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Characterization of Phorbol Ester Binding to Protein Kinase C Isotypes

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SUMMARY

A mixed micellar assay was used to study the *in vitro* binding of [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) to pure recombinant protein kinase C (PKC)-α, -β_1, -β_2, -γ, -δ, -ϵ, and -ζ isotypes expressed in the baculovirus/insect cell system. Scatchard analysis revealed that all isotypes except PKC-ζ were able to specifically bind PDBu, with K_d values ranging from 1.6 to 18 nM in the presence of calcium. In the absence of calcium PKC-α, -β_1, -β_2, and -δ were observed to have a 2–3-fold drop in affinity, although B_{max} values remained unchanged, at a stoichiometry of 1.4–2.8 mol of PDBu/mol of enzyme. Competition with specific [^{3}H]PDBu binding was assessed for the phorbol esters PDBu, 12-tetradecanoylphorbol-13-*O*-acetate, 12-deoxyphorbol-13-*O*-phenylacetate, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate, thymeleatoxin, resiniferatoxin, and sapintoxin A. Resiniferatoxin and 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate were found to compete effectively only with PDBu bound to the PKC-β_1 and -β_2 isotypes and were the least potent of the phorbol esters tested (IC_{50}, >5 μM). The phorbol esters sapintoxin A, 12-deoxyphorbol-13-*O*-phenylacetate, 12-tetradecanoylphorbol-13-*O*-acetate, and PDBu (in order of potency) competed for binding to all isotypes (IC_{50} values ranging from 2 to 70 nM), with unchanged or slightly decreased potency when calcium was replaced by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid. Thymeleatoxin, which was similar in other respects to these potent phorbol esters, was found to be less able to compete with binding to PKC-α and -ϵ isotypes (IC_{50}, 3–5 μM). It appears that, whereas the binding of phorbol esters to PKC depends primarily on the C20 substituent, other areas of the molecule have an influence on this interaction and the PKC isotypes themselves display heterogeneity in their phorbol ester-binding characteristics.

The tumor promoter TPA is one member of a growing class of diterpenoid esters (collectively termed the phorbol esters) that have been isolated from the *Euphorbiaceae* and *Thymelaeaceae* plant families and have been found to elicit a variety of biological and biochemical responses when applied to a wide range of cell systems (1–4). The biochemical mechanism of action of the phorbol esters is thought to be based on interaction with a cellular receptor protein, PKC (5–7).

Mammalian PKC has been found to comprise an expanding family of related, phospholipid-dependent, serine/threonine-specific protein kinases, which have been grouped into three classes (8–10). Both the cPKC isotypes (α, β_1, β_2, and γ isotypes, showing calcium dependence) and the nPKC isotypes (δ, ϵ, η, μ, and θ isotypes, showing no calcium dependence) are responsive to phorbol esters, whereas the atypical PKC isotypes (λ, ζ, and ι isotypes) are unresponsive to phorbol esters. All PKC polypeptides consist of two functional domains, i.e., a carboxyl-terminal catalytic domain involved in substrate phosphorylation and an amino-terminal regulatory domain involved in the binding of regulatory cofactors and activators. Within these two functional domains, four conserved regions (C1–C4) and five variable regions (V1–V5) are recognizable (11). PKC plays a central role in cellular transduction of extracellular signals to the interior of the cell and consequently can be pivotal in the regulation of cellular processes such as proliferation and differentiation (12). These isotypes provide the potential for divergence in signal transduction, in terms of both cell/tissue-specific distribution and enzymatic properties (13, 14). It is thought that phorbol esters exert their biological effects through interaction within the regulatory domain at the conserved C1 subdomain, which contains cysteine-rich repeated sequences (15, 16).

Recent results from our laboratories indicate substantial differences in the interactions between different phorbol es-

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ABBREVIATIONS: TPA, 12-tetradecanoylphorbol-13-*O*-acetate; PKC, protein kinase C; cPKC, classical protein kinase C; nPKC, novel protein kinase C; PDBu, phorbol-12,13-dibutyrate; DOPP, 12-deoxyphorbol-13-*O*-phenylacetate; DOPPA, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate; Thy, thymeleatoxin-A (9,13,14-*ortho*-benzoyl-6,7-epoxyresiniferonol-12-*O*-cinnamate); Rx, resiniferatoxin (9,13,14-*ortho*-phenylacetyl-resiniferonol-20-*O*-homovannillate); Sap A, sapintoxin A (12-*O*-2-methyaminobenzoyl-13-*O*-acetyl-4-deoxyphorbol); PS, phosphatidylserine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

ters and PKC isotypes *in vitro* (17, 18). In addition, it is known that the abilities of phorbol esters to induce different biological effects are strongly related to their structures (19, 20). Selective use of phorbol esters could therefore be useful for identifying the pathways in which PKC isotypes are involved (21, 22). In the present study we have examined the binding of [^{3}H]PDBu to pure recombinant PKC isotypes α, β_1, β_2, γ, δ, ϵ, and ζ *in vitro* and the inhibition of specific [^{3}H]PDBu binding to these isotypes by seven phorbol esters, of varied biological potential.

Experimental Procedures

Chemicals. [^{3}H]PDBu (20.7 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). PDBu and TPA were from Sigma (Poole, UK). The tigliane esters DOPP, DOPPA, and Sap A and the daphnane esters Thy and Rx were isolated in this laboratory, as described previously (23–25). Stock solutions of pure phorbol esters (1 mg/ml solutions in high performance liquid chromatography-grade acetone) were stored at −20°. PKC isotypes were isolated from Sf9 cells transfected with baculovirus vectors, as described below. Enhanced chemiluminescence detection agents were from Amersham (Amersham, UK). PS was obtained from Lipid Products (Nutfield Nurseries, Nutfield, UK). Cell culture plasticware and media were from Gibco-BRL (Paisley, UK). Hi-Trap heparin and Mono Q columns were from Pharmacia (Uppsala, Sweden), and DE-52 cellulose was from Whatman (Maidstone, UK). All other reagents were of analytical grade.

Purification of PKC isotypes. PKC isotypes α, β_1, β_2, γ, δ, ϵ, and ζ were expressed in Sf9 insect cells as described previously (26). For purification of individual isotypes, a cell pellet (~10^8 cells) was resuspended in ice-cold homogenization buffer (20 mM Tris·HCl, pH 8, 5 mM EGTA, 10 mM benzamidine, 0.3%, v/v, β-mercaptoethanol, 1%, v/v, Triton X-100, 50 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin) (Sigma) and disrupted by three freeze/thaw cycles with liquid nitrogen, and all further procedures were conducted at 4°. The homogenate was centrifuged at 40,000 × *g* for 15 min, and the supernatant was diluted 3-fold with homogenization buffer before being loaded onto a DE-52 column that had been previously equilibrated in homogenization buffer and linked to a fast protein liquid chromatography system (Pharmacia). Active fractions (see PKC assay) were eluted from this column with a linear gradient of 0–1 M NaCl in elution buffer (20 mM Tris·HCl, pH 7.5, 5 mM EDTA, 10 mM benzamidine, 0.3%, v/v, β-mercaptoethanol, 0.02%, v/v, Triton X-100). Pooled activity was applied successively to a Hi-Trap heparin column and then a Mono Q column, using the same NaCl gradient in elution buffer. Purified PKC isotypes were stored in storage buffer (20 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.02%, v/v, Triton X-100, 5 mM dithiothreitol) at −70°.

PKC assay. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into substrate, as described previously (27). The substrates used were salmon protamine sulfate, calf thymus histone III-S (Sigma), and PKC-δ, -ϵ, and -ζ pseudosubstrate site-based peptides, as described by Olivier and Parker (28) (synthesized by the Peptide Synthesis Laboratory, Imperial Cancer Research Fund). PKC isotypes were diluted in storage buffer before use. For binding/competition studies, 1–10 units of enzyme activity/assay were used (1 unit incorporated 1 pmol of ^{32}P into protamine sulfate per minute under kinase assay conditions in the absence of calcium, lipids, and phorbol esters), as specified in the figure legends.

Binding of [^{3}H]PDBu to recombinant PKC isotypes. [^{3}H]PDBu binding to PKC isotypes was measured using a combination of the mixed micellar protocol (28) and DE-52 binding protocol (7). The binding assay mixture (50 μl) contained 20 mM Tris·HCl, pH 7.5, 1 mg/ml bovine serum albumin, 0.5 mM $CaCl_2$ (or 1 mM EGTA), mixed Triton X-100/PS micelles (final concentrations of 2.3 mM and 1 mM, respectively, for 70/30 mol % mixed micelles), [^{3}H]PDBu (with or without nonradioactive PDBu), and enzyme. PS and PDBu (radioactive and nonradioactive) were incorporated into Triton X-100 micelles by first drying stock solutions under a stream of nitrogen gas and then adding Triton micelles (5.75 mM in 20 mM Tris·HCl, pH 7.5), with vortex-mixing (2 min) and bath sonication (2 min). Micelle solutions were clear. Incubation of PKC isotypes was carried out at room temperature for 20 min and terminated by addition of an ice-cold suspension of DE-52 anion exchange cellulose (20%, w/v, in 20 mM Tris·HCl, pH 7.5). The mixture was allowed to stand for 20 min at 4°, to allow the PKC/[^{3}H]PDBu to bind to the DE-52 resin. The suspension was then rapidly filtered through Whatman G/C glass fiber filters and washed (4 × 5 ml of 20 mM Tris·HCl, pH 7.5 at 4°). Filters were mixed with Ecoscint (National Diagnostics, Aylesbury, Bucks, UK) and counted for radioactivity by scintillation counting in a Beckman LS 6000IC counter. Each binding point was analyzed in triplicate assays in two groups to determine total binding and nonspecific binding (determined using a 1000-fold excess of nonradioactive PDBu). Specific binding was calculated as the difference between total and nonspecific binding. To determine the dissociation constants (K_d) and the maximum number of binding sites (B_{max}) for different PKC isotypes, Scatchard analysis was conducted for the specific binding curves at [^{3}H]PDBu concentrations varying between 4 and 200 nM. Lines of best fit for Scatchard plots were calculated using least-squares linear regression analysis.

Competition with binding of [^{3}H]PDBu to recombinant PKC isotypes. Competition with binding of [^{3}H]PDBu to recombinant PKC isotypes was performed under the same conditions as described above, using a fixed concentration of [^{3}H]PDBu (30 nM) and varying concentrations of the nonradioactive phorbol ester competitor. The phorbol esters used in competition were PDBu, TPA, Sap A, DOPP, and DOPPA from the tigliane series and Thy and Rx from the daphnane series (4). Varying concentrations of phorbol ester competitors were incorporated into the micelles with [^{3}H]PDBu (30 nM). The amount of specific [^{3}H]PDBu bound in the absence of competitor was taken as 100% binding, and the inhibition of this binding was calculated from this value for each isotype. Competition experiments were performed in triplicate for each point, and each experiment was conducted three times.

Results

Binding of [^{3}H]PDBu to recombinant PKC isotypes. The binding of [^{3}H]PDBu to purified recombinant PKC isotypes α, β_1, β_2, γ, δ, ϵ, and ζ was performed throughout with a modified mixed micellar system originally described by Hannun *et al.* (29). We decided to use this system because it appears to be a physically defined system consisting of approximately 140 molecules of Triton X-100/micelle, whereas sonic dispersion of phospholipids alone results in a heterogeneous mixture of unilammelar and multilamellar vesicles. This system was also used in our previous work on PKC isotype activation by phorbol esters (17). The retrieval of PKC from the binding assay was adapted from the method of Parker *et al.* (7), using DE-52 resin, and we found that all PKC activity was completely removed from solution under the conditions used. The binding of [^{3}H]PDBu to PKC isotypes was complete within 10 min at room temperature and was stable for at least 30 min. In the absence of PS, we found no binding of [^{3}H]PDBu to PKC in the presence of Triton X-100 micelles alone, irrespective of the calcium/EGTA concentration used. The total binding and nonspecific binding of [^{3}H]PDBu to PKC-α, in the presence or absence of calcium, are shown in Fig. 1A, and the specific binding of [^{3}H]PDBu to PKC-α is shown in Fig. 1B. [^{3}H]PDBu binding to PKC-α and -ϵ did not appear to be saturable at the doses of [^{3}H]PDBu

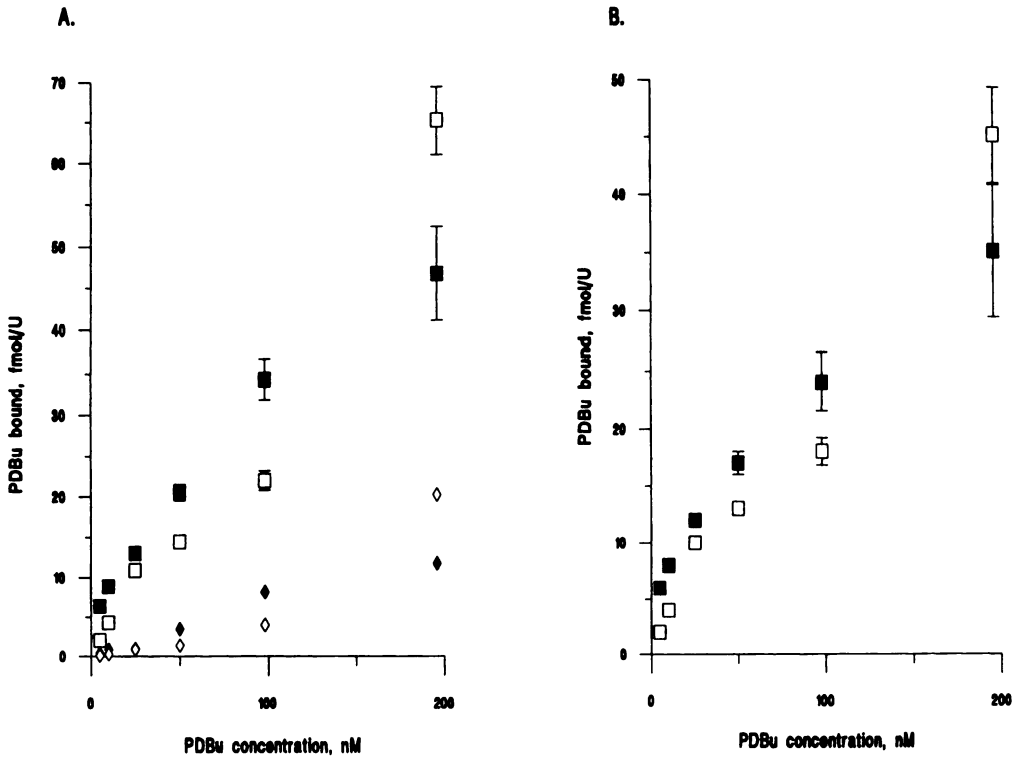

Fig. 1. Binding of [^{3}H]PDBu to PKC-α. A, Binding of increasing doses of [^{3}H]PDBu in the presence (*closed symbols*) and absence (*open symbols*) of calcium is shown. Total binding (*squares*) and nonspecific binding (determined with a 1000-fold excess of unlabeled PDBu) (*diamonds*) are shown for [^{3}H]PDBu doses up to 200 nM. B, From the values shown in A, the specific binding curves for [^{3}H]PDBu binding to PKC-α, in the presence (■) and absence (□) of calcium, were determined. The mean values and standard error ranges are shown for triplicate determinations of a representative experiment.

used here, reflecting what may be a second binding site. However, the limits of determination of the nonspecific binding at higher [^{3}H]PDBu concentrations precluded a quantitative analysis of any such low affinity binding site. PKC-ζ failed to show any binding activity above nonspecific binding even with 1000 nM [^{3}H]PDBu (data not shown). Scatchard analysis was conducted for the specific binding of [^{3}H]PDBu to PKC-α, -β_1, -β_2, -γ, -δ, and -ϵ (Fig. 2). These analyses showed that the K_d values for PDBu at equilibrium ranged from 1.6 to 18.5 nM in the presence of calcium and from 3.9 to 60 nM in the absence of calcium (Table 1), although PKC-α and -ϵ appeared to possess a possible second binding site. The specific PDBu binding to all PKC isotypes, as a percentage of total binding, was between 86 and 95% when determined for [^{3}H]PDBu concentrations at the K_d values (Table 1). The B_{max} values, based on kinase activity with protamine sulfate (requiring no lipid or calcium cofactors), were similar for all PKC isotypes (12–24 fmol of PDBu bound/unit of enzyme activity). When units of activity were calculated in terms of moles of enzyme present, the stoichiometry of the binding at the B_{max} values ranged from 1.4 to 2.8 molecules of PDBu/molecule of enzyme present.

Competition with binding of [^{3}H]PDBu to recombinant PKC isotypes. To compare the enzyme-ligand interactions for different PKC isotypes, we quantified the competition with specific [^{3}H]PDBu binding for a variety of naturally occurring tigliane and daphnane esters known to possess differing biological activities (1–4). The phorbol esters (Fig. 3) TPA, PDBu, Sap A, DOPP, DOPPA, Thy, and Rx were used at final concentrations of 1 nM to 50 μM (0.00003–1.5 mol % of micelle). A typical experiment measuring the competition with specific [^{3}H]PDBu binding to PKC-α, in the presence and absence of calcium, by these phorbol esters (1 nM to 10 μM) is shown in Fig. 4. Here TPA, PDBu, DOPP, and Sap A were potent competitors of [^{3}H]PDBu binding (~3–30 nM) (largely unaffected by the presence of calcium), whereas Thy, Rx, and DOPPA were only weakly able to compete at the doses tested. The concentrations at which 50% of specific [^{3}H]PDBu binding was abolished (IC_{50} values) by these phorbol esters for PKC-α and the other PKC isotypes are shown in Table 2. A comparison of the two 12-deoxyphorbol esters used in this study (DOPP and DOPPA) in competition with [^{3}H]PDBu binding to PKC-β_1, -β_2, -γ, -δ, and -ϵ is shown in Fig. 5. DOPP was able to fully compete for binding to all of these isotypes (IC_{50} range, 10–50 nM), whereas DOPPA was fully effective only for the PKC-β_1 and -β_2 isotypes and was much less potent than DOPP (IC_{50}, ~5–15 μM).

Discussion

The spectrum of biological effects elicited in mammalian cells by different phorbol esters is thought to be due mainly to their differential interaction with, and modification of, the cellular complement of PKC isotypes engaged in signal transduction. Initial studies using a range of phorbol esters to stimulate the *in vitro* kinase activity of PKC isotypes purified from bovine brain suggested that the ability to stimulate activity was related to the structure of substituent groups at the C20 and C13/14 areas of the phorbol ester nucleus (17). In the present study we have measured the binding of [^{3}H]PDBu to recombinant PKC-α, -β_1, -β_2, -γ, -δ, -ϵ, and -ζ isotypes, purified after baculoviral expression in the insect Sf9 cell

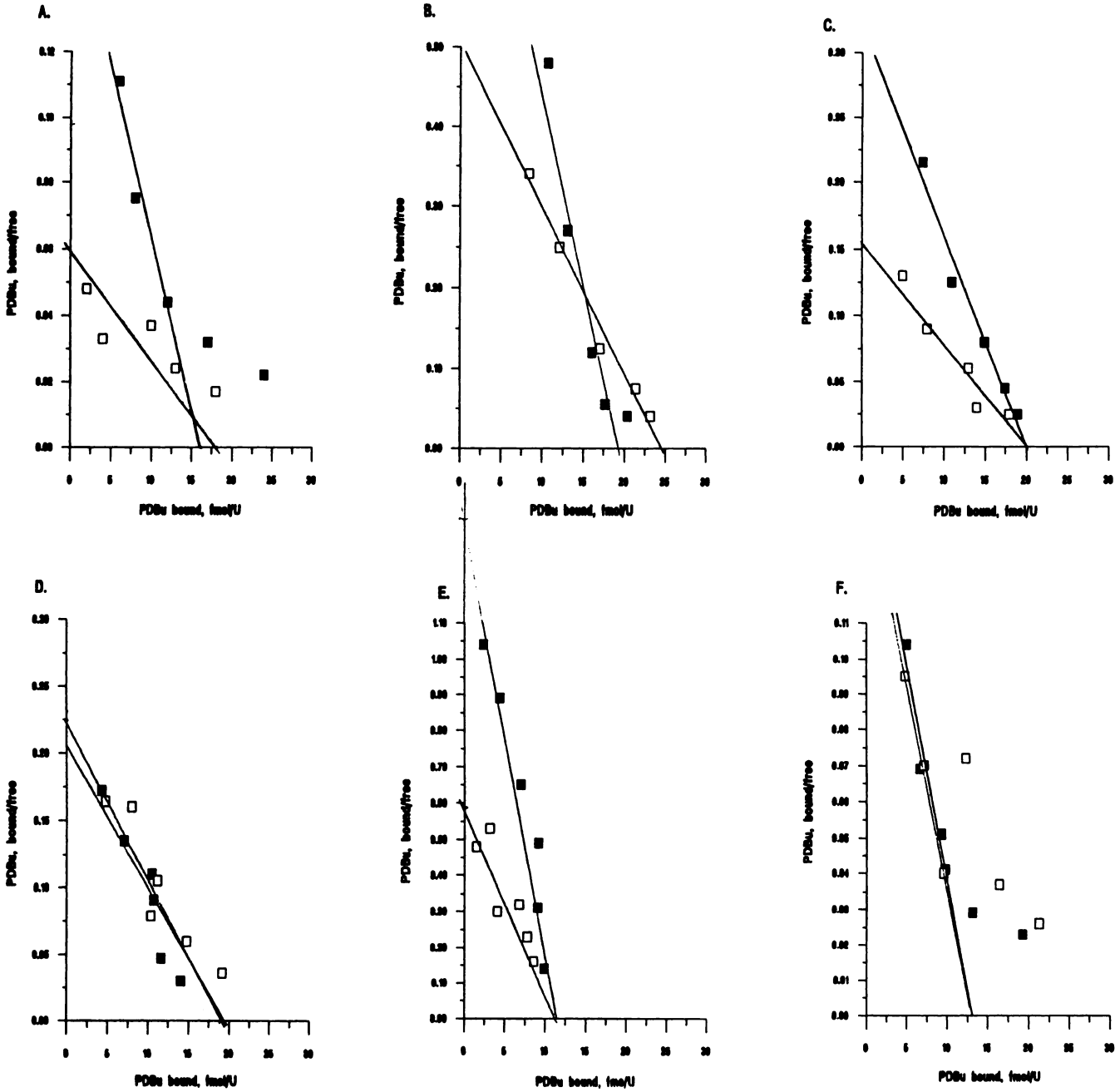

Fig. 2. Scatchard analysis of the specific binding of [^{3}H]PDBu to PKC isotypes. Scatchard plots (bound/free versus PDBu bound per PKC enzyme unit present in the assay) of mean values from three separate experiments, calculated from the specific binding curves in the presence (■) and absence (□) of calcium, are shown. A, PKC-α; B, PKC-β_1; C, PKC-β_2; D, PKC-γ; E, PKC-δ; F, PKC-ϵ. The K_d and B_{max} from these graphs are shown in Table 1.

system, and the inhibition of specific [^{3}H]PDBu binding to these isotypes by seven phorbol esters.

The conditions for [^{3}H]PDBu binding used an artificial Triton/PS micelle suspension, developed by Hannun and coworkers (27, 29), to simulate the requirements for phorbol ester binding previously used in the isotype kinase stimulation assays (17). Using this protocol, binding of [^{3}H]PDBu rapidly reached equilibrium and remained stable for at least 30 min, enabling Scatchard analysis to be performed. The DE-52 recovery step efficiently bound all PKC activity and limited nonspecific [^{3}H]PDBu binding (at a PDBu concentration equal to the K_d value) to <15% of total binding for PKC-α, -β_1, -β_2, -γ, -δ, and -ϵ. PKC-ζ did not show any [^{3}H]PDBu binding capacity above background, even when PDBu was used at 1000 nM, suggesting that even low affinity PDBu sites are not available with this isotype, in agreement with previous studies (30). Scatchard analysis of receptor-ligand interactions allows K_d and B_{max} values to be calculated for equilibrium conditions where ligand binding is not saturated. The concentration of each PKC isotype was not determined here but was instead expressed in terms of kinase activity when protamine sulfate was used as a substrate (requiring no lipid, calcium, or phorbol esters for maximum activity) (31)). Using this approach, we found that the B_{max}

TABLE 1

K_d and B_{max} values from Scatchard analyses of the specific binding of [^{3}H]PDBu to PKC isotypes, and percentages of specifically bound [^{3}H]PDBu at K_d

The values for PKC-α and -ϵ suggest that there may be two binding sites, of which only that with the higher affinity is shown. Each value represents the mean ± standard error of three separate experiments.

PKC isotype	+Calcium			−Calcium		
	K_d	B_{max}	Specific binding[a]	K_d	B_{max}	Specific binding[a]
	nM	fmol/unit	%	nM	fmol/unit	%
α	18 ± 2.1	16 ± 0.9	88 ± 6.4	60 ± 8.1	18 ± 0.7	90 ± 7.1
β_1	1.6 ± 0.4	19 ± 1.3	95 ± 7.3	3.9 ± 0.8	24 ± 4.2	89 ± 4.3
β_2	4.5 ± 0.6	20 ± 1.4	86 ± 4.2	9.5 ± 1.2	20 ± 3.1	85 ± 5.1
γ	16 ± 0.9	18 ± 0.7	89 ± 5.3	18 ± 3.1	18 ± 2.0	86 ± 2.1
δ	1.7 ± 0.2	12 ± 0.6	94 ± 7.8	4 ± 0.9	12 ± 0.9	92 ± 6.6
ϵ	17 ± 2.2	13 ± 2.1	89 ± 6.6	18 ± 1.1	13 ± 1.8	91 ± 8.1
ζ	No specific [^{3}H]PDBu binding					

[a] Specific binding as percentage of total binding at K_d.

values determined by Scatchard analyses for the PKC isotypes ranged from 12 to 24 fmol of PDBu/unit of activity and were unaffected by the presence of 0.5 mM calcium or 1 mM EGTA. PDBu binding to PKC-α and -ϵ was found to have incomplete saturation, and the resulting Scatchard plots suggested the presence of a second binding site with low affinity for PDBu. However, the technical limitations of determining nonspecific binding at higher [^{3}H]PDBu concentrations, under the conditions used here, precluded a quantitative analysis of any such low affinity binding site. When units of activity were calculated in terms of moles of enzyme present, the stoichiometry of the binding at the B_{max} values ranged from 1.4 to 2.8 molecules of PDBu/molecule of enzyme present.

The K_d values for specific PDBu binding, determined for each PKC isotype, ranged from 1.6 to 60 nM. The K_d values for the cPKC isotypes α, β_1, and β_2 were approximately 2–3-fold higher in the absence of calcium than in its presence, indicating that calcium can increase one or more parameters of the binding kinetics. PKC-γ showed no decrease in K_d with added calcium, although other researchers have found evidence for a calcium effect (32). The K_d observed for the nPKC isotype PKC-ϵ was also unaffected by the presence of calcium, in agreement with earlier [^{3}H]PDBu binding observations (33); however, calcium did appear to lower the K_d of PKC-δ in a manner similar to that seen with the cPKC forms α, β_1, and β_2. This was unexpected, in that the nPKC isotypes lack the calcium-binding ability associated with the C2 region and do not show calcium sensitivity in their kinase activity (9–12, 17).

The composition of the artificial membranes has been found to exert a great influence on the kinetics of [^{3}H]PDBu binding to PKC, and several laboratories using 100% PS micelles have found evidence of tighter binding in such an environment (34, 35). With these membranes the K_d values for cPKC isotypes were <1 nM and appeared to be unaffected by the presence of calcium. Work with artificial membranes composed of PS and phosphatidylcholine (at 20/80 mol %) found two types of PKC association with the membranes (34, 36). A strongly membrane-associated PKC was found to have kinase activity independent of calcium, diacylglycerol, and phorbol ester cofactors but was able to tightly bind PDBu, with a K_d of <0.5 nM, and this PKC was termed membrane inserted. The weaker and reversible binding of PKC to such membranes exhibited cofactor dependence for both phorbol ester binding and stimulation of kinase activity and could be converted to the membrane-inserted form by high concentrations of phorbol ester (37). With Triton/PS micelles, cofactor-sensitive, reversible association of PKC similar to that seen in PS/phosphatidylcholine vesicles and inverted erythrocyte vesicles (38, 39) is readily observed, whereas the membrane-inserted form has not been observed, even in the presence of PDBu (35–37). By using a Triton/PS mixture, we observe K_d values that are well above those seen in systems where membrane insertion occurs, and our PDBu binding appears to be cofactor sensitive for several of the PKC isotypes, suggesting that we are measuring the weaker reversible binding in our system.

We investigated the ability of seven different phorbol esters to compete with a fixed amount of [^{3}H]PDBu for specific binding to each PKC isotype. Competition studies with either cPKC or nPKC isotypes in the presence of calcium showed [^{3}H]PDBu titration curves similar to those seen in the absence of calcium. The functional group at the C20 carbon of these phorbol esters was found to be the most critical factor in the ability to compete with PDBu binding for all isotypes tested, with a free alcohol group conferring potent competition (seen with TPA, DOPP, and Sap A) and an acetyl group (e.g., DOPPA) or a bulky ester linkage (e.g., Rx) significantly decreasing or abolishing competing ability. The PKC-β isotypes (β_1 and β_2) were able to show DOPPA and Rx competition, however, suggesting that these isotypes have less definite requirements, compared with the other isotypes, for a free C20 for competition, and hence their kinase activity may be easier to stimulate with these less biologically potent phorbol esters. In the C12/C13 region, one aliphatic or aromatic ester group was adequate for potent competition with PDBu, which possesses butyrate groups at both of these positions. The presence of an hydroxyl group at the C4-position did not appear to affect the ability to compete with PDBu binding to the PKC isotypes.

Recently, Kazanietz *et al.* (35, 40) showed that DOPP had potent ability to compete with PDBu bound to all isotypes, in agreement with this report, but Rx had no competing ability. Those authors found that PDBu binding to the PKC isotypes was greater with 100% PS vesicles than with 20% PS vesicles, however, and we suggest that the difference between those reports and the present results may reflect the tighter membrane-inserted binding of PDBu in the former system.

Although findings for competition with specific PDBu bind-

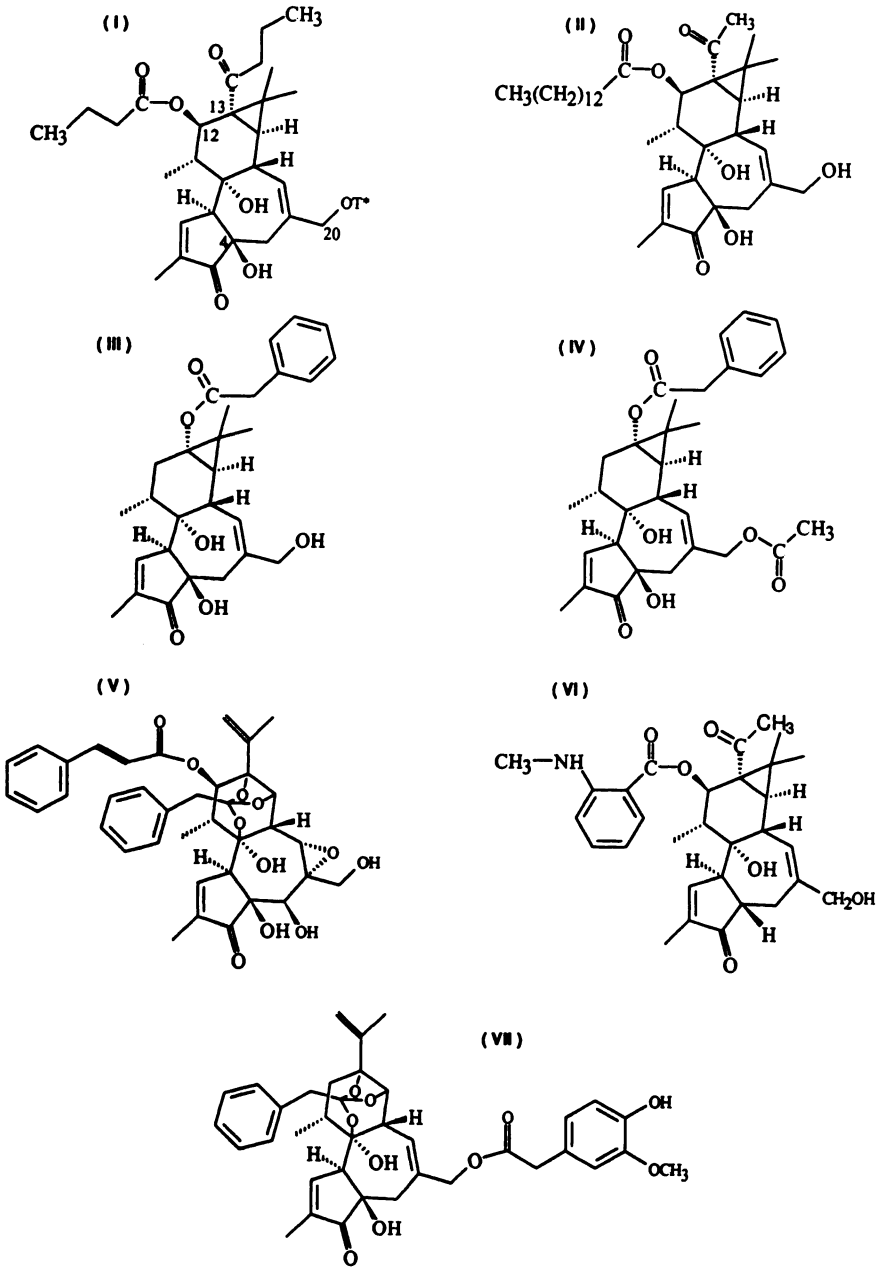

Fig. 3. Structures of the phorbol esters used in this study. **I**, PDBu; **II**, TPA; **III**, DOPP; **IV**, DOPPA; **V**, Thy; **VI**, Sap A; **VII**, Rx. The C20, C12, C13, and C4 carbon atoms and the location of the tritiated label (*T**) on PDBu are shown.

ing to the PKC isotypes are not directly comparable to results from earlier activation studies in the same system, the potencies of TPA, DOPP, DOPPA, and Rx to compete for binding were similar to their activation potencies in the presence of calcium (i.e., TPA and DOPP showed similar potent activation of all isotypes, but both DOPPA and Rx were able to activate only β_1, with β_2 being untested) (17). The tigliane Sap A and the daphnane Thy did not conform to their kinase activation profiles, however, in that Sap A was able to potently compete with PDBu bound to PKC-δ and Thy was a poor competitor with PDBu bound to PKC-α. Further investigation of the kinase activities of these isotypes was conducted with purified recombinant proteins from Sf9 cells. The PKC-δ preparation was subsequently found to be able to phosphorylate histone III-S, in a calcium-independent manner, and to be stimulated by Sap A (data not shown), indicating that it appeared to be different from the COS cell-expressed preparation used in previous work (28). We found that with the preparations of PKC-α from recombinant Sf9 cells a rapid loss of phorbol ester-stimulated kinase activity occurred, although no evidence of proteolysis to protein kinase M or loss of PDBu binding ability was seen. The discrepancy in PKC-δ and -α behavior may lie in differences in post-translational processing of these isotypes between the two systems from which these isotypes were purified; this may also explain the shift in K_d for PDBu binding to PKC-δ that is apparent with calcium. It has recently been discovered, using fusion proteins with the C1/C2 domains of cPKCs and nPKCs, that the C1 domain (rather than the C2 region) of PKC-ε can bind calcium in place of magnesium as a divalent cation, whereas the C2 region imparts calcium selectivity (41). The divalent metal ion dependence of the C1 region may offer an explanation for the change in K_d for PKC-δ in the presence of calcium.

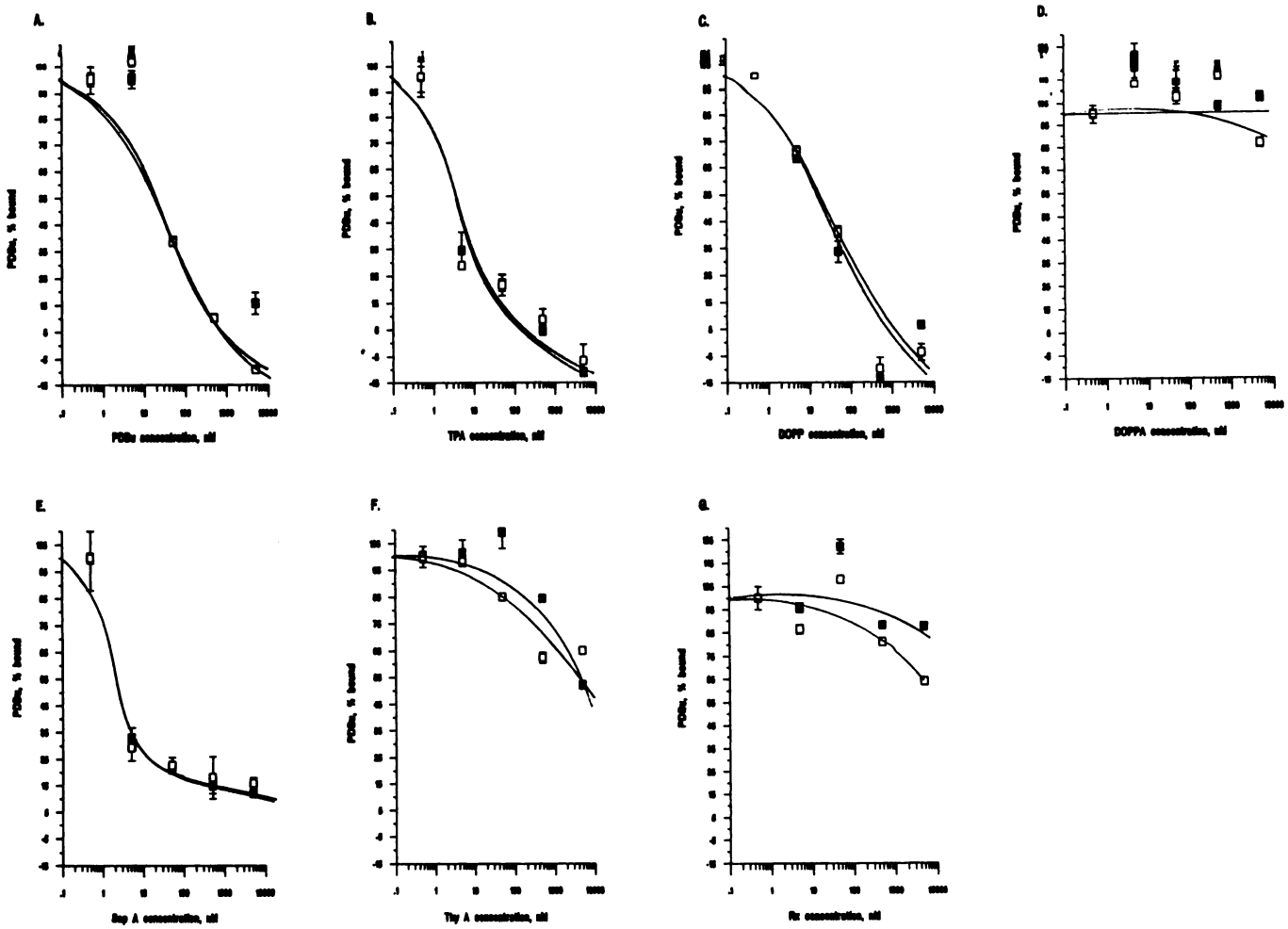

Fig. 4. Competition with specific binding of [^{3}H]PDBu to PKC-α by phorbol esters. [^{3}H]PDBu (present at 30 nM throughout) was competed with by increasing concentrations of the phorbol esters PDBu (A), TPA (B), DOPP (C), DOPPA (D), Thy (E), Sap A (F), and Rx (G), in the presence (■) and absence (□) of calcium. The specifically bound PDBu was termed 100%, and the competition is depicted as a reduction from this value. The mean values and standard error ranges are shown for triplicate determinations of a representative experiment.

TABLE 2

IC_{50} values for phorbol esters competing for specific [^{3}H]PDBu binding sites (30 nM) on PKC isotypes

Each value represents the mean ± standard error of three separate experiments.

PKC isotype	Ca^{2+}	IC_{50}						
		PDBu	TPA	DOPP	DOPPA	Sap A	Thy	Rx
α	+	25 ± 3.1 nM	4 ± 0.6 nM	25 ± 3.1 nM	>10 μM	2.5 ± 0.4 nM	5 ± 0.4 μM	>10 μM
	−	25 ± 2.0 nM	4 ± 0.7 nM	25 ± 2.4 nM	>10 μM	2.5 ± 0.7 nM	5 ± 0.4 μM	>10 μM
β_1	+	75 ± 4.2 nM	70 ± 7.1 nM	10 ± 1.1 nM	9 ± 1.1 μM	3 ± 0.1 nM	20 ± 4.1 nM	9 ± 2.1 μM
	−	75 ± 8.4 nM	70 ± 4.2 nM	10 ± 1.0 nM	6 ± 0.8 μM	3 ± 0.6 nM	20 ± 3.8 nM	11 ± 3.4 μM
β_2	+	7 ± 1.0 nM	8 ± 1.1 nM	20 ± 1.0 nM	5 ± 1.0 μM	2.5 ± 0.5 nM	50 ± 7.2 nM	45 ± 3.2 μM
	−	16 ± 2.4 nM	17 ± 2.3 nM	20 ± 0.8 nM	15 ± 2.3 μM	2.5 ± 0.1 nM	50 ± 8.1 nM	45 ± 4.1 μM
γ	+	100 ± 15 nM	50 ± 1.2 nM	50 ± 4.2 nM	>50 μM	2 ± 0.4 nM	300 ± 45 nM	>50 μM
	−	400 ± 24 nM	250 ± 43 nM	50 ± 7.1 nM	>50 μM	2 ± 0.4 nM	500 ± 61 nM	>50 μM
δ	+	25 ± 1.1 nM	30 ± 5.1 nM	25 ± 0.9 nM	>50 μM	35 ± 5.1 nM	95 ± 11 nM	>50 μM
	−	25 ± 2.4 nM	30 ± 2.3 nM	25 ± 3.2 nM	>50 μM	35 ± 4.3 nM	95 ± 15 nM	>50 μM
ϵ	+	60 ± 8.1 nM	25 ± 3.1 nM	15 ± 2.1 nM	>50 μM	2 ± 0.9 nM	3 ± 0.7 μM	>50 μM
	−	60 ± 4.2 nM	40 ± 5.1 nM	15 ± 3.4 nM	>50 μM	2 ± 0.3 nM	3 ± 0.6 μM	>50 μM
ζ	+/−	No specific [^{3}H]PDBu binding						

The physiological significance of different states of PKC association with membranes is not known. Although transient translocation of cytosolic PKC can be observed with a variety of agonists, this does not exclude the possibility of a membrane-inserted PKC playing a role in the PKC signaling system. Binding studies with a fluorescent phorbol ester added to cells *in vivo* or to 100% PS micelles *in vitro* have shown both high and low affinity sites (possibly corresponding to both the membrane-inserted and reversible PKC states) (42). Phorbol esters may change the amount of membrane-inserted PKC *in vivo*, and there remains the possibility that some biological effects of potent phorbol esters may

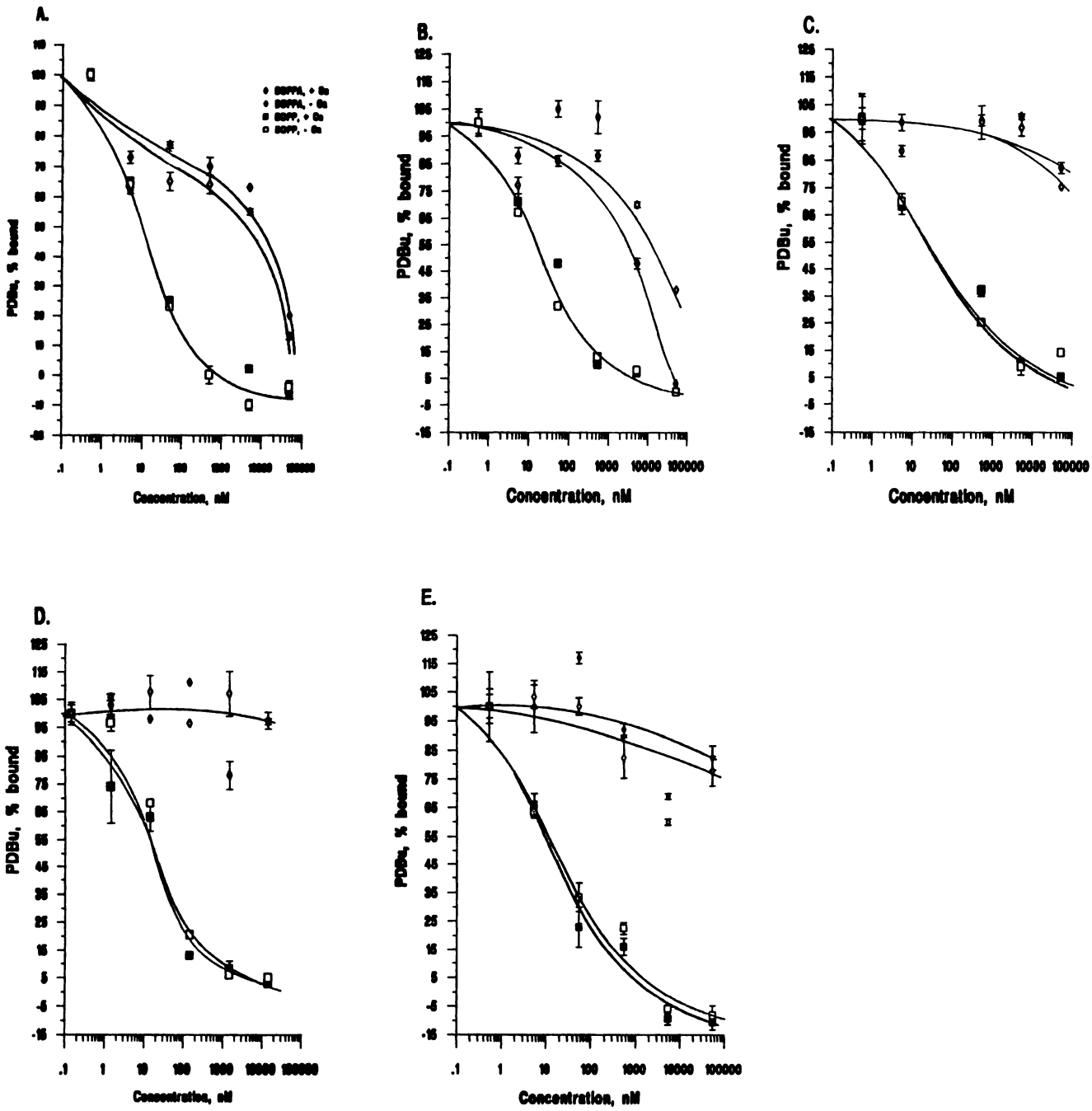

Fig. 5. Competition with specific binding of [^{3}H]PDBu to PKC isotypes by the 12-deoxyphorbol esters DOPP and DOPPA. Increasing concentrations of DOPP (*squares*) or DOPPA (*diamonds*) were added to [^{3}H]PDBu (30 nM) bound to PKC-β_1 (A), PKC-β_2 (B), PKC-γ (C), PKC-δ (D), or PKC-ϵ (E), in the presence (*closed symbols*) and absence (*open symbols*) of calcium. The specifically bound PDBu was termed 100%, and the competition is depicted as a reduction from this value. The mean values and standard error ranges are shown for triplicate determinations of a representative experiment.

be mediated through their ability to irreversibly insert PKC into cellular membranes. Ultimately, detailed understanding of the interaction of phorbol esters with the regulatory domain of PKC might permit the development of agents that interfere with PKC activity and thereby function as regulators of altered pathways in pathological states.

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