Selective binding of phorbol esters and diacylglycerol by individual C1 domains of the PKD family

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The PKD (protein kinase D) family are novel DAG (diacylglycerol) receptors. The twin C1 domains of PKD, designated C1a and C1b, have been shown to bind DAG or phorbol esters. However, their ligand-binding activities and selectivities have not been fully characterized. Here, binding activities of isolated C1a, C1b and intact C1a–C1b domains to DAG and phorbol esters were analysed. The isolated C1b domains of PKD isoforms bind [3H]PDBu ([20-3H]phorbol 12, 13-dibutyrate) with similar high affinities, while they exhibit weaker affinities towards a synthetic DAG analogue, DOG (1,2-dioctanoyl-sn-glycerol), as compared to the control. Mutating a conserved lysine residue at position 22 to tryptophan in C1b of PKD3 fully restores its affinity to DOG, indicating that this residue accounts for its weaker affinity to DOG. In contrast, the non-consensus residues in the isolated C1a domain of PKD mainly contribute to maintaining the protein’s structural fold, since converting these residues in C1a of PKD3 to those in PKD1 or PKD2 drastically reduces the maximal number of active receptors, while only minimally impacting ligand-binding activities. Moreover, ligand-binding activities of C1a and C1b are sensitive to the structural context in an intact C1a–C1b domain and exhibit unique patterns of ligand selectivity. C1a and C1b in the intact C1a–C1b of PKD1 are opposite in selectivity for PDBu and DOG. In contrast, C1a of PKD3 exhibits 48-fold higher affinity to DOG as compared to C1b, although both domains bind PDBu with equivalent affinities. Accordingly, mutating C1a of a full-length PKD3–GFP greatly reduces DOG-induced plasma membrane translocation, but does not affect that induced by PMA. In summary, individual C1 domains of PKD isoforms differ in ligand-binding activity and selectivity, implying isoform-selective regulation of PKD by phorbol esters and DAG.

Key words: C1 domain, diacylglycerol, ligand-binding affinity, phorbol esters, protein kinase D (PKD), selectivity.

INTRODUCTION

The PKD (protein kinase D) family are a novel serine/threonine kinase family that belongs to the Ca2+-calmodulin kinase superfamily [1]. Three isoforms have been identified, including PKD1/PKCθ, PKD2 [4] and PKD3 (PKCν) [5]. PKD1 was the first isoform discovered and the best characterized. Members of the PKD family are highly homologous, particularly in their catalytic domain, which is more closely related to myosin light chain kinase and Ca2+-calmodulin-dependent kinases, and is only distantly related to PKC. PKD has been implicated in a variety of cellular processes including the regulation of Golgi function, cell proliferation, apoptosis and cell migration [6,7]. PKD1 localizes in multiple subcellular compartments such as cytosol, nucleus, Golgi and mitochondria [8], and is activated in intact cells by tumour-promoting phorbol esters, GPCR (G-protein coupled receptor) agonists and certain growth factors [6,7]. In most cells, the activity of PKD isoforms is controlled through a PKC-dependent mechanism. The PKC family, particularly the novel members, directly bind, phosphorylate and activate PKD [9,10].

The regulatory domain of PKD comprises two structural motifs, a C1 domain and a PH (pleckstrin homology) domain. The C1 domain in PKD, similar to that in PKC, confers high-affinity binding to DAG (diacylglycerol) and phorbol esters. The C1 domain is a highly conserved structural motif that exists either as a single motif or as twin motifs designated as C1a and C1b in a variety of proteins distinct in structure and function [11,12]. It consists of 50 amino acids with a consensus sequence of HX12CX3CX13/14CX3CX4HX2CX7C (C, cysteine; H, histidine; X, any other amino acid) [12]. The two histidines and all but one of the six conserved cysteines co-ordinate two Zn2+ ions. A number of residues, such as those at the 3, 8, 11, 21, 24, 27 and 38 positions, have been shown to be critical for maintaining the overall structure and ligand binding by the C1 domain [11–13]. The C1 domain of PKD contains twin C1a and C1b motifs and a linker sequence between them. The binding to DAG and phorbol esters by individual C1a or C1b domains has been reported, although their relative activities remain controversial. It has been shown that C1a and C1b of PKD1 and PKD2 are dissimilar in ligand-binding activities and that the C1b domain is mainly responsible for the binding to phorbol esters in vivo and in vitro [14,15]. However, interestingly, the C1a domain of PKD1 has been shown to bind DAG and is required for the recruitment of PKD1 to the Golgi. The molecular basis underlying their differential interactions with ligands is unknown, and whether PKD2 and PKD3 C1 domains behave similarly remains to be determined.

The C1 domain, upon binding to DAG/phorbol ester, serves as an important membrane-targeting module for a variety of proteins in intact cells. For classical and novel PKCs, membrane targeting is essential for PKC activation [16]. For PKD, the binding of DAG and phorbol esters to the C1 domain mainly functions to recruit PKD to membrane compartments. It has been shown that the interaction of the C1 domain of PKD1 with DAG in the Golgi is essential for the recruitment of PKD1 to the Golgi and the regulation of Golgi function by PKD1. DAG generated through the activation of GPCRs triggers plasma membrane translocation of PKD1, which allows PKD1 to propagate signals from the cell surface to the cell interior [17].

Abbreviations used: CHO, Chinese hamster ovary; DAG, diacylglycerol; DOG, 1,2-dioctanoyl-sn-glycerol; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; GST, glutathione transferase; IFTG, isopropl β-D-thiogalactoside; MDBu, phorbol 12, 13-dibutyrate; PKC, protein kinase C; PKD, protein kinase D; PS, l-α-phosphatidylserine.

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In this study, we evaluated the ligand-binding activities of individual C1 domains of the PKD family. When comparing the activities of the isolated C1b and C1a domains of PKD, we found that the C1b domain of PKD exhibited 4-fold less affinity for DAG. Mutating a conserved lysine residue at position 22 to tryptophan fully restored the affinity for DAG without significant impact on its affinity for PDBu (phorbol 12, 13-dibutyrate). Further study using the intact full-length C1a–C1b domain demonstrated marked ligand selectivity between individual C1 domains of PKD isoforms. Taken together, our findings show unique structure-activity requirements for ligand binding to C1 domains of PKD, implying ligand-mediated selective regulation of PKD isoforms.

**EXPERIMENTAL**

**Materials**

PDBu was obtained from LC Laboratories. [3H]PDBu ([20-3H]PDBu) (20 Ci/μmol) was purchased from PerkinElmer Life Sciences. PS (l-α-phosphatidylserine) and DOG (1,2-dioctanoyl-sn-glycerol) were obtained from Avanti Polar Lipids. Reagents for expression and purification of GST (glutathione transferase) fusion proteins were purchased from GE Healthcare Bio-Sciences Corp.

**Cloning and Mutagenesis of PKD3**

The PKD C1 domain constructs were generated by PCR using the full-length PKD cDNAs as template and were subsequently cloned into a pGEX-4T3 vector (vector; Roche Diagnostics) or a pGEX-2TK vector (vector; GE Healthcare, UK) which was performed by the DNA Sequencing Core Facilities at the University of Pittsburgh (Pittsburgh, PA, U.S.A.) or Genewiz, Inc. (South Plainfield, NJ, U.S.A.).

**Expression and purification GST-tagged PKD C1 proteins in Escherichia coli**

The expression of GST fusion proteins was induced by the addition of 0.5 mM IPTG (isopropyl β-D-thiogalactoside). The bacteria were harvested after a 5 h induction at 37°C and the recombinant proteins were purified to homogeneity using glutathione–Sepharose 4B beads as described previously [18].

**Binding of [3H]PDBu**

[3H]PDBu binding to PKD C1 domains and PKCδ-C1b was measured using the polyethylene glycol precipitation assay developed in the Blumberg laboratory with minor modifications [19]. For the determination of dissociation constants (Kd) and number of sites (Bmax), 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/HCl (pH 7.4), 100 μg/ml 100% phosphatidylserine, 4 mg/ml bovine immunoglobulin G and variable concentrations of [3H]PDBu. Incubation was carried out at 37°C for 5 min. Samples were chilled to 0°C for 10 min, then 200 μl of 35% polyethylene glycol in 50 mM Tris/HCl (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 (12000 rev./min, 15 min). A 100 μl aliquot of the supernatant was removed for the determination of the free concentration of [3H]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 [3H]PDBu. CytoScint™ was added both to the aliquots of the supernatants and to the pellets, and radioactivity was determined in a Wallac 1409 scintillation counter. Non-specific 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 [3H]PDBu binding by DOG, assays were performed under similar conditions but used a fixed concentration of [3H]PDBu (2 nM) and increasing concentrations of the nonradioactive ligand. In a typical competition assay, six to eight different concentrations of DOG were used, ID50 [50% inhibitory concentration (dose)] values were determined from the competition curve, and the Kd for the competing ligand was calculated from the ID50 using the relationship Kd = ID50/(1+L/Kd), where L is the concentration of free [3H]PDBu at the ID50 and Kd is the dissociation constant.

**Western blot analysis**

Equal amounts of cell lysates or purified GST fusion proteins were subjected to SDS/PAGE and electro-transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and 2% BSA in Tris-buffered saline. Membranes were then probed with a primary antibody, followed by anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories). Bands were visualized by the ECL® (enhanced chemiluminescence) Western blotting detection system (Amersham Biosciences). The primary antibodies used were anti-GST (Santa Cruz Biotechnology) and anti-GFP (green fluorescent protein; Roche Diagnostics) antibodies.

**Cytosol/plasma membrane fractionation**

CHO (Chinese hamster ovary) cells were transiently transfected with wild-type PKD3–GFP or C1a(P165G)-PKD3–GFP. On day two after transfection, the transfected cells were harvested by scraping in ice-cold TE buffer containing 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5, supplemented with 10 μg/ml leupeptin and 5 mM phenylmethylsulfonyl fluoride. Cells were homogenized by passing through a 22G1 needle 35 times. The homogenate was centrifuged at 750 g for 15 min at 4°C twice to pellet nuclei and unbroken cells, leaving a membrane-containing supernatant. Plasma membrane was collected by centrifugation of the supernatant for 30 min at 31 000 rev./min in a Beckman TLA100.3 rotor at 4°C. Proteins in the resulting supernatant were subjected to Western blotting analysis.

**Time-lapse fluorescent confocal microscopy in live cells**

The 4-well chambered cover glass slide containing the transfected CHO cells was attached to the microscope stage with a custom stage adapter. Initially, cells were imaged in imaging medium: DMEM (Dulbecco’s modified Eagle’s medium) with high glucose, 2 mM L-glutamine, 25 mM Hepes, without sodium.
Ligand-binding activities of PKD C1 domains

Figure 1 Amino acid sequence alignment of PKD C1 domains

The non-consensus amino acids in the C1 domains (including C1a, linker region and C1b) of mouse PKD1, human PKD2 and human PKD3 as compared to that of human PKD1 (PKCμ) are in bold and underlined. Proline residues at position 11 in the boxes were mutated to inactivate C1a or C1b. Amino acids introduced through mutation are indicated below the mutated residues. Asterisks indicate the critical lysine (K) at position 22 of the C1b domains. Percentages of amino acid homology between each region of the C1 domains of PKD are to the right of the sequences. Numbers in parentheses to the right of the human PKD1 C1 sequences indicate the position in the full-length protein. The secondary protein structures are indicated on top of the aligned sequences.

Figure 2 Purified GST-tagged C1 proteins of PKD isoforms

(A) Isolated C1a or C1b domains of PKD or (B) C1a–C1b of PKD were expressed and purified from bacteria culture. Their sizes were confirmed by Western blotting analysis. The apparent [3H]PDBu binding affinities of isolated C1a and C1b proteins were first determined in the presence of glutathione–Sepharose 4B beads. The size of purified proteins was confirmed on SDS/PAGE. As shown in Figure 2, GST–C1a or GST–C1b exhibited an apparent molecular mass of approximately 33 kDa, whereas GST–C1a–C1b proteins were approx. 45–47 kDa.

RESULTS

Isolated C1a and C1b domains of PKD differ in their affinities for DOG

The twin C1 domains of PKD are highly conserved. As depicted in Figure 1, based on their sequence alignment, PKD isoforms share over 90% amino acid identity at the C1a domain and over 80% identity at the C1b domain. Higher homology is noted between C1 domains of PKD1 and PKD2, while those of PKD3 are more divergent. The linker regions between C1a and C1b are least conserved and show an approximately 40% amino acid identity as compared to that of PKD1. To obtain C1 domain proteins for structure–activity analysis, plasmids expressing wild-type and mutant C1a, C1b and C1a–C1b domains of PKD were constructed as described in the Experimental section. All constructs and point mutations were confirmed by sequencing. The truncated C1 domains were expressed in E. coli as GST-fusion proteins. Protein expression was induced by the addition of IPTG as previously described [20]. The fusion proteins were predominantly expressed in the cytoplasm and were isolated from the soluble fraction by binding to glutathione–Sepharose 4B beads. The size of purified proteins was confirmed on SDS/PAGE. As shown in Figure 2, GST–C1a or GST–C1b exhibited an apparent molecular mass of approximately 33 kDa, whereas GST–C1a–C1b proteins were approx. 45–47 kDa.

The apparent [3H]PDBu binding affinities of isolated C1a and C1b proteins were first determined in the presence of phosphatidylserine and compared to those of PKCδ-C1b, a previously well-characterized active C1 domain. The affinity for DAG was evaluated by competition of [3H]PDBu binding by the synthetic DAG analogue, DOG. As shown in Table 1, [3H]PDBu binding to C1a-C1b was approximately 33 kDa, whereas GST–C1a–C1b proteins were approx. 45–47 kDa.

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Table 1 Comparison of the binding activities of the isolated single C1 domains of PKD for PDBu and DOG

<table>
<thead>
<tr>
<th>C1a and C1b of PKD</th>
<th>PDBu</th>
<th>DOG</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (nM)</td>
<td>Ratio (PKD/a)</td>
</tr>
<tr>
<td>PKCδ-C1b</td>
<td>0.49 ± 0.03 (3)</td>
<td>1.0/1.0</td>
</tr>
<tr>
<td>PKD1-C1a</td>
<td>0.37 ± 0.14 (3)</td>
<td>1.0/1.3</td>
</tr>
<tr>
<td>PKD2-C1b</td>
<td>0.32 ± 0.09 (5)</td>
<td>1.0/1.5</td>
</tr>
<tr>
<td>PKD3-C1b</td>
<td>0.28 ± 0.04 (3)</td>
<td>1.0/1.8</td>
</tr>
<tr>
<td>PKD1-C1a</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td>PKD2-C1a</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td>PKD3-C1a</td>
<td>1.62 ± 0.03 (5)</td>
<td>3.3/1.0</td>
</tr>
</tbody>
</table>

Table 2 Comparison of the ligand binding affinities of wild-type and mutants of PKD3-C1b domain

<table>
<thead>
<tr>
<th>Mutants of PKD3-C1b</th>
<th>PDBu</th>
<th>DOG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (nM)</td>
<td>Ratio (mut/D3-C1b)</td>
</tr>
<tr>
<td>Wild-type PKD3-C1b</td>
<td>0.28 ± 0.04 (3)</td>
<td>1.0/1.0</td>
</tr>
<tr>
<td>I13V</td>
<td>0.34 ± 0.05 (3)</td>
<td>1.2/1.0</td>
</tr>
<tr>
<td>K22W</td>
<td>0.38 ± 0.04 (3)</td>
<td>1.4/1.0</td>
</tr>
</tbody>
</table>

Figure 3 K22W mutation restores the DAG binding affinity of PKD3-C1b

Binding affinity to DOG was measured by inhibiting [3H]PDBu binding to GST–PKD3-C1b ( ), GST–PKD3-C1b(K22W) mutant (■ and broken line), and PKD3-C1a ( ) with increasing doses of DOG. The K22W mutation shifted the inhibition curve to the left, overlapping with that of PKD3-C1a. Three independent experiments were performed and a representative one is shown. $K_i$ values are the mean ± S.E.M. of three experiments. Each point on the curve represents the mean ± S.E.M. of triplicate determinations.
Table 3 Comparison of the PDBu and DOG binding activities of the intact C1a–C1b domains of PKD
Ratios between the K_d or K_i of PKD2-C1a–C1b and PKD3-C1a–C1b to those of PKD1-C1a–C1b were calculated. Values represent the mean ± S.E.M. of the number of experiments in parentheses.

<table>
<thead>
<tr>
<th>PKD isoform</th>
<th>PDBu</th>
<th>DOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_d (nM)</td>
<td>Ratio (mut/C1a)</td>
<td>B_max (pmol/mg)</td>
</tr>
<tr>
<td>PKD1-C1a–C1b</td>
<td>3.08 ± 0.79 (3)</td>
<td>(1.0/1.0)</td>
</tr>
<tr>
<td>PKD2-C1a–C1b</td>
<td>1.51 ± 0.84 (6)</td>
<td>1.0/2.0</td>
</tr>
<tr>
<td>PKD3-C1a–C1b</td>
<td>0.40 ± 0.11 (3)</td>
<td>1.0/7.7</td>
</tr>
</tbody>
</table>

Table 4 Comparison of wild-type and mutants of PKD3-C1a domain for binding to PDBu and DOG
Ratios of mutants against wild-type PKD3-C1a were calculated. Values represent the mean ± S.E.M. of the number of experiments in parentheses.

<table>
<thead>
<tr>
<th>Mutants of PKD3-C1a</th>
<th>PDBu</th>
<th>DOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_d (nM)</td>
<td>Ratio (mut/C1a)</td>
<td>B_max (pmol/mg)</td>
</tr>
<tr>
<td>Wild-type PKD3-C1a</td>
<td>1.62 ± 0.32 (5)</td>
<td>(1.0/1.0)</td>
</tr>
<tr>
<td>T2A/Y4F</td>
<td>2.63 ± 0.81 (3)</td>
<td>1.6/1.0</td>
</tr>
<tr>
<td>T12A</td>
<td>4.72 ± 0.71 (3)</td>
<td>2.9/1.0</td>
</tr>
<tr>
<td>Y16H</td>
<td>1.56 ± 0.14 (3)</td>
<td>1.0/1.0</td>
</tr>
<tr>
<td>PKD2-C1a–C1b</td>
<td>8.1 (3)</td>
<td>(1.0/1.0)</td>
</tr>
</tbody>
</table>

Contribute to specific interactions with DAG. Mutating this residue back to valine (V) as in the C1b domain of PKD1 or PKD2 (T13V) caused a 2.5-fold increase in PKD3-C1b affinity for DOG, reflecting weaker binding to this ligand, but did not impact the binding activity for PDBu. However, since the affinities for DOG were similar among the C1b domains of the PKD family, the negative effect as a result of altering this residue may have been compensated for by other non-conserved amino acids in the C1b domains of PKD1 and PKD2.

Mutating the non-identical residues in the C1a domain of PKD3 back to those in PKD1 negatively affects protein stability
The lack of [3H]PDBu binding by the C1a domains of PKD1 and PKD2 may be due to either the inactivity of the C1 domain or the lack of protein stability that affects folding. The amino-acid sequences of the PKD-C1a domains are almost identical, with only 3 amino acids out of 50 for PKD2 or 5 out of 50 for PKD3 being non-identical. Initial analysis of these residues showed that they were not in positions that will dramatically affect ligand-binding affinity. To further determine their functions, we back-mutated the non-identical residues in the C1a domain of PKD3 to those in the C1a domain of PKD1. Three mutants were generated: PKD3-C1a(T2A/Y4F) that contains mutations T2A and Y4F at positions 2 and 4, PKD3-C1a(T12A) that contains a T12A mutation at position 12, and PKD3-C1a(Y16H) that contains a Y16H mutation at position 16. As shown in Table 3, a large decrease in B_max was noted for all three mutants (5- to 8-fold reduction), indicating that the majority of receptors were inactive, most likely due to lack of protein stability and loss of structural folding. In contrast, we observed only a small drop in B_max for the binding of PKD3-C1a by [3H]PDBu agreed with the value obtained in a previous study [21]. The DOG-binding affinity of PKD3-C1a–C1b was comparable to that of PKCδ-C1b. Taken together, our results demonstrate that the PKD3-C1a–C1b domain may be a preferred high-affinity receptor for phorbol esters and DAG as compared to the C1α–C1β domains of PKD1 and PKD2.

The combined C1α–C1β domains of PKD3 bind phorbol esters and DAG with stronger affinity as compared to those of PKD1 and PKD2
The C1 domain is thought to be modular and could function independently of other structural domains. Although we had determined the ligand-binding activities of isolated individual C1 modules, their contributions to the overall activity of the intact PKD C1α–C1β domains remain to be determined. To address this question, we constructed the intact twin C1 domain of each PKD isoform, designated C1α–C1β, which contains C1α and C1β and the linker sequence between them. The GST–C1α–C1β domains were expressed and purified from bacterial culture. The sizes of the C1α–C1β proteins were confirmed by Western blotting (Figure 2B). Their ligand-binding activities were subsequently determined. Significant differences in affinities for phorbol esters and DAG were detected among the C1α–C1β regions of the PKD isoforms (Table 4). In general, the C1α–C1β domain of PKD3 exhibited stronger affinities for PDBu (8-fold more) and DOG (4-fold more) as compared to the C1α–C1β domain of PKD1. The K_i for the binding of PKD3-C1a–C1b by [3H]PDBu agreed with the value obtained in a previous study [21]. The DOG-binding affinity of PKD3-C1a–C1b was comparable to that of PKCδ-C1b. Taken together, our results demonstrate that the PKD3-C1a–C1b domain may be a preferred high-affinity receptor for phorbol esters and DAG as compared to the C1α–C1β domains of PKD1 and PKD2.

The C1α and C1β domains in the intact PKD-C1α–C1β domain bind phorbol esters and DAG with distinct selectivity
To determine whether structural context impacts the binding activities of individual C1α/b motifs in a C1α–C1β domain, the activity of each C1 module was evaluated by inactivating the other C1 motif through introduction of a P11G mutation. As a result, two mutants were generated for each PKD isoform: the C1α mutant, C1α(P11G)–C1β, and the C1β mutant, C1α-C1β(P11G). The mutants were first analysed for binding by [3H]PDBu. As shown in Table 5, all mutants were active, with the exception of the C1α mutant of PKD2, PKD2-C1α(P11G)–C1β that did not bind [3H]PDBu up to a receptor concentration of 4 μg assay. Importantly, the C1β mutants, C1α-C1β(P11G), were all active,
indicating that the C1a domains of PKD1 and PKD2 are indeed functional and their structural folding can be stabilized in an intact C1a–C1b domain. When DOG binding activities were evaluated, consistent with our previous findings obtained using the isolated C1a and C1b proteins, the C1a mutants, C1a(P11G)–C1b, bound DOG more weakly as compared to the C1b mutants [note that PKD2-C1a(P11G)–C1b could not be assessed due to a lack of binding activity under our assay conditions].

In general, the $K_d$ and $K_i$ values of the PKD1-C1a–C1b mutants were much higher as compared to those of PKD3-C1a–C1b, indicating weaker binding, which coincides with the weaker affinities of wild-type PKD1-C1a–C1b for PDBu and DOG (Table 4). When comparing the C1a and C1b mutants of each PKD isoform, we noted different patterns of ligand selectivity for each C1 domain. For PKD1, the C1a mutant bound DOG with 9-fold weaker affinity as compared to the C1b mutant, although this number may have underestimated the actual difference in affinities, since the $K_d$ for the C1a mutant, 1100 nM, may have exceeded our assay detection limit. However, interestingly, the C1a mutant bound PDBu with a 3-fold stronger affinity than that of the C1b mutant, indicating that the C1a and C1b domains in the intact C1a–C1b domain of PKD1 possess opposite ligand selectivity. The C1a domain showed relative greater affinity for DOG, while the C1b domain bound PDBu preferentially. Taken together, our findings suggest that the C1a and C1b domains of PKD1 exhibit differential selectivity for phorbol ester and DAG analogues.

In contrast, for the PKD3 mutants, the C1b mutant showed remarkably stronger affinity for DOG ($K_d = 7.95 \text{nM}$), the greatest among all the C1 domains analysed. The difference in affinity for DOG between the C1b and C1a mutants was 48-fold. However, the PDBu binding affinities for both mutants were equivalent. Meanwhile, the ligand-binding affinities of C1a in the intact C1a–C1b domain were greater as compared to the isolated C1a domain (Table 1), reflecting the impact of context within an intact C1a–C1b domain on the activities of C1 modules. To confirm that the stronger binding of the C1b mutant of PKD3 to DOG was not an artifact of the P11G mutation, we evaluated a set of alternative mutants, PKD3-C1a–C1b(G23A) and PKD3-C1a(G23A)–C1b, generated in a previous study [21]. Similar to PKD3-C1a–C1b(P11G), PKD3-C1a–C1b(G23A) bound [$^3$H]PDBu with high affinity [$K_d = 0.63 \pm 0.04$ (n = 3)]. Its $K_d$ for DOG was $26.8 \pm 5.5$ (n = 3), comparable with that of PKD3-C1a–C1b(P11G), confirming that the C1a domain in intact PKD3-C1a–C1b possesses high affinity for DAG. In contrast, the C1a mutant, PKD3-C1a(G23A)–C1b, bound DOG with a $K_d$ of $359 \pm 8.5$ (n = 2), comparable to that of PKD3-C1a(P11G)–C1b. Taken together, our data show that the C1a and C1b domains in intact C1a–C1b differ in ligand-binding affinities as compared to their behaviours as isolated single domains, emphasizing that context affects the interactions of C1 modules and ligands. For PKD, this context-dependent modulation confers isoform-specific selectivity to different ligands, providing the basis for differential regulation of PKD isoforms by phorbol esters and DAG.

### Table 5 Analysis of the binding activities of the C1a-C1b mutants of PKD to PDBu and DOG

<table>
<thead>
<tr>
<th>C1a–C1b mutants of PKD</th>
<th>PDBu $K_d$ (nM)</th>
<th>Ratio (C1a mut /C1b mut)</th>
<th>DOG $K_i$ (nM)</th>
<th>Ratio (C1a mut /C1b mut)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKD1-C1a–C1b</td>
<td>3.08 ± 0.79 (3)</td>
<td>1.0/3.2</td>
<td>224 ± 36 (3)</td>
<td></td>
</tr>
<tr>
<td>PKD1-C1a(P11G)–C1b</td>
<td>0.90 ± 0.07(3)</td>
<td>1.0/3.2</td>
<td>1100 ± 53 (3)</td>
<td>8.9/1.0</td>
</tr>
<tr>
<td>PKD1-C1a–C1b(P11G)</td>
<td>2.88 ± 0.38 (4)</td>
<td>1.0/3.2</td>
<td>123 ± 5.1 (3)</td>
<td></td>
</tr>
<tr>
<td>PKD2-C1a–C1b</td>
<td>1.51 ± 0.84 (6)</td>
<td>1.0/3.2</td>
<td>404 ± 85 (4)</td>
<td></td>
</tr>
<tr>
<td>PKD2-C1a(P11G)–C1b</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td>PKD2-C1a(C1b(P11G)</td>
<td>0.44 ± 0.12 (4)</td>
<td>1.0/3.2</td>
<td>208 ± 30 (5)</td>
<td></td>
</tr>
<tr>
<td>PKD3-C1a–C1b</td>
<td>0.40 ± 0.11 (3)</td>
<td>1.0/3.2</td>
<td>57.2 ± 9.7 (4)</td>
<td></td>
</tr>
<tr>
<td>PKD3-C1a(P11G)–C1b</td>
<td>0.49 ± 0.11 (4)</td>
<td>1.0/3.2</td>
<td>456 ± 72 (5)</td>
<td>48.2/1.0</td>
</tr>
<tr>
<td>PKD3-C1a(C1b(P11G)</td>
<td>0.51 ± 0.08 (3)</td>
<td>1.0/3.2</td>
<td>7.95 ± 2.4 (3)</td>
<td></td>
</tr>
</tbody>
</table>
time in cytosolic fractions in response to DOG treatment, while they increased in plasma membrane fractions in a time-dependent fashion. In contrast, levels of the C1a mutant of PKD3–GFP were only slightly increased in the plasma membrane fractions in response to DOG treatment, indicating a significantly weakened response to DOG. These results corroborate our data obtained by live cell imaging and the findings obtained using truncated C1a–C1b mutants, indicating that the C1b domain of PKD3 is a weaker receptor for DOG but not for PDBu, and DOG binding requires an intact C1a motif. Consequently, mutating C1a significantly reduces DOG-induced plasma membrane translocation, while not affecting that induced by PMA. Taken together, our findings further support the selective bindings of individual PKD C1 domains by DAG and phorbol esters.

**Full-length PKD3–GFP translocates more readily to the plasma membrane in response to PMA and DOG treatment**

Our results in Table 4 show differences in affinities of PKD isoforms to phorbol esters and DAG may selectively regulate PKD isoforms. To address the possibility, we evaluated the PMA- and DOG-induced plasma membrane translocation of full-length PKD isoforms in intact cells. The plasma membrane translocation of PKD1–GFP induced by PMA and DOG was visualized by live-cell imaging, and compared to that of PKD3–GFP. As shown in Figure 5, PMA induced time-dependent plasma membrane translocation of PKD3–GFP with concomitant depletion of cytosolic PKD3–GFP, similar to that observed in Figure 4(A). In contrast, PKD1–GFP was translocated to a lesser extent as compared to PKD3–GFP in response to PMA, and by the end of the treatment, the majority of PKD1–GFP remained in the cytosol or was associated with the internal membranes, while little was present on the plasma membrane. Similarly, more rapid and apparent translocation of PKD3–GFP was observed in response to DOG treatment as compared to that of PKD1–GFP. These data indicate that PKD3–GFP is more readily translocated by PMA and DOG to the plasma membrane as compared to PKD1–GFP in intact cells, suggesting that DAG/phorbol esters may interact with PKD3 preferentially.
**DISCUSSION**

The existence of multiple DAG/phorbol ester receptors raises the question of their roles in DAG/phorbol ester-induced cellular responses. Thus, understanding how DAG and phorbol esters regulate these novel receptors will provide important clues as to their biological functions in cells and strategies for their selective manipulation in therapeutics. In this study, we evaluated the structural activity requirements for the binding of C1 domains of PKD by phorbol esters and DAG. Using wild-type and mutants of isolated C1a, C1b or C1a–C1b domains of PKD isoforms, we demonstrated that the C1a and C1b domains were different in their affinities for DAG. The C1b domains of PKD isoforms had weaker affinities for the DAG analogue DOG, and Lys$^{22}$ in C1b was responsible for the reduced binding activity. Our results also demonstrated isoform-specific differences in ligand-binding activities of PKD C1a–C1b domains, with PKD3-C1a–C1b being most active. Accordingly, the full-length PKD3 was more readily translocated to the plasma membrane by PMA and DOG as compared to PKD1. Moreover, the binding activity of individual C1 modules was sensitive to the structural context in which it resided and exhibited unique ligand-binding properties and selectivities in an intact C1a–C1b domain. Studies using full-length protein in intact cells demonstrated that mutating C1a of a full-length PKD3–GFP greatly reduced DOG-induced plasma membrane translocation, but did not significantly impact that induced by PMA. Our study, for the first time, demonstrated differential ligand selectivities of individual C1 domains of PKD, providing the molecular basis for potential isoform-specific regulation of PKD isoforms by C1 ligands, implying a possible functional difference between members of the PKD family in response to phorbol esters and DAG.

The activities of C1a and C1b domains in PKD have been evaluated in several studies, with those in PKD1 being the most studied. Although phorbol ester binding activities of the PKD1 C1 domain have been demonstrated in these studies, it remained uncertain which domain was active. Dissimilar phorbol ester binding activities of the C1a and C1b domains of PKD1 have been described in vivo or in vitro, with cys2 (C1b) responsible for the majority of $[^3]$H]PDBu binding activity [14]. However, analysis of synthetic C1 domains of PKD indicated that the two cysteine-rich motifs of PKD were functionally equivalent ($K_d = 2.5$ or $2.7 \text{ nM}$) [22]. Our data clearly showed that the C1b domains of all PKD isoforms in an isolated state were functional and bound $[^3]$H]PDBu with high affinity, which agrees with most of the studies. Although initial analysis of isolated C1a domains of PKD1 and PKD2 found them to be inactive, they were later confirmed to be active in the intact C1a–C1b domain, and bound phorbol ester with high affinity, implying that the C1a domain actively contributes to ligand binding. Our data clearly show that, despite some sequence divergence from that of a typical C1 domain, the twin C1 modules in the PKD family are both functional and actively engaged in ligand binding.

Using isolated C1a, C1b or C1a–C1b mutants, we have demonstrated consistently reduced binding affinity of the C1b domain of PKD to DOG. The isolated C1b domains of PKD showed a 4-fold weaker affinity for DOG as compared to that of the isolated C1a domains and were even weaker in the intact C1a–C1b domains. A key conserved lysine residue at position 22 was responsible for the reduced DOG affinity. Lys$^{22}$ in PKD C1b represents one of the major structural divergences from a typical C1 domain. Highly-conserved hydrophobic amino acids such as tryptophan, tyrosine or phenylalanine are commonly present at this position. It has been demonstrated that the
backbone of this residue is part of the ligand-binding pocket and its flexible side-chain interacts with different ligands or phospholipids [12,20,23,24]. Thus introducing different side chains at this position could affect ligand selectivity. The W22G mutant of PKCδ-C1b showed reduced ligand/phospholipid-binding affinity [13]. Our findings coincide with a previous study from Blumberg and co-workers showing that Lys22 mutants of PKDδ-C1b (W22Y and W22K) bound DAG analogues with weaker affinities but retained affinities for PDBu [20]. Our findings also coincide with previous research of the binding activity of a full-length PKD1, in which PKD1 was shown to bind the two DAG analogues B8-DL-B8 and 97D76 with over 3-fold weaker affinity as compared to PKCα and PKCδ, a difference that is enhanced under low PS conditions [25].

To evaluate the activities of individual C1 modules in intact C1α–C1b domains, we introduced a P11G mutation to inactivate each C1 domain. The P11G mutation has been widely used to probe the binding activities of individual C1 domains in DAG/phorbol ester receptors. It has been shown that this mutation caused a 125-fold loss in binding affinity in the isolated PKCδ-C1b [13]. The same mutation, when introduced into the C1 domains of full-length PKC (α or δ) shifted the dose–response curve for PMA-induced translocation by approx. 20–30-fold [26,27]. Thus, the P11G mutation causes effective inactivation of C1 domains. However, it is worth noting that the impact of mutating P11 in the C1 domain depends on the residue with which it is replaced. It has been shown that mutating P11 to an arginine in PKCδ-C1b only caused a 4-fold drop in binding activity [28].

To confirm our results obtained using the P11G mutant, we tested an alternative inactivating mutation, G23A. The results obtained using the G23A mutants corroborate those obtained using the P11G mutants, providing further support for our conclusions.

Through mutating the C1 modules in intact C1α–C1b domains, we found that the context in which a C1 module resides has a significant impact on its ligand-binding activity. When comparing the activities of isolated C1 domains and C1 modules in a C1α–C1b domain, we noted that the C1b domain in general exhibited weaker ligand-binding affinities in intact C1α–C1b domains as compared to when it was in an isolated state. In contrast, the C1α domains showed stronger affinities in intact C1α–C1b domains than in an isolated state. This was most apparent in terms of the binding to DOG. When comparing the mutants to the wild-type C1α–C1b domains, it appears that C1b becomes a “less favourable” domain for DAG when C1α is lost, though correspondingly, C1a becomes a “more favourable” binding domain when C1b is lost. Thus, C1a and C1b domains seem to exert opposite effects on each other, i.e. ligand binding to C1a facilitates the binding to C1b, while ligand binding to C1b limits the binding at C1a. However, in determining which domain is more critical in the intact twin C1 domain for ligand binding, our data suggest that C1a is more important, since losing C1a has a more detrimental effect on the overall DAG-binding activities of the twin C1 domain. Our data from analysis of PKD3 C1 domains support this notion since mutants of C1a bound DOG poorly in vitro and translocate less efficiently in response to DOG in cells in vivo.

Our data also indicate that the C1α and C1β domains of PKD1 are opposite in selectivity to phorbol esters and DAG. Differential selectivity of the C1α and C1β domains of PKD1 to DAG and phorbol esters has been implied in several studies, but has not been investigated. It has been demonstrated that the C1β domain bound PDBu with high-affinity and was responsible for the majority of phorbol ester binding [14] and the C1β mutant of PKD1 (P287G) prevented PDBu-induced membrane translocation [29]. Conversely, the C1α domain of PKD1 was required for localization of PKD1 to the trans-Golgi network, and the P155G mutation (equivalent of P11G) in C1α of PKD1 prevented the recruitment of PKD1 to the Golgi [30,31]. Overall, these studies fit with our model that the C1β of PKD1 preferentially mediates a phorbol ester-induced response while the C1α domain is essential for DAG-mediated processes. Our findings are also in line with those of Oancea et al. [32], who demonstrated that the GFP-tagged truncated C1α domain exhibited complete, rapid and reversible plasma membrane translocation in response to stimulation by the GPCR agonist carbachol, indicative of a high-affinity DAG receptor response, whereas the GFP–C1β domain showed only partial, slow and persistent translocation, indicative of a low-affinity receptor response. Thus, the difference in DAG binding affinity between the C1α and C1β domains of PKD1 may provide an additional explanation for the different behaviours of these domains in intact cells. Above all, our study provides the molecular basis for the selective interaction of individual C1 domains of PKD1 with phorbol esters and DAG, implying a potential functional difference as a result of the differential regulation by phorbol esters and DAG.

In the present study, our in vitro binding data based on the truncated C1α–C1β domains indicate that the PKD C1α–C1β domains bind PDBu and DOG with different affinities, with PKD3 C1α–C1β exhibiting the greatest affinities for both ligands (Table 4). These results raise the possibility that the PKD isoforms may be differentially regulated by ligands. Does this occur in vivo to full-length PKD proteins? To address this question, we evaluated the plasma membrane translocation of ectopically-expressed full-length PKD isoforms in response to PMA and DOG treatment. Our results showed that PKD3 was more readily translocated by PMA and DOG as compared to PKD1 in terms of the onset and/or the extent of translocation to the plasma membrane. We also observed a small increase in levels of FLAG–PKD3 in the plasma membrane fractions upon DOG treatment as compared to those of FLAG–PKD2 (J. Chen and Q. J. Wang, unpublished work). These results corroborate our in vitro data and support the notion that PKD3 is a preferred receptor for PDBu and DOG among members of the PKD family. However, since the ectopically-expressed PKD isoforms may behave differently from those of endogenous proteins, and a synthetic DAG analog (DOG) might not fully recapitulate the responses induced by the endogenously generated DAG, it will be important in the future to determine whether DAG generated endogenously acts in a similar manner.

In summary, individual C1 domains of PKD isoforms are functional and exhibit differential selectivity for phorbol esters and DAG. The C1α domain of PKD is more critical for the DAG binding of the twin C1 domains, while the C1β domain in PKD is a low-affinity receptor for DAG but a high-affinity receptor for phorbol esters. Our results also indicate an important role of structural context in modulating the activities of C1 modules. The difference in ligand-binding activity and selectivity for individual C1 domains of PKD may provide a mechanism for selective control of these DAG targets.

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