was similar in the presence of either calcium or EGTA (data not shown; see also Fig. 3). Interestingly, all the calcium-dependent isozymes (PKC-α, -β_1, and -γ) showed higher affinities for [^{3}H]PDBu than did the calcium-independent isozymes (PKC-δ, -ϵ, and -η), regardless of the presence or absence of calcium. The composition of the phospholipid vesicles strongly affected the efficiency of reconstitution, as is shown in Fig. 3 for a calcium-dependent isozyme (PKC-α) and a calcium-independent isozyme (PKC-δ). In both cases, [^{3}H]PDBu binding in the absence of calcium was substantially lower when PS was reduced from 100% to 20% (in the latter case the remaining phospholipid was PC). However, only binding to PKC-α was restored by addition of the divalent cation. [^{3}H]PDBu binding to PKC thus has different phospholipid cofactor requirements for the calcium-dependent and calcium-independent isozymes. Earlier experiments in our laboratory using a mixture of PKC isozymes from brain also showed that calcium increased the potency of phospholipid for reconstitution of binding activity without substantially affecting the binding affinity of the receptor-lipid complex for [^{3}H]PDBu (21). In contrast, indirect experiments by Bazzi and Nelsestuen (22) suggested an effect on affinity.

Structure-activity relationships of PKC activators. To compare the enzyme-ligand interactions for the different PKC isozymes, we quantitated competition with [^{3}H]PDBu binding for a range of analogs known to be activators of PKC, which differed substantially in their spectra of biological activities. The issue we wished to test was whether the difference in biological activities was reflected in different selectivities for the PKC isozymes. The series of compounds studied included 12-deoxyphorbol esters, mezerein and mezerein analogs, indole alkaloids, diacylglycerols, and resiniferatoxin (Table 2).

DPP was extremely potent at competing for [^{3}H]PDBu binding to the PKC isozymes. The K_i for inhibition of binding to PKC-α, determined from the ID_{50}, was 0.14 ± 0.02 nM. Prostratin (12-deoxyphorbol-13-acetate) was approximately 35-fold less potent than DPP in binding to the same isozyme and, like PDBu, both 12-deoxyphorbol esters showed preference for the calcium-dependent isozymes. The affinity of prostratin for PKC-δ, -ϵ, and -η was 7–9-fold lower than that for PKC-β_1.

Mezerein bound with high affinity to the PKC isozymes (Table 2) and, as was the case for phorbol esters and 12-deoxyphorbol esters, mezerein bound with higher affinity to PKC-α, -β_1, and -γ than to the calcium-independent PKC isozymes δ, ϵ, and η. Unsaturation is thought to be the critical structural feature of mezerein that is responsible for its behavior as an incomplete tumor promoter. Octahydromezerein, a compound that differs from mezerein by saturation of the lipophilic chain at the C12-position, is thus a complete tumor promoter (23). Octahydromezerein bound with 3–4-fold lower affinity than mezerein, but the pattern of selectivity remained the same; octahydromezerein showed higher affinity for binding to the calcium-dependent isozymes, compared with the calcium-independent isozymes. We conclude that differences in isozyme selectivity cannot account for the different biological activities of the two compounds. On the other hand, the most striking difference between PKC isozymes was found for thymeleatoxin. This compound differs from mezerein in that the lipophilic chain at the C12-position is shorter by two carbons (24). Thymeleatoxin was as potent as mezerein for binding to PKC-α, -β_1, and -γ, but its reduced affinity for the calcium-independent isozymes, mainly PKC-ϵ and -η, was even more pro-

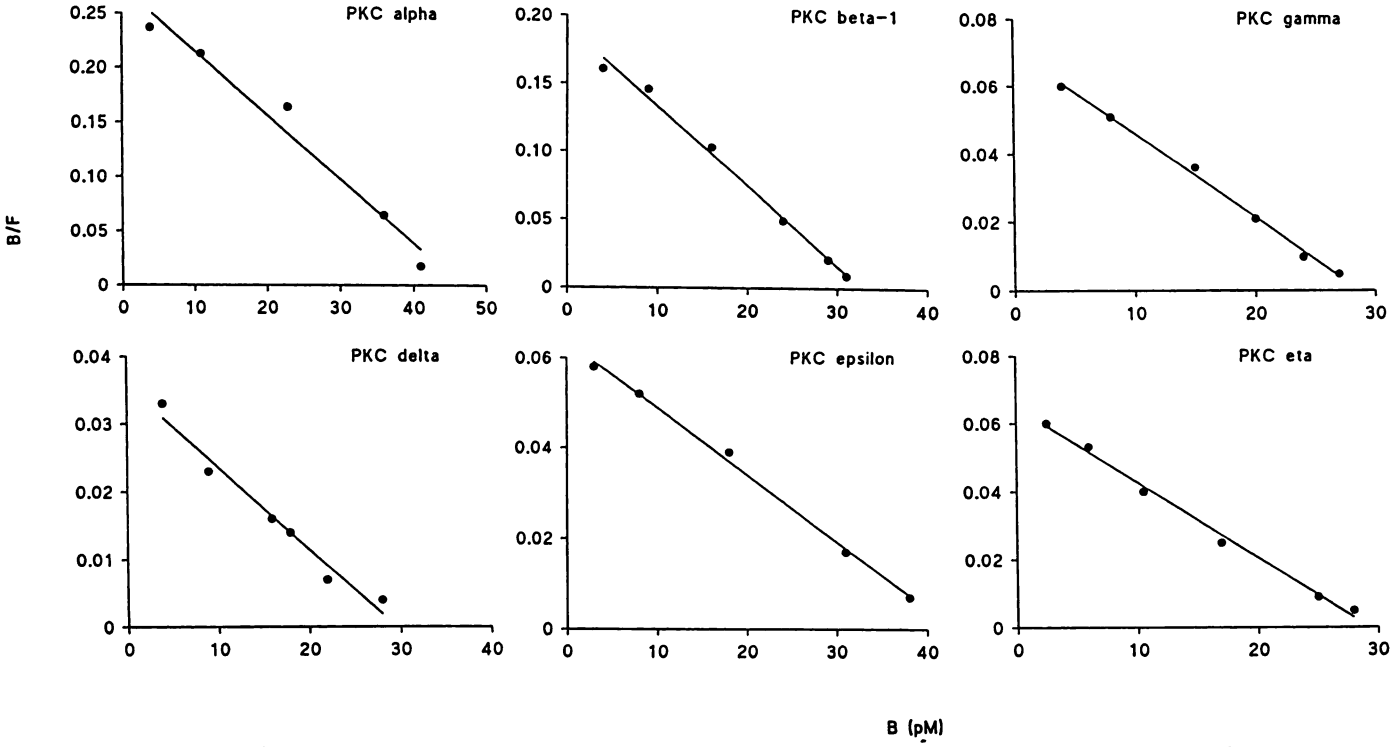

Fig. 2. Scatchard plot for [^{3}H]PDBu binding to PKC isozymes. Each PKC isoform was incubated with increasing concentrations of [^{3}H]PDBu (0.125–8 nM) for 5 min, using 100% PS vesicles and 1 mM EGTA, and binding was measured using the polyethylene glycol precipitation assay, as described in Experimental Procedures. A representative experiment for each PKC isozyme is shown. Each experiment was performed two to six times. Each *point* represents the mean of three experimental values, generally with a standard error of <2%.

TABLE 1

Dissociation constant (K_d) for [^{3}H]PDBu for the different PKC isozymes

Binding was performed in the presence of 100% PS vesicles and either 0.1 mM calcium or 1 mM EGTA. Each K_d value represents the mean ± standard error of the number of experiments in parentheses.

PKC isozyme	K_d	
	+Ca^{2+}	−Ca^{2+}
	nM	
α	0.20 ± 0.03 (3)	0.15 ± 0.02 (3)
β	0.20 ± 0.02 (3)	0.14 ± 0.01 (3)
γ	0.33 ± 0.03 (5)	0.37 ± 0.03 (2)
δ	0.94 ± 0.09 (5)	0.71 ± 0.10 (6)
ϵ	0.81 ± 0.09 (3)	0.63 ± 0.07 (4)
η	0.87 ± 0.15 (4)	0.58 ± 0.11 (4)
ζ	No binding (3)	No binding (3)

nounced. Thymeleatoxin displaced [^{3}H]PDBu binding from PKC-η and -ϵ with 16- and 20-fold lower affinity, respectively, than from PKC-β_1.

The indole alkaloids (−)-indolactam V and (−)-octylindolactam V are synthetic analogs related to the tumor promoter teleocidin, and they differ from one another by the presence of an *n*-octyl chain in the C7-position of the molecule (25). Indole alkaloids bind to PKC and share most of the biological properties with the tumor-promoting phorbol esters (26). (−)-Octylindolactam V inhibited [^{3}H]PDBu binding to the different PKC isozymes with higher affinity than did (−)-indolactam V, reflecting the importance of the lipophilic alkyl chain for the interaction. Interestingly, and unlike the phorbol esters and mezerein-like compounds, the indole alkaloids did not show a preference for the calcium-dependent isozymes. The largest difference between isozymes was a 3-fold lower affinity for PKC-γ and -ϵ, compared with PKC-β_1 (Table 2). The indole alkaloids thus presented a different recognition pattern for the PKC isozymes than did the other compounds.

The same pattern of isozyme specificity was also found for *sn*-1,2-diacylglycerols, the postulated physiological activators of PKC. The relative potency of OAG was substantially lower than those of the phorbol esters. The K_i for PKC-α was 230 ± 28 nM. However, analysis of the binding properties of OAG revealed that it displaced [^{3}H]PDBu binding from all PKC isozymes with very similar potencies (Table 2). Therefore, the pattern of binding of the postulated endogenous activators of PKC differs significantly from those of the "nonphysiological" activators, i.e., phorbol esters and mezerein-related compounds.

The last compound we tested in this study was resiniferatoxin, a naturally occurring phorbol-related diterpene that has a homovanillyl substitution at the C20-position that confers capsaicin-like activity (27). As shown in Table 2, resiniferatoxin was very poor as a PKC ligand. Less than 20% inhibition of [^{3}H]PDBu binding for all the PKC isozymes was obtained at a concentration of 10 μM. The hydroxyl group in the C20-position of the phorbol nucleus is thought to be essential for binding of phorbol esters and related compounds to PKC (28). A summary of the relative potencies of the different compounds tested in this study is shown in Fig. 4.

Substrate specificity of PKC isozymes. We compared the ability of the recombinant PKC isozymes to phosphorylate a large series of peptides and proteins as substrates. In these experiments we used 20% PS/80% PC vesicles, standard conditions under which the effects of activators can be evaluated. In the presence of 1 μM PMA maximal activation was observed. In experiments using histone H1 and MBP as substrates,

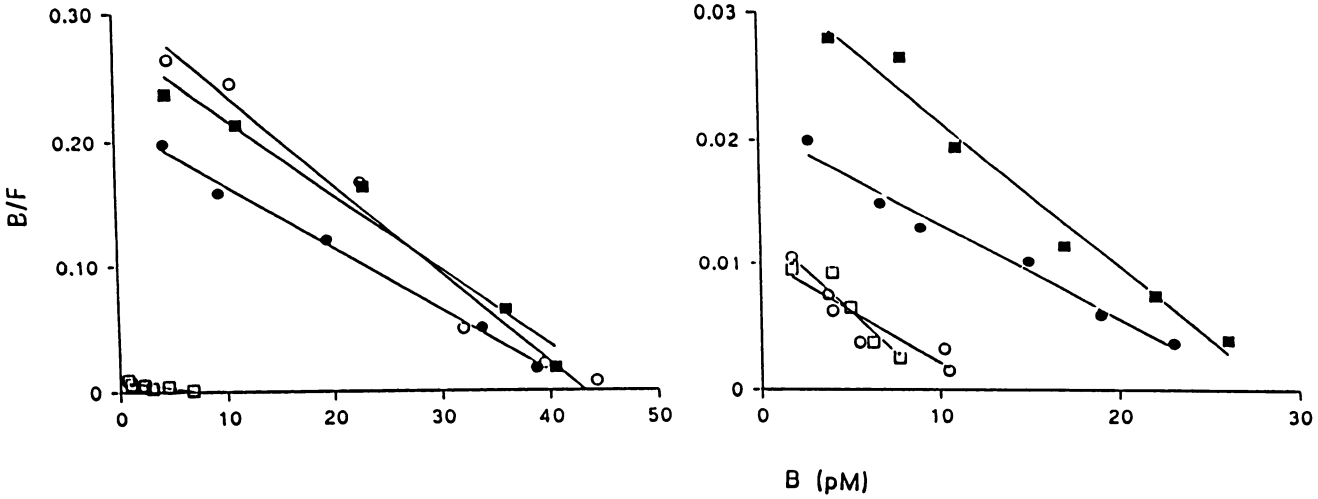

Fig. 3. Effect of phospholipid composition on [^{3}H]PDBu binding to calcium-dependent and calcium-independent isozymes. Scatchard analysis of binding to PKC-α (*left*) and PKC-δ (*right*) was performed using 100% PS vesicles (●, ■) or 20% PS vesicles (○, □), in the presence of either 0.1 mM calcium (●, ○) or 1 mM EGTA (■, □). Experiments (in triplicate) were repeated at least two times, with similar results.

TABLE 2

Structure-activity analysis of binding to PKC isozymes

Enzyme-ligand interactions were analyzed by competition with [^{3}H]PDBu binding to PKC isozymes, using 100% PS vesicles and 1 mM EGTA. A fixed concentration of the radioactive ligand (3 nM) and six to eight increasing concentrations (in triplicate) of the competing ligand were used. The ID_{50} values were determined from the competition curves, and the corresponding K_i values for the ligands were calculated from the ID_{50} values as described in Experimental Procedures. Values represent the mean ± standard error (n > 2) or range (n = 2) of the number of experiments in parentheses. For resiniferatoxin, limits of inhibition of binding at 10 μM are indicated.

Compound	K_i					
	α	β	γ	δ	ϵ	η
	nM					
DPP	0.14 ± 0.02 (3)	0.11 ± 0.01 (3)	0.28 ± 0.03 (3)	0.56 ± 0.03 (2)	0.62 ± 0.03 (3)	0.61 ± 0.23 (2)
Prostratin	4.83 ± 0.44 (5)	3.05 ± 0.52 (3)	9.81 ± 0.57 (2)	24.59 ± 4.40 (3)	27.47 ± 3.34 (5)	20.89 ± 3.12 (3)
Mezerein	0.27 ± 0.04 (4)	0.16 ± 0.01 (3)	0.21 ± 0.02 (3)	0.55 ± 0.18 (3)	1.20 ± 0.22 (3)	1.27 ± 0.24 (2)
Octahydromezerein	0.82 ± 0.11 (4)	0.51 ± 0.07 (2)	0.80 ± 0.17 (3)	2.17 ± 0.31 (4)	3.13 ± 0.59 (3)	2.48 ± 0.01 (2)
Thymeleatoxin	0.29 ± 0.03 (3)	0.16 ± 0.03 (3)	0.26 ± 0.03 (3)	1.31 ± 0.22 (4)	3.11 ± 0.20 (3)	2.63 ± 0.83 (3)
(−)-Indolactam V	10.96 ± 1.32 (3)	6.06 ± 0.96 (2)	19.44 ± 3.69 (3)	8.16 ± 0.65 (3)	21.86 ± 1.10 (3)	15.56 ± 0.05 (3)
(−)-Octylindolactam V	0.53 ± 0.06 (3)	0.39 ± 0.06 (3)	1.19 ± 0.33 (3)	0.77 ± 0.08 (3)	0.95 ± 0.09 (3)	0.55 ± 0.09 (2)
OAG	230 ± 28 (3)	218 ± 41 (3)	373 ± 55 (3)	224 ± 27 (3)	235 ± 33 (3)	269 ± 56 (3)
Resiniferatoxin (10 μM)	<20% (3)	<20% (3)	<20% (3)	<20% (3)	<20% (3)	<20% (3)

similar levels of PKC activity were observed with 100% PS vesicles in the absence of phorbol ester. Initial attempts at kinetic analysis revealed that, because in a number of cases the curves did not clearly fit a Michaelis-Menten plot, accurate K_m values could not be obtained consistently. Therefore, we used a fixed high concentration of substrate, as was done previously by Nishizuka and co-workers (29). Substantial differences among the PKC isozymes were found (Table 3). In general, peptides derived from the pseudosubstrate region (in which an alanine was replaced by a serine) were the best substrates for all of the PKC isozymes, although the degree of phosphorylation differed in each case. Of this class of substrates, the PKC-α pseudosubstrate peptide was the best phosphate acceptor for the calcium-dependent isozymes as well as for PKC-δ and -η. As was also recently described by Ways *et al.* (30), the highest levels of phosphorylation by PKC-ζ were obtained with the peptide derived from the pseudosubstrate region of PKC-ϵ. A peptide derived from the sequence of the enzyme glycogen synthase was a good phosphate acceptor for PKC-β_1; PKC-ϵ, on the other hand, was relatively inefficient at phosphorylating this synthetic peptide. A synthetic peptide corresponding to the carboxyl-terminal region of the interleukin-2 receptor was a very good phosphate acceptor for the PKC isoforms α, β, γ, δ, and η. MBP represented a very good substrate for PKC-δ. Unexpectedly, an undecapeptide derived from the sequence of MBP, AcMBP (see Experimental Procedures), was a better substrate for those isozymes that were not very efficient in phosphorylating the complete protein. Another interesting finding was that histone H1, the classical PKC substrate, as well as a heptapeptide derived from its sequence (Hi-7), were very poor substrates for all seven PKC isozymes. Analysis of the phosphotransferase activity using protamine sulfate as a substrate revealed that high activity was obtained for most of the isozymes. This polyanionic protein was definitely the best substrate for PKC-ϵ. Two commercially available substrates with potential sites for PKC phosphorylation (KRTLRR and VRKRTLRRL) revealed that the peptide length might be critical for enzyme recognition, because only the longer of the two was a good phosphate acceptor, although not for PKC-ϵ and -ζ. None of the PKC isozymes was able to phosphorylate the peptide substrates for the myosin light chain kinase, cAMP-dependent kinase (Kemptide), or insulin receptor kinase. Low phosphorylation levels were obtained for the Ca^{2+}/calmodulin-dependent protein kinase II substrate (Syntide 2) and a peptide derived from the sequence of the ribosomal protein S6.

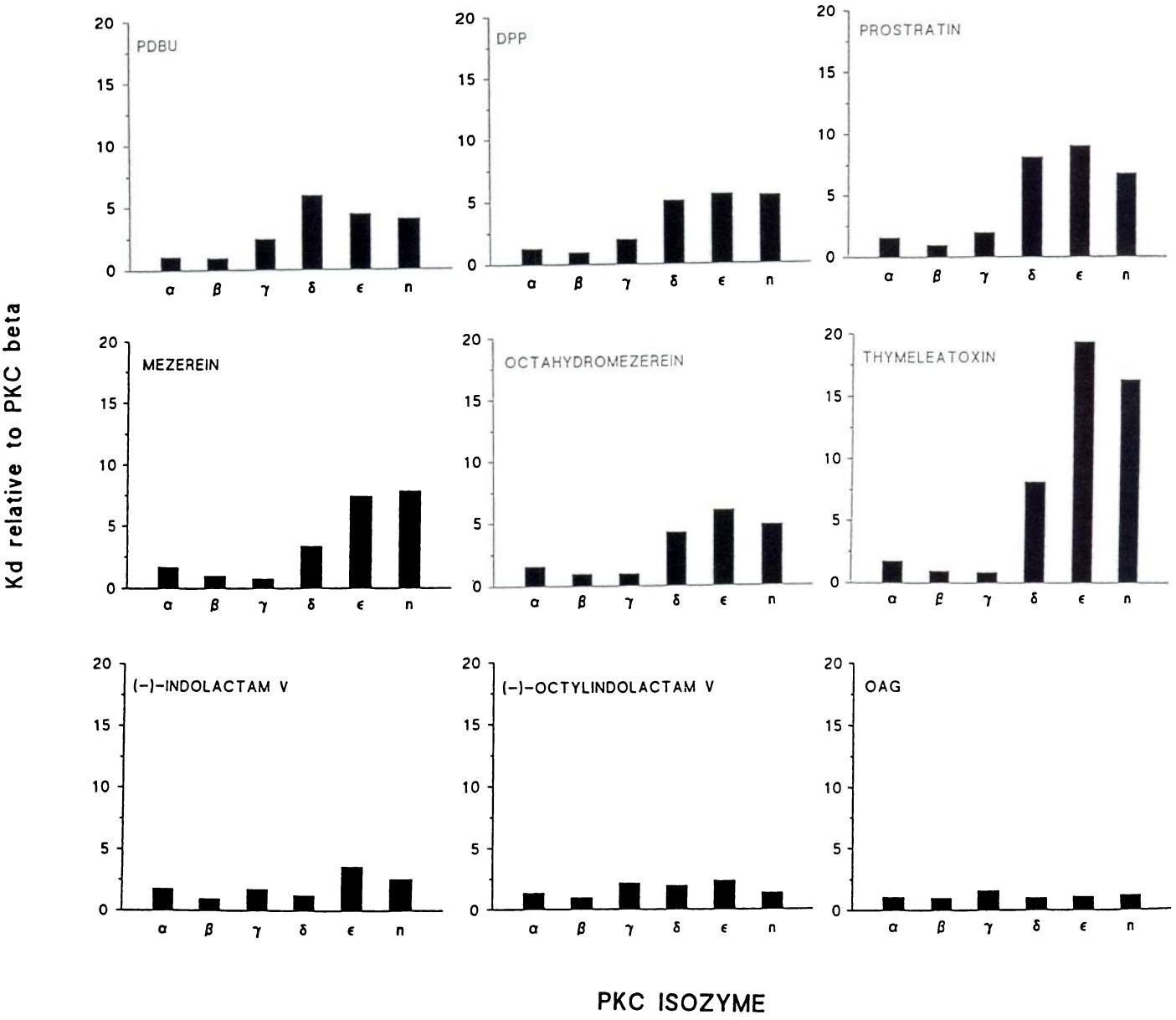

Fig. 4. Relative affinities of PKC ligands for the different PKC isoforms. Affinities of the different ligands for each PKC isozyme are expressed as the ratio to those for PKC-β_1.

Discussion

The evidence for heterogeneity in the responses to different PKC activators in a variety of biological systems could not easily be explained by different structure-activity requirements for PKC isozymes α, β, and γ (16). To assess the potential role of binding heterogeneity of the novel PKC isozymes, we have examined here the structure-activity relationships for binding of different classes of PKC activators to the PKC isoforms α through η.

To prepare the individual PKC isozymes, we expressed the proteins in the baculovirus expression system. By expressing genetically defined PKC species from their corresponding cDNAs in Sf9 insect cells, we overcame some of the limitations inherent in the isolation of PKC isozymes from tissues, mainly contamination with other known or unknown isozymes, and we were able to obtain large quantities of protein. As an additional advantage, post-translational processing of PKC isozymes in Sf9 cells most likely reflects processing events similar to those that occur in mammalian tissues (16). This is critical because phosphorylation is thought to play an important role in the regulation of PKC function, and PKC isozymes isolated from expression systems such as bacteria are not phosphorylated.

Characterization of [^{3}H]PDBu binding using 100% PS vesicles revealed that PKC-α, -β_1, -γ, -δ, -ϵ, and -η bind PDBu with high affinity. Although binding to the calcium-independent isozymes PKC-δ, -ϵ, and -η expressed in COS cells (31–33) or Sf9 cells (17–19) had already been demonstrated qualitatively, binding affinities for single isozymes had not been determined quantitatively. In the only reported attempt to characterize [^{3}H]PDBu binding to a calcium-independent isozyme, Akita *et al.* (34) found a higher K_d for PKC-ϵ than for the calcium-dependent isozymes, a result that we confirmed. However, because the former experiments were performed in homoge-

TABLE 3

Relative kinase activities of PKC isozymes with various peptide and protein substrates

PKC activities were assayed in the presence of 100 μg/ml phospholipid vesicles (20% PS/80% PC), 100 μM calcium chloride, 1 μM PMA, and 1 nM enzyme. For PKC-ζ, 2.5 μl/tube of the enzyme preparation were used. Substrates were added at 50 μM (peptides) and 1 mg/ml (proteins). Measurements performed in duplicate generally differed by <10%, and the results presented are the mean of the two values. Results were normalized to the activity with the PKC-α pseudosubstrate peptide, as 100% (PKC-α, 170; PKC-β_1, 130; PKC-γ, 170; PKC-δ, 72; PKC-ε, 20; PKC-η, 57; PKC-ζ, 61; values represent pmol of phosphate transferred in 10 min). Specific activity of [γ-^{32}P]ATP ranged between 380 and 600 cpm/pmol. A second experiment gave similar results. Peptides are as described in Experimental Procedures.

Substrate	PKC activity						
	α	β	γ	δ	ε	η	ζ
				%			
PKC-α peptide	100	100	100	100	100	100	100
PKC-ε peptide	72	58	92	77	147	79	151
PKC-η peptide	51	77	76	50	180	80	92
PKC-ζ peptide	42	56	56	48	125	69	77
Glycogen synthase peptide	28	107	22	40	15	48	30
Interleukin-2 receptor peptide	58	74	77	91	12	71	29
Protamine sulfate	42	79	139	100	393	60	55
Histone H1	5	3	10	16	18	6	10
Hi-7 peptide	0	0	0	0	0	0	0
MBP	19	22	57	86	36	17	34
AcMBP peptide	32	37	38	22	8	56	3
Syntide 2	22	27	13	22	10	33	14
S6 peptide	6	8	8	7	7	11	4
KRTLRR	4	5	4	2	5	7	1
VRKRTLRRL	40	72	58	57	14	68	6
Myosin light chain kinase substrate	1	0	0	0	0	3	3
Insulin receptor substrate	0	0	0	0	0	0	0
Kemptide	0	0	1	4	1	1	0

nates of PKC-ε-transfected COS cells, where more than one PKC isozyme is present, the conclusion had been ambiguous.

Our data with recombinant PKCs showed that PKC-α and -β_1 had the highest affinities for [^{3}H]PDBu, either in the presence or in the absence of calcium. Scatchard plots of [^{3}H]PDBu binding to both calcium-dependent and calcium-independent isozymes were linear, suggesting that under our binding conditions only high affinity binding sites could be detected. Although two binding sites corresponding to the two zinc fingers in the C1 domain have been demonstrated (35), a low affinity interaction between the ligands and PKC can regulate the irreversible insertion of the enzyme into membranes, and only a high affinity interaction is involved in the reversible binding (14). Although the issue of the stoichiometry of phorbol ester binding to PKC isozymes remains unresolved, PKC-ζ, the only isotype with a single zinc finger, was not able to bind [^{3}H]PDBu. The fact that a very high concentration of [^{3}H]PDBu (50 nM) and the ultrapotent PKC ligand [^{3}H]bryostatin 1[3] both failed to bind specifically to PKC-ζ indicates that this isozyme does not bind, as opposed to simply binding with low affinity. We assume that critical residues for the interaction with the ligand or the phospholipid might be altered. Sequence aligment of PKC-ζ with the postulated binding consensus for PKCs and the novel phorbol ester receptor neuronal chimaerin reveals that 14 of 15 of the consensus amino acids are present in PKC-ζ. The missing amino acid (proline) is also absent in the zinc finger region of certain proteins that are not phorbol ester receptors, such as the *raf* and *vav* protooncogenes (see Ref. 36 for sequence aligment). Site-directed mutagenesis experiments on PKC-ζ are currently being performed in our laboratory to resolve this issue.

A decrease in the PS concentration in the phospholipid vesicles from 100% to 20% substantially reduced the binding of [^{3}H]PDBu to both calcium-dependent and calcium-independent isozymes in the presence of 1 mM EGTA under our assay conditions, and binding could be restored by the cation only in the case of the calcium-dependent isozyme. Scatchard plots for [^{3}H]PDBu binding to calcium-dependent or calcium-independent PKCs in 100% PS vesicles revealed similar K_d and B_{max} values in the presence of calcium and in the EGTA-induced absence of calcium. This suggests that the binding itself is a calcium-independent phenomenon and that the role of the divalent cation must be limited to facilitation of protein-phospholipid interaction (for the calcium-dependent isozymes), as shown previously by Bazzi and Nelsestuen (37). At the intracellular level such a difference in cofactor requirements could constitute an important regulatory factor for ligand-protein interactions for the different PKC isozymes.

A major issue that we address in this paper is the structure-activity relationship of PKC activators for binding to the different PKC isozymes. Previous studies of the structure-activity relationship for binding have generally been performed using a mixture of PKC isozymes. A comparison of activation of kinase activity of different isozymes by different compounds was described by Ryves *et al.* (38). Phorbol esters (PDBu) and 12-deoxyphorbol esters (prostratin and DPP) both showed a slight preference for the calcium-dependent isozymes. Although these differences were in some cases relatively small, it is possible that differential activation of PKC isozymes that results from certain concentrations of these compounds could alter the ratio of particular isozyme-specific pathways and be responsible for specific physiological changes at the cellular level. The unusual biological properties of 12-deoxyphorbol esters, on the other hand, probably reflect factors other than isozyme selectivity. For example, the 12-deoxyphorbol esters are not very efficient at inserting PKC-α into membranes (14), although they bind with high affinity to the enzyme. Differential insertion of each PKC isozyme by different activators might represent a mechanism that explains the changes in biology, and this postulate is currently under evaluation.

Mezerein and mezerein-like compounds also showed preference for PKC-α, -β_1, and -γ. The most dramatic example of isozyme selectivity was found for thymeleatoxin, which had almost 20-fold less affinity for PKC-ε and -η than for PKC-β_1. Changes in structure of the lipophilic chain of the C12 ester in these derivatives, therefore, select for isozyme recognition, and synthesis of new derivatives with structural modifications at this position may yield compounds with even greater selectivity for PKC isozymes. In a previous report, thymeleatoxin was also described as a poor activator of kinase activity of PKC-δ and -ε (38); therefore, it could represent a useful tool to explore biological roles of selective PKC isozymes in cellular systems. Unlike the aforementioned classes of compounds, the indole alkaloids were not very efficient at distinguishing between the two groups of isozymes, suggesting that different structural classes of PKC activators interact in different fashions with

[3] M. G. Kazanietz, X. R. Bustels, M. Barbacid, W. Kulch, H. Mischak, G. Wong, J. D. Bruns, and P. M. Blumberg. Zinc finger domains and the phorbol ester pharmacophore: analysis of binding to a mutated form of Pkc *zeta*, and the vav and c-raf oncogene products. Manuscript submitted for publication.

the binding sites of the PKC isozymes. In addition, it is quite interesting that 1,2-diacylglycerol showed very similar K_i values for all the PKC isozymes. Many biological differences between phorbol esters and 1,2-diacylglycerols have been described (39, 40) and may reflect either selectivity in isozyme activation by phorbol esters or, as we described recently, inefficient insertion of PKC into membranes by the diacylglycerols (14). Interestingly, a third pattern of isozyme selectivity was found for bryostatins, macrocyclic lactones that produce only a subset of the responses typical of the phorbol esters and that block those responses to phorbol esters that they themselves fail to induce. Bryostatin 1 is less potent in binding to PKC-β and -γ than to the other isozymes, although the assays could be carried out only under reconstitution conditions different from those used here.[4]

Several models for the interaction between PKCs and their ligands have been postulated, including analysis of the pharmacophore groups for phorbol esters (41, 42), indole alkaloids (42), bryostatins (43), and diacylglycerols (43). In light of the present findings we believe that the molecular modeling studies should be re-evaluated for single purified isozymes, because we have demonstrated that their pharmacophores differ. An attractive model is that the rotatable bonds in *sn*-1,2-diacylglycerols allow a correspondingly good interaction between the active residues of the pharmacophore and the protein binding site for each of the PKC isozymes. Phorbol esters and mezerein-like compounds represent "conformationally constrained" analogs of *sn*-1,2-diacylglycerol in which a complete interaction with the active site for some of the PKC isozymes is compromised, resulting in a reduced affinity for those isoforms.

Comparison of peptides and proteins as substrates of PKCs also revealed striking differences among the PKC isozymes. Although a unique substrate for a single isoform could not be found, the relative efficacies for different PKC isozymes differed significantly from one another. It is thus likely that at the intracellular level each isozyme might have a unique phosphorylation pattern. Recently, McGlynn *et al.* (18) showed that cell extracts of Sf9 insect cells that overexpressed PKC-δ or -ζ exhibited qualitatively and quantitatively different endogenous phosphorylation. The particular substrate specificity in the cell could yield activation of selective intracellular pathways and different functional responses mediated by each isozyme. For example, we recently found selective roles for PKC isozymes in phospholipase C inhibition (44) and antigen-mediated exocytosis (45) in RBL-2H3 cells, as well as in myeloid differentiation (46).

In summary, our findings show that the PKC isozymes differ in their patterns of ligand and substrate preference. We believe that the differences found can contribute to an understanding of the mechanistic basis for the functional heterogeneity within the PKC family of closely related protein kinases.

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