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Ligand Structure-Activity Requirements and Phospholipid Dependence for the Binding of Phorbol Esters to Protein Kinase D

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ABSTRACT

Although protein kinase D (PKD), like protein kinase C (PKC), possesses a C1 domain that binds phorbol esters and diacylglycerol, the structural differences from PKC within this and other domains of PKD imply differential regulation by lipids and ligands. We characterized the phorbol ester and phospholipid binding properties of a glutathione *S*-transferase–tagged full-length PKD and compared them with those of PKC-α and -δ. We found that PKD is a high-affinity phorbol ester receptor for a range of structurally and functionally divergent phorbol esters and analogs and showed both similarities and differences in structure-activity relations compared with the PKCs examined. In particular, PKD had lower affinity than PKC for certain diacylglycerol analogs, which might be caused by a lysine residue at the 22 position of the PKD-C1b domain in place of the tryptophan residue at this position conserved in the PKCs. The membrane-targeting domains in PKD are largely different from those in PKC; among these differences, PKD contains a pleckstrin homology (PH) domain that is absent in PKC. However, phosphatidylinositol-4,5-bisphosphate PIP_2, a lipid ligand for some PH domains, reconstitutes phorbol 12,13-dibutyrate (PDBu) binding to PKD similarly as it does to PKC-α and -δ, implying that the PH domain in PKD may not preferentially interact with PIP_2. Overall, the requirement of anionic phospholipids for the reconstitution of [^{3}H]PDBu binding to PKD was intermediate between those of PKC-α and -δ. We conclude that PKD is a high-affinity phorbol ester receptor; its lipid requirements for ligand binding are approximately comparable with those of PKC but may be differentially regulated in cells through the binding of diacylglycerol to the C1 domain.

Phorbol esters, natural products from plants of the family *Euphorbiaceae*, were first described as potent tumor promoters in mouse skin and were later found to induce divergent cellular responses. Although protein kinase C (PKC) is the primary target of phorbol esters, it is not solely accountable for the heterogeneous responses of phorbol esters in cells and animal models. Additional families of phorbol ester receptors have emerged such as the chimaerins (Areces et al., 1994; Caloca et al., 1997), the Munc-13 proteins (Kazanietz et al., 1995a), a guanyl nucleotide-releasing protein for the small guanosine triphosphatase Ras (Ebinu et al., 1998), diacylglycerol kinase-γ (Shindo et al., 2001), and PKD (Rozengurt et al., 1995; Van Lint et al., 1995).

The PKD family is a newly identified serine/threonine kinase family that belongs to a subfamily of the Ca-Calmodulin kinase superfamily (Manning et al., 2002), including PKD1 (mouse PKD, its human homolog PKC-μ), PKD2, and PKD3 (human PKC-ν) (Valverde et al., 1994; Hayashi et al., 1999; Sturany et al., 2001). Studies on PKD1 point to its role in regulating Golgi function, cell proliferation, and apoptosis (Van Lint et al., 2002). Although similar to PKC at the C1 domain, PKD is markedly divergent from PKC in other structural features: 1) the catalytic domain of PKD/PKC-μ is distantly related to Ca^{2+}-calmodulin–regulated protein kinases and shows little homology to the catalytic domains of the

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ABBREVIATIONS: PKC, protein kinase C; PH, pleckstrin homology; PKD, protein kinase D; [^{3}H]PDBu, [^{3}H]phorbol 12,13-dibutyrate; PIP_2, phosphatidylinositol-4,5-bisphosphate; POPS, *sn*-1-palmitoyl-2-oleoylphosphatidylserine; POPC, *sn*-1-palmitoyl-2-oleoylphosphatidylcholine; LUV, large unilamellar vesicles; OAG, 1-oleoyl 2-acetyl glycerol; GST, glutathione *S*-transferase; 97D76, 3-hydroxy-2-[4-methyl-3-(methylethyl)pentanoyloxy]propyl 4-methyl-3-(methylethyl)pentanoate; B8-DL-B8, {2-(hydroxymethyl)-4-[4-methyl-3-(methylethyl)pentylidene]-5-oxo-2-2,3-dihydrofuryl}methyl 4-methyl-3-(methylethyl)pentanoate; DAG, diacylglycerol; PS, phosphatidylserine; GST-PKD, glutathione *S*-transferase protein kinase D; 97F31, 1-(4-methyl-3-(methylethyl)pentanoyl)-2-(3-methylbut-2-enoyl)-*sn*-glycerol.

PKC family (23.6% homology to that of PKC-δ) (Rozengurt et al., 1995); 2) PKD possesses a putative transmembrane sequence and a pleckstrin homology (PH) domain, both lacking in PKC; and 3) conversely, PKD lacks the pseudosubstrate domain found in the PKCs.

Functionally, the activity of PKD in cells is dependent on PKC (Zugaza et al., 1996), and PKC was found to directly bind, phosphorylate, and activate PKD (Waldron et al., 1999). Moreover, although the C1 domain of PKD possesses the structural motifs of a functional C1 domain and binds diacylglycerol with high affinity, it functions mainly as a membrane-targeting module to shuttle PKD between different subcellular compartments (Matthews et al., 1999, 2000; Maeda et al., 2001). The binding of diacylglycerol was necessary for the recruitment of PKD to the Golgi, and inhibition of this interaction by mutation or by reducing the levels of diacylglycerol blocked the PKD-mediated protein transport from Golgi to the plasma membrane (Baron and Malhotra, 2002). Therefore, understanding the structure-activity requirements of the C1 domain of PKD will provide insights into the regulation of PKD by ligands and the lipid membrane and will contribute significant structural information for the design of selective activators and inhibitors of PKD.

The C1 domain of PKD and PKC comprises a conserved-structure motif: HX12CX2CX13/14CX2CX4HX2CX7C (C, cysteine; H, histidine; X, any other amino acid). Structural analysis of the complex between PKCδ-C1b and phorbol 13-acetate by Hurley and collaborators (Zhang et al., 1995) indicates that the C1 domain functions as a hydrophobic switch, in which the binding of phorbol ester or diacylglycerol completes a hydrophobic surface and contributes further hydrophobicity, triggering the membrane association of the domain. The C1 domains of PKD differ from those of PKC in that there is a much longer space between the two C1 domains in PKD (95 residues versus 14–22 residues for the PKCs) and there are alterations at several generally conserved sites in the C1 domain (Valverde et al., 1994; Iglesias et al., 1998). In particular, a lysine (K) residue at position 22 of PKD-C1b appears in all PKD isoforms, whereas it is a well-conserved tryptophan (W) in PKCs. The mutation of position 22 in PKCδ-C1b from tryptophan to the basic residue lysine or the hydrophilic residue tyrosine (Y) showed greater selectivity between PDBu and the diacylglycerol analog 97F31 (a synthetic unconstrained branched diacylglycerol with hydrophobicity similar to that of PDBu), as described in our previous study (Wang et al., 2001), which implies that K22 in the PKD-C1b domain may confer selectivity to PDBu compared with diacylglycerol.

Membrane anchoring is critical for the activation and regulation of PKC signaling. The domains in PKC that contribute to membrane association include the pseudosubstrate, C1, and C2 domains (Newton and Johnson, 1998). PKD lacks the pseudosubstrate and C2 domains but possesses a PH domain that binds to a range of negatively charged lipids such as phosphatidylinositol-4,5-bisphosphate (PIP_2) and inositol 1,4,5-trisphosphate and plays a role in membrane association and subcellular localization (Harlan et al., 1994; Hyvonen et al., 1995; Touhara, 1997). Therefore, we evaluated the lipid dependence for the binding of PDBu to PKD as a function of both phosphatidylserine and PIP_2.

Materials and Methods

Materials. [^{3}H]PDBu (20 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). [^{3}H]Bryostatin 1 was prepared as described previously (Lewin et al., 1992). L-α-[1-^{14}C]Dipalmitoylphosphatidylcholine was obtained from Amersham Biosciences Inc. (Piscataway, NJ). PDBu, mezerein, thymeleatoxin, prostratin, 12-deoxyphorbol 13-phenylacetate, 1-oleoyl 2-acetyl glycerol, (−)-indolactam V and (−)-octylindolactam V were obtained from LC Laboratories (Woburn, MA). *sn*-1-Palmitoyl-2-oleoylphosphatidylserine (POPS), *sn*-1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and brain PIP_2 were purchased from Avanti Polar Lipids (Birmingham, AL). Phosphatidyl-L-serine was obtained from Sigma Chemical Co. (St. Louis, MO). Unconstrained DAG analog 97D76 and the corresponding constrained analog B8-DL-B8 bearing equivalent 2,3,4-trimethylpentane branched chains were synthesized as reported previously (Nacro et al., 2000).

Glutathione *S*-transferase–PKD, PKC-α, and PKC-δ. Glutathione *S*-transferase (GST)-PKD was expressed in human embryonic kidney 293T cells and purified to homogeneity. Recombinant PKC-α and PKC-δ were expressed in Sf9 insect cells by infection with recombinant baculovirus that contained the desired construct and were purified as described previously (Kazanietz et al., 1993).

Binding of [^{3}H]PDBu and [^{3}H]Bryostatin 1. [^{3}H]PDBu binding to PKD, PKC-α, and PKC-δ was measured using the polyethylene glycol precipitation assay developed in our laboratory (Lewin and Blumberg, 2003) with minor modifications. For the determination of dissociation constants (K_d) and number of sites (B_{max}), typical saturation curves with increasing concentrations of the radioactive ligand (between 0.125 and 4 nM) were performed in triplicate. The assay mixture (250 μl) contained 50 mM Tris, pH 7.4, 100 μg/ml 100% phosphatidylserine, 4 mg/ml bovine immunoglobulin G, and variable concentrations of [^{3}H]PDBu. Incubation was carried out at 37°C for 5 min. Samples were chilled to 0°C for 10 min, 200 μl of 35% polyethylene glycol in 50 mM Tris-Cl, pH 7.4, was added, and the samples were incubated at 0°C for an additional 15 min. The tubes were centrifuged in a Beckman 12 microcentrifuge at 4°C (12,000 rpm for 15 min). A 100-μl aliquot of the supernatant was removed for the determination of the free concentration of [^{3}H]PDBu, and the pellet was carefully dried. The tip of the centrifuge tube containing the pellet was cut off and transferred to a scintillation vial for the determination of the total bound [^{3}H]PDBu. CytoScint (ICN, Costa Mesa, CA) was added both to aliquots of the supernatants and to the pellets, and radioactivity was determined with the use of a Wallac 1409 scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Nonspecific binding was measured using an excess of nonradioactive PDBu (30 μM). Specific binding was calculated as the difference between total and nonspecific binding. Nonspecific binding was typically less than 20% of the total binding observed in the assays for PKD and PKC.

To measure competition of [^{3}H]PDBu binding by different compounds, assays were performed under similar conditions but with the use of a fixed concentration of [^{3}H]PDBu (2 nM) and increasing concentrations of the nonradioactive ligand. In a typical competition assay, six to eight different concentrations 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} value using the relationship $K_i = ID_{50}/(1 + L/K_d)$, where L is the concentration of free [^{3}H]PDBu at the ID_{50} and K_d is the dissociation constant. When diacylglycerol analogs were assayed, they were mixed with the phospholipids in organic solvents, the solvent was removed under a stream of nitrogen, and the mixed liposomes were prepared as described previously (Kazanietz et al., 1992).

To assay [^{3}H]PDBu binding under low phosphatidylserine (PS) conditions, the assay mixture (250 μl) contained 50 mM Tris-HCl, pH 7.4 at 37°C, 20 μg/ml POPS, 80 μg/ml POPC, 2.8 mg/ml of bovine immunoglobulin G, 100 μM calcium chloride or 10 mM EGTA, and variable concentrations of [^{3}H]PDBu for determining K_d or 2 nM

[^{3}H]PDBu for the competition study. The binding assay was carried out as described above. [26-^{3}H]Bryostatin 1 binding was measured by a filtration assay with the use of Triton X-100/phosphatidylserine–mixed micelles in a final volume of 250 μl as described previously (Kazanietz et al., 1994).

Lipid. Aliquots of lipids in chloroform were mixed and dried under a stream of nitrogen. The lipids were subsequently resuspended in 170 mM sucrose in 20 mM Tris-Cl, pH 7.4, and subjected to five freeze-thaw cycles by alternately placing them in a 42°C water bath and in dry ice. Large unilamellar vesicles (LUV) were obtained by 40 rounds of extrusion through a 100-nm polycarbonate membrane in a LipoFast LIPOSOME "factory" (Sigma). Lipid concentrations were monitored by including trace amounts of L-α-[1-^{14}C]dipalmitoylphosphatidylcholine before extrusion, and concentrations could thereby be corrected for losses during preparation.

Vesicle Binding Assay. PKD and PKC were incubated with 2 nM [^{3}H]PDBu and 100 μM sucrose-loaded POPS/POPC vesicles and 20 μM PIP_2/POPC vesicles of different compositions in the presence of 50 mM Tris-Cl buffer, pH 7.4, 100 mM KCl, 100 μM $CaCl_2$, and 1 mg/ml γ-globulin. Incubations were carried out at 22°C for 5 min, followed by polyethylene glycol precipitation as described above for the usual [^{3}H]PDBu binding assay.

Results

Characterization of [^{3}H]PDBu and [^{3}H]Bryostatin 1 Binding to PKD. The binding of [^{3}H]PDBu to GST-PKD and the isoforms of PKC was carried out at 37°C for 5 min. Scatchard analysis showed that [^{3}H]PDBu bound to GST-PKD with high affinity in the presence of phosphatidylserine (Fig. 1A). The dissociation constant (K_d) of PKD for [^{3}H]PDBu was 0.80 ± 0.35 nM (n = 3 experiments), which is similar to that for PKC-δ (0.94 ± 0.09 nM, n = 5 experiments) but which is approximately five times weaker than that obtained for PKC-α (0.15 ± 0.02 nM, n = 3 experiments).

Bryostatin 1 is a macrocyclic lactone that induces a pattern of biological responses somewhat different from those of the typical phorbol esters. It is an ultrapotent PKC activator with antitumor activity and is currently in multiple clinical trials as an antitumor agent (Clamp and Jayson, 2002). We measured the binding of GST-PKD to [^{3}H]bryostatin 1 in the presence of phosphatidylserine/Triton X-100 micelles at several different temperatures. At 0°C, 80% of maximal binding was attained after 45 min of incubation, and binding did not diminish for at least 2 h. The rate of binding was faster at 18°C; 65% maximal binding was reached after 10 min of incubation. At 37°C, maximal binding was obtained with 5 min of incubation (data not shown). Therefore, 5 min of incubation at 37°C was used for the Scatchard analysis. We found that [^{3}H]bryostatin 1 bound to PKD with high affinity (K_d = 0.77 ± 0.11 nM, n = 3 experiments), which is slightly higher than the findings observed for PKC-α (K_d = 1.6 ± 0.1 nM, n = 3 experiments) and PKC-δ (K_d = 1.2 ± 0.1 nM, n = 4 experiments) (Fig. 1B), whereas the average binding sites (B_{max}) for [^{3}H]bryostatin 1 (2360 ± 320, n = 3 experiments) was three times lower than that for the [^{3}H]PDBu binding (7190 ± 690, n = 3 experiments).

Structure-Activity Analysis of Phorbol Esters and Related Ligands Binding to PKD. To determine the structure-activity requirements for ligand recognition by PKD, we evaluated the competition of [^{3}H]PDBu binding to PKD by a

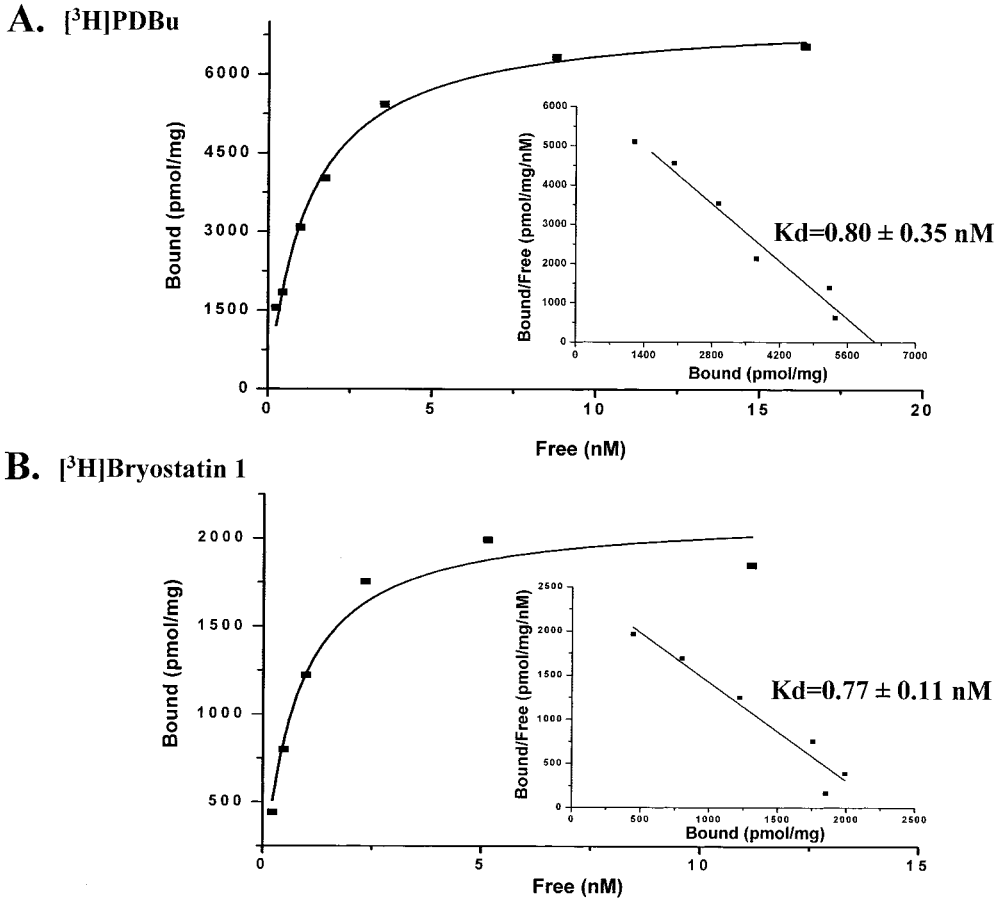

Fig. 1. [^{3}H]PDBu and [^{3}H]bryostatin 1 binding to PKD. A, binding to [^{3}H]PDBu was measured using increasing concentrations of the radioligand (8–90.25 nM) in the presence of 100 μg/ml of phosphatidylserine and 1 mM EGTA. B, binding to [^{3}H]bryostatin 1 was determined in the presence of phosphatidylserine/Triton X-100–mixed micelles and 1 mM EGTA. Insets, Scatchard plots derived from the corresponding binding curves. Data shown are from a representative experiment; similar results were obtained in two additional experiments.

group of structurally divergent phorbol ester analogs. These compounds bind to PKC with high affinity and induce a range of different biological responses. Different PKC isoforms display unique patterns of recognition for these compounds, and a range of three orders of magnitude in binding affinity for the PKC isoforms was reported previously (Kazanietz et al., 1993). The compounds tested in the study include two 12-deoxyphorbol esters (12-deoxyphorbol-13-phenylacetate and prostratin), two indole alkaloids [(−)-indolactam V and (−)-octylindolactam V], mezerein, a mezerein analog (thymeleatoxin), a diacylglycerol (OAG), and a novel constrained diacylglycerol (B8-DL-B8) and its unconstrained form (97D76).

The binding affinities of PKD to these compounds in general were similar to those reported earlier for PKC-δ (Kazanietz et al., 1993), although they were slightly weaker than those of PKC-α, with the exception of OAG and (−)-octylindolactam V (Table 1) (Kazanietz et al., 1993). The mezerein analog thymeleatoxin showed dramatic selectivity between PKC isozymes (20-fold lower affinity for PKC-ε and -η compared with PKC-β1) (Kazanietz et al., 1993) and between β2-chimaerin and PKC-α (56-fold) (Caloca et al., 1997). In this study, an 11-fold lower binding affinity to thymeleatoxin was detected for PKD compared with PKC-α, which was the greatest difference among all compounds (Table 1). In addition, a large reduction in binding affinity to (−)-indolactam V was also noted for PKD (38 ± 18, $n = 3$ experiments) compared with PKC-α (11.0 ± 1.3, $n = 3$ experiments) and PKC-δ (8.16 ± 0.65, $n = 3$ experiments). This decrease in affinity of PKD for (−)-indolactam V resulted in an 85-fold higher selectivity toward the lipophilic (−)-octylindolactam V (Table 1). This is the greatest difference in affinity reported for recognizing the two indole alkaloids [(−)-indolactam V and (−)-octylindolactam V] by different phorbol ester receptors.

The presence of a lysine (K) instead of a tryptophan (W) at position 22 of the PKD-C1b domain implies that PKD may recognize diacylglycerol differently than PKC. As shown in Table 1, GST-PKD bound to the unconstrained diacylglycerol analog 97D76 with much lower affinity compared with binding to PKC-α and -δ. Similarly, the constrained diacylglycerol analog B8-DL-B8 also showed significant lower affinity to GST-PKD compared with PKC-α but not with PKC-δ. Overall, PKD showed an approximately 3-fold decrease in affinity for 97D76 and B8-DL-B8 compared with PKC-α and, for 97D76, compared with PKC-δ. Although PKD, PKC-α, and PKC-δ did not show a significant difference in affinity for OAG, it might be caused by the limited sensitivity of the binding assay under the low-affinity range at which OAG binds to the C1 domains. Our findings show that PKD binds with reduced affinities to diacylglycerol compounds, suggesting that PKD might be differentially regulated by the endogenous diacylglycerol.

The ligand binding affinities of phorbol ester receptors are modulated in part by the specific lipid environment with which they associate. It is possible that the relatively small differences in affinity to phorbol ester and derivatives were caused by the nonphysiological lipid conditions used in the assays. To address this issue, we determined the affinity of selected compounds for PKD, PKC-α, and PKC-δ in the presence of 20 μg/ml of POPS and 80 μg/ml of POPC instead of 100 μg/ml of PS. These conditions should better mimic the physiological lipid condition (Table 1). PKD and PKC-α in general bound the compounds with similar affinities in the presence of 20% POPS:80% POPC and in the presence of 100% PS. The one exception was B8-DL-B8, for which the affinity was reduced by an additional 2.4-fold, further emphasizing that PKD may be differentially regulated by the endogenous diacylglycerol. For PKC-δ, thymeleatoxin and (−)-indolactam V showed appreciably decreased affinities.

Lipid Requirements for Phorbol Ester Binding to PKD. Phospholipids are major regulators of membrane localization and subsequent activation of PKC. Unlike PKC, PKD lacks the pseudosubstrate and C2 domains, the two major membrane-targeting modules in the classic PKCs that act together with the C1 domains; instead, PKD possesses a PH domain. Therefore, it is highly plausible that PKD differs from PKC in its lipid requirements for membrane binding. To test this hypothesis, we examined the ability of phospholipids to reconstitute PDBu binding to PKD with the use of LUVs at different phospholipid concentrations and compositions. Be-

TABLE 1

Structure-activity analysis of binding to PKD

Receptor-ligand interaction was studied by competition of [^{3}H]PDBu (2 nM) binding with each compound in the presence of 100 μg/ml of PS and 100 μM (in the case of PKC-α) [Ca^{2+}]. Seven 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 from the ligands were calculated from the ID_{50} as described under *Materials and Methods*. The K_i values for PKC-α and PKC-δ were reported earlier by Kazanietz et al. (1993). To determine binding properties under conditions of a reduced proportion of PS, the K_d for PDBu and the K_i for selected compounds as specified in the table were determined in the presence of 20 μg/ml of POPS and 80 μg/ml of POPC. Values represent the mean ± S.E. of the number of experiments in parentheses.

Compounds	K_d or K_i			Ratio	
	GST-PKD	PKC-α	PKC-δ	PKD/PKC-α	PKD/PKC-δ
		nM			
PDBu (K_d)	0.80 ± 0.35 (3)	0.15 ± 0.02 (3)	0.94 ± 0.09 (5)	5.3	0.9
PDBu (K_d, low PS)	1.62 ± 0.05 (3)	0.22 ± 0.01 (3)	1.67 ± 0.06 (3)	7.4	0.9
12-Deoxyphorbol 13-phenylacetate	0.35 ± 0.06 (3)	0.14 ± 0.02 (3)	0.56 ± 0.03 (2)	2.5	0.6
Thymeleatoxin	3.2 ± 1.1 (3)	0.29 ± 0.03 (3)	1.31 ± 0.22 (3)	11.0	2.4
Thymeleatoxin (low PS)	3.85 ± 0.25 (3)	0.22 ± 0.01 (3)	10.7 ± 0.4 (3)	17.5	0.4
Prostratin	12.9 ± 1.4 (3)	4.83 ± 0.44 (5)	24.6 ± 4.4 (3)	2.7	0.5
Mezerein	0.43 ± 0.10 (3)	0.27 ± 0.04 (4)	0.55 ± 0.18 (3)	1.6	0.8
(−)-Indolactam V	38 ± 18 (3)	11.0 ± 1.3 (3)	8.16 ± 0.65 (3)	3.5	4.7
(−)-Indolactam V (low PS)	48.1 ± 2.8 (3)	12.6 ± 1.1 (3)	43.1 ± 1.4 (3)	3.8	1.1
(−)-Octylindolactam V	0.45 ± 0.08 (3)	0.53 ± 0.06 3)	0.77 ± 0.08 (3)	0.9	0.6
OAG	146 ± 26 (3)	230 ± 28 (3)	224 ± 27 (3)	0.6	0.7
B8-DL-B8	10.8 ± 2.7 (3)	2.9 ± 0.2 (3)	11.6 ± 3.1 (3)	3.7	0.9
B8-DL-B8 (low PS)	26.1 ± 1.1 (3)	3.57 ± 0.16 (3)	10.0 ± 0.2 (3)	7.3	2.6
97D76	90 ± 14 (3)	28.6 ± 3.2 (3)	30.9 ± 4.2 (3)	3.2	2.9

cause phosphatidylinositols are reported to be natural ligands for the PH domains, the ability of PIP_2 to reconstitute PDBu binding was also evaluated.

Our findings revealed differences in lipid requirements for ligand binding by PKD compared with PKC-α and -δ. The ability of different mol% of POPS to reconstitute [^{3}H]PDBu binding to PKD was first evaluated using 100 μM large unilamellar vesicles (Fig. 2A). The mol% of POPS required for half-maximal binding (ED_{50}) was 7.0 $\pm$ 1.5, 28.4 $\pm$ 1.7, and 61.7 $\pm$ 2.0 mol% for PKC-α, PKD, and PKC-δ, respectively (Fig. 2A). The concentrations of LUV (POPS:POPC = 1:1) required for half-maximal reconstitution were 0.57 $\pm$ 0.11, 5.14 $\pm$ 0.96, and 179 $\pm$ 21 μM for PKC-α, PKD, and PKC-δ, respectively (Fig. 2B). Compared with POPS, similar relative patterns of dependence for PIP_2 were found for PKC-α, PKD, and PKC-δ (Fig. 3A). Briefly, the mol% of PIP_2 required for half-maximal reconstitution (ED_{50}) was 5.1 $\pm$ 1.4, 20.5 $\pm$ 3.7, and 54.4 $\pm$ 5.4 mol% for PKC-α, PKD, and PKC-δ, respectively (Fig. 3A). The concentrations of LUV (PIP_2:POPC = 10:90) required for half-maximal reconstitution were 2.61 $\pm$ 0.27, 21.7 $\pm$ 1.6, and 91.1 $\pm$ 7.0 μM for PKC-α, PKD, and PKC-δ (Fig. 3B). Overall, the three enzymes showed greater difference in their dependence on anionic phosphatidylserine to support PDBu binding. There was a 10- to 30-fold difference in the ED_{50} concentrations between PKD and PKCs for POPS containing LUVs (POPS:POPC = 1:1) versus a 4- to 6-fold difference for PIP_2 containing LUVs (PIP_2:POPC = 10:90). PKC-α had the lowest ED_{50} values for POPS, and PIP_2 and PKC-δ had the highest; the ED_{50} values for PKD were intermediate between those of PKC-α and PKC-δ. Because the ligand binding affinities of phorbol ester receptors reflect in part their ability to interact with lipid membranes, the lack of appreciable difference for the reconstitution of PDBu binding to PKD and PKC by PIP_2 suggests that PIP_2 might not be a lipid that selectively regulates the membrane-targeting and ligand recognition of PKD.

Discussion

The presence of multiple phorbol ester receptors in addition to PKC raises the possibility that some of the phorbol ester-mediated responses in cells are in fact mediated by these novel phorbol ester receptors. Thus, understanding the interaction of these proteins with phorbol esters, diacylglycerol, and cofactors is of great importance to dissect the complex diacylglycerol signaling networks in cells.

Previously, PKD was demonstrated to be a phorbol ester binding protein in vitro using purified protein. It was reported that an N-terminal domain of PKD purified from bacteria exhibited a K_d of 35 nM, and its binding to [^{3}H]PDBu was inhibited dose-dependently by OAG (Valverde et al., 1994). An analysis of the individual C1 domains of PKD in vivo or in vitro indicated that the cys1 (C1a) and cys2 (C1b) motifs of PKD were functionally dissimilar, with cys2 responsible for the majority of the [^{3}H]PDBu binding activity (Iglesias et al., 1998). On the contrary, an analysis of synthetic C1 domains of PKD indicated that the two cysteine-rich motifs of PKD were functionally equivalent, with a K_d of 2.5 or 2.7 nM (Irie et al., 1999). In these studies, either bacterially expressed or synthetic PKD C1 domains were used, which may not exclude the possible defects in protein folding or differential posttranslational modifications in the bacterial system. Of the studies using purified mammalian PKD, Van Lint et al. (1995) reported a K_d of 2.2 nM for [^{3}H]PDBu binding to immunopurified PKD from COS7, which is comparable with the K_d of the purified mammalian GST-PKD used in our study. However, in all reports, the structure-activity requirements and the cofactor-dependence of PKD to phorbol esters are not fully characterized. Here, we conducted a detailed analysis on a GST-tagged full-length PKD for its interaction with PDBu.

A broad spectrum of structurally and biologically divergent phorbol esters and derivatives were tested for their binding to PKD. The binding of PKD to phorbol esters was comparable with those of the classic and novel PKC isoforms, PKC-α and PKC-δ (Kazanietz et al., 1993). Likewise, bryostatin 1, an atypical activator of PKC with high affinity, binds to PKD with a K_d similar to those of the PKC isozymes. We noted that octyl-indolactam V binds to PKD with 85-fold higher affinity than indolactam V, the largest difference in affinity for indolactams among all phorbol ester receptors examined. Understanding the structure basis underlying this difference

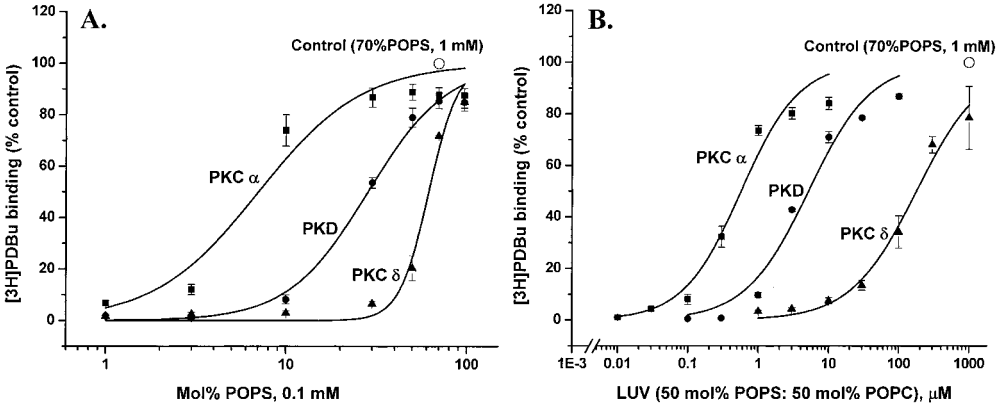

Fig. 2. Reconstitution of PDBu binding to PKD, PKC-α, and PKC-δ by phosphatidylserine. Binding was performed using large unilamellar vesicles with 5 min of incubation at 22°C as described under *Materials and Methods*. A, [^{3}H]PDBu binding was carried out using 100 μM LUV with increasing mol% of POPS with the remainder being POPC. Each point represents the mean $\pm$ S.E. of five experiments. B, [^{3}H]PDBu binding was performed using increasing concentrations of LUV composed of 50 mol% POPS with the remainder being POPC. Values were normalized to the maximal reconstitution at 1 mM LUV (POPS:POPC = 70:30), as indicated with a single point at the top of the figure. Results represent the mean $\pm$ S.E. of three independent experiments.

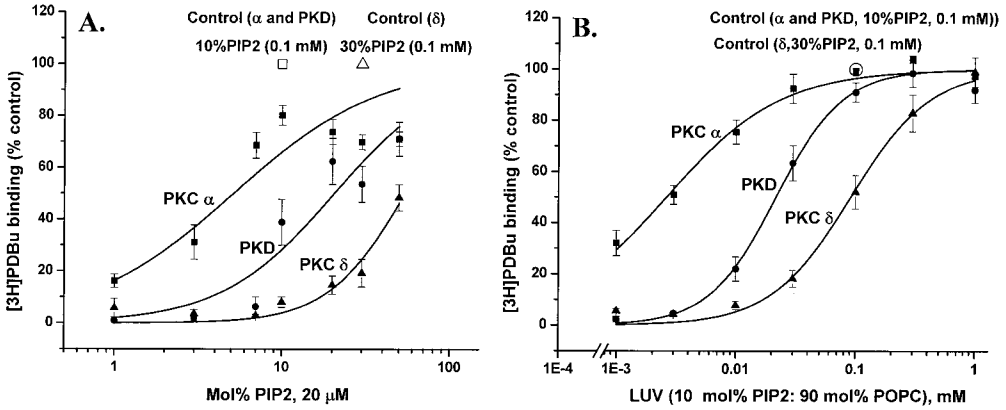

Fig. 3. Reconstitution of PDBu binding to PKD, PKC-α, and PKC-δ by PIP_2. A, [^{3}H]PDBu binding was carried out using 20 μM LUV with increasing mol% of PIP_2 with the remainder being POPC. Each point represents the mean ± S.E. of three experiments. B, [^{3}H]PDBu binding was performed using increasing concentrations of LUV composed of 10 mol% PIP_2 with the remainder being POPC. Values were normalized to maximal reconstitution at 0.1 mM LUV (PIP_2:POPC = 10:90) for PKD and PKC-α and at 0.1 mM LUV (PIP_2:POPC = 30:70) for PKC-δ, as indicated with two single points at the top of the figure. Results represent the mean ± S.E. of three independent experiments.

in activity may provide clues to the design of selective ligands for PKD.

The lysine residue at position 22 of the PKD-C1b domain represents one of the major structural divergences from the C1 domain of PKC isoforms. In PKC, this residue is a highly conserved hydrophobic amino acid, a tryptophan in PKC-δ, -ε, -η, and -θ, or a tyrosine in PKC-α, -β, and -γ. Its backbone is part of the ligand binding pocket, whereas its side chain is exposed to the external environment and is believed to interact with phospholipids (Zhang et al., 1995; Wang et al., 1996; Pak et al., 2001). Indeed, mutant W22G of PKC-δC1b showed reduced ligand/phospholipid binding affinity (Kazanietz et al., 1995b). However, in our previous study, the mutations at W22 (W22Y and W22K) of PKC-δC1b, instead of impairing the interaction with phospholipids, caused a large reduction in affinity to DAG but not to PDBu (Wang et al., 2001). Our molecular dynamics simulation revealed that the side chain of W22 was very flexible (Pak et al., 2001), which would allow the side chain to easily adopt different conformations when interacting with different ligands. Thus, introducing different side chains at this position could affect the ligand selectivity. The fact that the same mutated residue (K22) occurs in all members of the PKD family implies a potentially different selectivity for DAG and consequently a possible functional difference compared with the members of the PKC family. Our findings indicate that PKD binds to the two diacylglycerol analogs, B8-DL-B8 and 97D76, with 3-fold lower affinity compared with PKC-α and -δ, and this difference is enhanced under low PS conditions, which agrees with our hypothesis that diacylglycerol differentially interacts with PKD and PKC.

We have summarized the substantial structural differences between PKD and PKC, which implies altered phospholipid requirements for PKD compared with PKC isoforms. Our findings showed approximately comparable differences between PKD and PKC isoforms in both phosphatidylserine and PIP_2 requirements for PDBu binding. Although our results suggest that PIP_2 might not have selectively regulated the membrane association and ligand recognition of PKD in our study, it remains possible that phosphatidylinositols other than PIP_2 may be the preferred lipid ligands for the PH domain in PKD. However, more studies are required to identify such lipid factors with specificity for the PH domain of PKD.

In summary, PKD is a high-affinity phorbol ester receptor that may be differentially regulated in cells by diacylglycerol. The phospholipid requirement is approximately comparable with those of the PKC isoforms, despite the presence of dissimilar membrane-targeting modules. The divergent requirements of PKD compared with PKC for diacylglycerol may assist the cells in controlling the relative engagement of these signaling pathways.

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