The Role of C2 Domains in Ca\(^{2+}\)-activated and Ca\(^{2+}\)-independent Protein Kinase Cs in Aplysia*

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In the nervous system of the marine mollusk Aplysia there are two protein kinase C (PKC) isoforms, the Ca\(^{2+}\)-activated PKC Apl I and the Ca\(^{2+}\)-independent PKC Apl II. PKC Apl I, but not PKC Apl II is activated by a short-term application of the neurotransmitter serotonin. This may be explained by the fact that purified PKC Apl II requires a higher mole percentage of phosphatidylserine to stimulate enzyme activity than does PKC Apl I. In order to understand the molecular basis for this difference, we have compared the ability of lipids to interact with the purified kinases and with regulatory domain fusion proteins derived from the kinases using a variety of assays including kinase activity, phorbol dibutyrate binding, and liposome binding. We found that a C2 domain fusion protein derived from PKC Apl I binds to lipids constitutively, while a C2 domain fusion protein derived from PKC Apl II does not. In contrast, fusion proteins containing the C1 domains of PKC Apl I and PKC Apl II showed only small differences in lipid interactions. Thus, while the presence of a C2 domain assists lipid-mediated activation of PKC Apl I, it inhibits activation of PKC Apl II.

Protein kinase Cs (PKC) are a family of lipid-activated enzymes that play important roles in many cellular processes including regulating the strength of synapses in the nervous system (1, 2). This role of PKC has been well conserved over evolution with activation of PKC causing similar changes in vertebrate and invertebrate synapses (1, 3–6). The structure of PKCs has also been well conserved. Ca\(^{2+}\)-activated PKCs (cPKCs) in vertebrates and invertebrates (PKC Apl I and in PKC Apl I in Aplysia) have two C1 domains and a C2 domain. Ca\(^{2+}\)-independent or novel PKCs (nPKCs) in vertebrates (δ, ε, η, and θ) and invertebrates (PKC Apl II in Aplysia) also have two C1 domains and a C2 domain. However, the C2 domain in these kinases is at the N-terminal side of the C1 domain and lacks several of the critical aspartic acids that are required for Ca\(^{2+}\) binding (7).

In cPKCs, the C2 domain confers the ability to bind to acidic lipids in the presence of Ca\(^{2+}\) ions, while the C1 domain confers the ability to bind to lipid in a diacylglycerol (DAG)-dependent manner (8, 9). The DAG-dependent lipid binding to the C1 domain is specific for phosphatidylserine (PS) while binding to other lipids is independent of DAG (10). The two lipid-binding domains act in concert to activate cPKCs by causing a conformational change leading to the release of kinase inhibition by the pseudosubstrate. In contrast to cPKCs, a detailed model for the activation of nPKCs has not been presented. Removing the C2 domain of PKCη does not affect substrate specificity or the ability to be activated by PIP\(_2\) or PIP\(_3\) (11, 12). However, removing the C2 domain of PKC Apl II reduces the levels of PS required for enzyme activation, suggesting that the C2 domain plays an inhibitory role (13). As well, peptides derived from the C2 domains of both cPKCs and nPKCs act as isoform-specific dominant negative inhibitors in cells, suggesting that the physiological function of both kinases requires specific binding of factors to these domains (14, 15).

C2 domains are thought to be important in the translocation of proteins to membranes (7, 8, 16). In Aplysia, short-term treatments with the facilitating transmitter 5-HT can translocate PKC Apl I to membranes, but not PKC Apl II (17). In contrast, inulin can persistently translocate PKC Apl II, but not PKC Apl I to membranes (18). These results suggest that the regulation of enzyme translocation is different in these two PKC isoforms, perhaps due to their distinct C2 domains.

One way to determine the roles of C1 and C2 domains is to study these domains in isolation using fusion proteins containing one or both of these domains. These studies have illustrated that the C1 domains are sufficient to bind to phorbol dibutyrate (PDBu, a pharmacological analog of DAG) in a lipid-dependent manner, and that some C2 domains bind to lipids in a Ca\(^{2+}\)-dependent manner (16, 19–22). Furthermore, there is some evidence that these two domains may interact since addition of the C2 domain to a single C1 domain of PKCγ leads to a decrease in PDBu affinity (21). Similarly, a fusion protein containing both the C2 domain and C1 domains of PKC Apl II has a lower affinity for PDBu than a fusion protein containing only the C1 domains (23). Small amounts of phosphatidic acid (PA) increase the affinity of the C2 domain containing construct for PDBu, suggesting that PA might act specifically to remove the effect of the C2 domain of PKC Apl II (23).

In this paper we have examined lipid interactions of fusion proteins containing C1 or C2 domains of the cPKC Apl I and the nPKC Apl II, using either a direct liposome binding assay or measuring the ability of lipids to stimulate PDBu binding to the fusion proteins. We have compared these results to the ability of lipids to stimulate either PDBu binding or kinase activity of purified preparations of PKC Apl I, PKC Apl II, and
PKC Apl II without its C2 domain. Our results indicate that the major difference between cPKCs and nPKCs is that lipids act through the C2 domain to enhance activity of cPKCs but not nPKCs.

**MATERIALS AND METHODS**

**Reagents**—[4β-Phorbol 12,13-dibutyrate was from LC Services; [3H]β-phorbol 12,13-dibutyrate (19.6 Ci/mmol), NEN Life Science Products Inc.; 1,2-dipalmitoyl-3-[N-methyl-3H]-nicotinic acid (100 mCi/mg), Amersham, Oakville, ON, Canada; dioleoyl phosphatidylserine (PS), dioleoyl phosphatidylethanolamine (PE), dioleoyl phosphatidic acid (PA), and dioctylglycerol (DOG) were from Avanti Polar Lipids Inc., Alabaster, AL; phosphatidylserine (PS), phosphatidylinositol (PI) (mostly linoleic and palmitic acid) from Sigma; Triton X-100 (Pierce); Sephacryl S-100 HR from Pharmacia, Uppsala, Sweden; prestained molecular weight markers, Amersham Corp., and trypsin from Worthington Biochemicals, Freehold, NJ. All other reagents were of the highest grade available.

**Construction of Plasmids Encoding Fusion Proteins**—The generation of GST-Apl II-C1 and GST-Apl II-C2 has been described (23). GST-Apl I C1 domain was made using polymerase chain reaction with VENT polymerase (New England Biolabs, Beverly, MA) from the plasmid expressing PKC Apl I. The 5′-primer was GGGATCCTTATGAGAAGCGCGAGTTGGATCC and the 3′-primer was GACAGTTGCTTGGACGCTCCTTGTGTAG. The amplified product was cut with BamHI and EcoRI and inserted into pGEX-3X (Pharmacia) which had been cut with the same enzymes. This generated a fusion protein with PKC sequences beginning from the initiator methionine of PKC Apl I Met-Glu-Lys-Arg and ending with Gly-Ala-Val-Lys, which included the pseudosubstrate of PKC Apl I at the 5′-terminal strand I of the C2 domain at the 3′-terminal. The pseudosubstrate in PKC Apl I is immediately after the initiator methionine and there is no V1 region in this kinase. Earlier constructs made with shorter boundaries did not produce stable fusion proteins. We had previously made a GST-Apl II-C1 construct including the pseudosubstrate and its behavior was identical to that of the construct without this domain (23). For GST-Apl I-C2, an EcoH1111 fragment excised from the PKC Apl I plasmid and inserted into pGEX3X after cutting the vector with EcoRI and filling in with Klencow. The fusion protein contains PKC sequences beginning with Cys-Glu-Lys-Asn from near the end of the C1 domain to Ser-Ser-Glu-Lys in the hinge region of PKC Apl I, 30 amino acids after the end of strand 8 from the crystal structure. Expression of fusion proteins was as described (23). For each fusion protein preparation, gels were scanned and analysis performed using the public-domain image processing program developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ (NIH-image) to determine the percentage of purified fusion protein at the correct molecular weight and these values (Apl I-C1, 19 ± 3; Apl I-C2, 81 ± 10; Apl II-C1, 26 ± 11; Apl II-C2, 82 ± 20; errors are S.D., n = 3) were used to calculate the stochiometries for fusion protein binding. Much of this degradation occurred during or after proteins from the bacteria were subjected to digestion and was initiated by incubation of the fusion protein in proteolysis buffer and the reaction was allowed to proceed at 30°C for 5 min. The final reaction contained either 50 or 200 ng of protein, 0.1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 2 mM 2-mercaptoethanol, and 0.4, 1, 2, 5, or 10 μg/ml N-tosyl-l-phenylalanyl-chloromethyl ketone-treated trypsin. Proteolysis was quenched by addition of 20 μl of Laemmli sample buffer. Samples were analyzed by SDS-PAGE and Coomassie staining.

**Liposome Binding Assay**—Phospholipids were prepared as described (22). Liposome mixtures consisted of either 1.75 mg of PC or 1.4 mg of PC and 0.35 mg of PA, PS, PI, or PE. Phospholipids were mixed in a 15-m1 tube and dried under a stream of nitrogen. All experiments measuring phospholipid binding used liposome mixtures containing 5% of lipids of 1,2-dipalmitoyl-3-[N-methyl-3H]-nicotinic acid (specific activity 108 mCi/mg) as a tracer. Dried phospholipid mixtures were resuspended in 5 ml of binding buffer (50 mM HEPES, pH 7.2, 100 mM NaCl) by vortexing for 3 min. The phospholipid suspensions were then sonicated for 30 s using a probe sonicator (Vibracell) and centrifuged before use to pellet aggregates. The liposome suspensions were stored at 4°C and used within 1 month.

Recombinant proteins (5 or 30 μg of protein) bound to glutathione-Sepharose beads (standardized to 15 μl wet volume) were prewashed with binding buffer and then added to a reaction mixture containing either 1 mM CaCl2 or 1 mM MgCl2 and 1 mM EGTA, 50 mM HEPES, pH 7.2, 100 mM NaCl and 3H-labeled liposomes (approximately 200,000 cpm) in a total assay volume of 400 μl. The mixture was incubated at room temperature for 15 min, vortexed vigorously and then briefly centrifuged in a tabletop centrifuge. The bound beads were then washed three times with 1 ml of the binding buffer and liposome binding was quantified by liquid scintillation counting of the beads. We confirmed that equal amounts of fusion proteins were used in the assay by visual inspection of Coomasie-stained gels.

**Kinase Assays**—The kinase assay using mixed micelles and an Apl II-derived pseudosubstrate peptide was as described (13). The dependence of phorbol ester binding or kinase activity on the mole % resulting in half-maximal binding, and is the maximum value, k is the mole % resulting in half-maximal binding, and is the Hill coefficient, y = (value)/(x^a + x^n).

**RESULTS**

**Lipid Specificity for Enzyme Activation of PKC Apl I and PKC Apl II**—PKC Apl II requires more PS for activation than does PKC Apl I (13; Table I). To examine the ability of other lipids to activate the two enzymes, we determined the lipid specificity of enzyme activation of PKC Apl I and PKC Apl II in the presence of 100 mM CaCl2 using the 3H-labeled phospholipid assay (27). PS was the most effective lipid for both kinases (Fig. 1A). PA activated PKC Apl I better than PKC Apl II, although the concentration of PI required was significantly higher than that of PS (Fig. 1B). PA only stimulated kinase activity of PKC Apl I and PKC Apl II at high concentrations (Fig. 1, B and C). Neither PE nor PC could stimulate kinase activity.
One possible explanation for the difference between PKC Apl I and PKC Apl II in the amount of PS required for activity could be that PKC Apl I has a higher affinity for DOG than PKC Apl II, since the concentration of DOG affects the amount of PS required for maximal activity (Table I). However, in the presence of saturating amounts of DOG, or using PDBu (150 nM) instead of DOG, the difference in lipid required by the two enzymes is not diminished (Fig. 1D and data not shown).

Although the results above suggested that for both PKC Apl I and PKC Apl II only PS could effectively synergize with DOG to stimulate enzyme activity, it is possible that in the presence of some PS, other lipids would become more effective. We used levels of PS (5 mol % for PKC Apl I and 10 mol % for PKC Apl II) that gave 10–20% of the maximal activity with 1 mol % DOG and compared addition of more PS to addition of other lipids (Fig. 1, E and F). For PKC Apl I, addition of PA was more effective than addition of PS, despite the poor ability of PA to activate PKC Apl I on its own. PI could replace PS at an equimolar level, while PC and PE were ineffective (Fig. 1E).

For PKC Apl II, PA was equivalent to PS, PI was poorer than PS, and PC and PE had no ability to replace PS (Fig. 1F).

These experiments demonstrate several similarities between the isoforms. For both enzymes, PS was the most effective lipid at increasing PKC activity, while PC and PE had no effects on

![Role of C2 Domains in PKC Activation](http://www.jbc.org/)

**TABLE I**

<table>
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<tr>
<th>Enzyme</th>
<th>DOG</th>
<th>Lipid</th>
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<td>Apl I</td>
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<td>PS</td>
<td>1 ± 0.2</td>
<td>1 ± 0.5</td>
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<tr>
<td>Apl II</td>
<td>10</td>
<td>PS</td>
<td>9.2 ± 1</td>
<td>5 ± 1</td>
<td>3</td>
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<tr>
<td></td>
<td>(0.5 mole % PA)</td>
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<tr>
<td>Apl IIΔC2</td>
<td>10</td>
<td>PS</td>
<td>6.5 ± 0.6</td>
<td>4 ± 1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(0.5 mol % PA)</td>
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**FIG. 1. Lipid specificity of enzyme activation.** A, activation of PKC Apl I (white bars) and PKC Apl II (dark bars) in the presence of 1 mol % DOG and 30 mol % of PS, PC, PI, PE, or PA. Activity in the absence of DOG was subtracted. Values are mean ± S.E., n = 3. Activation curves of PKC Apl I (B) or PKC Apl II (C) in the presence of 1 mol % DOG and various concentrations of PS (open circles), PI (open squares), or PA (closed circles). Activity in the absence of DOG was subtracted. Values are mean ± S.E., n = 2–4. D, activation of PKC Apl I (open circles) and PKC Apl II (closed circles) in the presence of 10 mol % DOG and increasing amounts of PS. Activity in the absence of PS was subtracted. Values are mean ± S.E., n = 3. Activation curve of PKC Apl I (E) in the presence of 1 mol % DOG, 5 mol % PS or PKC Apl II (F) in the presence of 1 mol % DOG, 10 mol % PS and increasing amounts of either PS (open circles), PI (open squares), PA (closed circles), PE (closed squares), and PC (open diamonds). The amount of additional lipid added is indicated on the x axis and is different for PKC Apl I and PKC Apl II. The levels of PA added were different than the other lipids, but were the same for PKC Apl I and PKC Apl II. Values are means ± S.E., n = 3–5.
Values are mean ± S.D., n = 3. B, binding of [3H]PDBu (150 nM) to PKC Apl I (open circles) or PKC Apl II (closed circles) at different mole % PS. Values are mean ± S.E., n = 3.

either enzyme. These experiments also revealed several differences between the enzymes: 1) PI was more effective at stimulating PKC Apl I kinase activity than PKC Apl II; 2) PA was more effective at stimulating kinase activity in the presence of small amounts of PS in PKC Apl I than PKC Apl II; and 3) PKC Apl I required less lipid for activation than PKC Apl II.

PKC Apl I and PKC Apl II Require Similar Levels of Lipid to Induce PDBu Binding.—To determine the lipid specificity for PKC binding, we compared the ability of lipids to induce PDBu binding to PKC Apl I and PKC Apl II using the mixed micelle PDBu binding assay (24), since this assay allows us to use the same lipid compositions used to measure kinase activity. In this assay bound [3H]PDBu is separated from free [3H]PDBu by gel filtration since micelles bound to protein and PDBu are larger than micelles bound to PDBu alone. This assay will only detect protein bound to PDBu that is also bound to micelles and thus PDBu binding in this assay is directly related to the ability of lipids to support enzyme binding to the mixed micelles (25, 26, 28).

We found that the lipid specificity for PDBu binding differed from that for kinase activation. In contrast to the superiority of PS in stimulating kinase activity, PA and PI were equally effective at inducing PDBu binding (Fig. 2A). PI supported PDBu binding to a greater extent for PKC Apl II than PKC Apl I, while the reverse was true for enzyme activity. Neither PC nor PE could support PDBu binding.

There was no difference in the $K_{1/2}$ for PS to stimulate PDBu binding between the two isoforms, as opposed to the large difference in the $K_{1/2}$ for PS measured for enzyme activity (Fig. 2B; Tables I and II). Thus, the difference in the amount of PS needed to stimulate enzyme activity of PKCs Apl I and Apl II was not reflected in the ability of PS to stimulate PDBu binding to the two enzymes.

Lipid Specificity for PDBu Binding Can be Attributed to Lipid Specificity of the C1 Domain.—In order to determine whether the lipid specificity of the enzymes could be explained by the lipid specificity of the isolated regulatory domains, we measured PDBu binding to fusion proteins containing the C1 or C2 domains of PKC Apl I and PKC Apl II. Proteins of the correct molecular weight were observed for all constructs (Fig. 3), along with several degradation products the size of GST. Since GST does not bind to PDBu (data not shown), these proteins should not interfere with the PDBu binding assays.

The lipid specificity for PDBu binding to the C1 domains was very similar to that of the purified enzymes (Fig. 4A). The only exception was that PDBu binding to the C1 domains of PKC Apl II was induced more strongly by PA than PS compared with the full-length enzyme. The GST-C2 domain fusion proteins did not bind to PDBu (data not shown). Similar amounts of PS were required for PDBu binding to the C1 domains of PKC Apl I and PKC Apl II. Proteins of the correct molecular weight were observed for all constructs (Fig. 3), along with several degradation products the size of GST. The liposomes interacted with the C1 domains of PKC Apl I and PKC Apl II in a similar manner.

The C2 Domain of PKC Apl I But Not PKC Apl II Binds Directly to Lipid.—Since the C2 domains do not bind to PDBu, we determined their lipid specificity by measuring the ability of liposomes to bind to fusion proteins that were still attached to glutathione beads. This is a well characterized assay that has been used previously to measure Ca$^{2+}$-dependent lipid binding to the synaptotagmin C2 domain and the C2 domain of PKCβ (22, 29). The liposomes are made up of 80% PC and 20% of each of the other lipids. The C2 domain of PKC Apl I bound liposomes containing PS or PA, but not other lipids, while the C2 domain of PKC Apl II bound no liposomes better than the GST control (Fig. 5A). The ability of the PKC Apl I C2 domain to bind to liposomes did not require Ca$^{2+}$, but could be increased by Ca$^{2+}$ ions (Fig. 5A). However, the concentration of Ca$^{2+}$ ions required to stimulate additional lipid binding to the C2 domain was extremely high (1–2 mM Ca$^{2+}$) (Fig. 5B). Nevertheless, lipid binding to the GST control was not affected by this concentration of Ca$^{2+}$ (Fig. 5B). We also compared the ability of the C1 domains to bind liposomes using this assay. Both GST-C1 domain fusion proteins had higher background binding to PC liposomes than did the GST-C2 domain fusion proteins (Fig. 5C). However, the ability of the C1 domains to bind the different liposomes broadly agreed with the specificity of lipids to stimulate PDBu binding to the C1 domains. Liposomes containing PI and PA bound better to the PKC Apl I C1 domains than to the PKC Apl I C1 domains, similar to results with PDBu binding. Lipo-
somes containing PS bound equally well to PKC Apl I and PKC Apl II and PS was also equipotent at inducing PDBu binding to the two C1 domain fusion proteins. The studies of fusion protein-lipid interactions can be summarized as follows. First, the C1 domains of PKC Apl I and PKC Apl II had similar affinities for PS, suggesting that differences in the C1 domains could not explain the differential activation of the two enzymes by PS. Second, PS bound to the C2 domain of PKC Apl I, but not of PKC Apl II, possibly explaining why less PS is required to stimulate PKC Apl I activity than PKC Apl II.

The Role of C2 Domains in PKC Apl I and PKC Apl II—Binding of lipids to the C2 domain could remove an inhibitory effect of the C2 domain, facilitate the effects of PS acting through the C1 domain, or do both. To differentiate between these models we compared the amount of PS required to activate PKC Apl I, PKC Apl II, and a PKC Apl II construct with the C2 domain removed (Apl IIΔC2) (13). If the C2 domain is simply an inhibitory domain, activation of PKC Apl IIΔC2 should require the same amount of PS as that of PKC Apl I, since the C1 domains of PKC Apl I and PKC Apl II have similar interactions with PS. In fact, PKC Apl IIΔC2 required an intermediate amount of PS, less than that of PKC Apl I, but more than that of PKC Apl II, both at low and high concentrations of DOG (Table I). This finding suggests that the C2 domain has both effects, inhibiting PKC Apl II but also facilitating activation of PKC Apl I. This analysis was complicated, however, by the fact that PS is acting both at the C1 domain and the C2 domain of PKC Apl I.

PA specifically reduces the inhibitory effect of the C2 domain in PDBu binding experiments with a fusion protein containing both the C1 and C2 domains of PKC Apl II (23), but our data suggest that PA does not interact with the C2 domain directly (Fig. 5A). To investigate whether PA binding to the C1 domain of PKC Apl II could specifically remove the inhibition of enzyme activity mediated by the C2 domain, we examined the ability of PA to decrease the concentration of DOG required to activate PKC Apl II and PKC Apl IIΔC2. Indeed, deletion of the C2

FIG. 3. Expression of C1 and C2 domain fusion proteins. A, Coomassie-stained gel of fusion proteins after elution from glutathione beads. 6 μg of each purified protein preparation was run in each lane. Lane 1, GST-Apl I C1; Lane 2, GST-Apl II C1; Lane 3, GST-Apl I C2; Lane 4, GST-Apl II C2. B, Coomassie-stained gel of fusion proteins before elution from glutathione beads. 10 μg of each purified protein preparation was run in each lane. Lane 1, GST; Lane 2, GST-Apl I C1; Lane 3, GST-Apl II C1; Lane 4, GST-Apl I C2; Lane 5, GST-Apl II C2.

FIG. 4. Phospholipid dependence of PDBu binding to GST-C1 fusion proteins. A, binding of [3H]PDBu (150 nM) to GST-Apl I-C1 (white bars) or GST-Apl II-C1 (dark bars) in the presence of 20 mol % of PS, PC, PI, PE, or PA. Values for GST-Apl II-C1 are from Pepio and Sossin (23). Values are mean ± S.D., n = 3. Binding of [3H]PDBu to GST-Apl I-C1 (open circles) and GST-Apl II-C1 (closed circles) at various concentrations of PS (B). Values are mean ± S.E., n = 3.

FIG. 5. Phospholipid dependence of liposome binding by the C1 and C2 domains of PKC Apl I and PKC Apl II. A, GST (open bars), GST-Apl I-C2 (hatched bars), or GST-Apl II-C2 (dark bars) were incubated with 3H-labeled liposomes composed of PC only or PC mixed with 20% PS, PA, PI, or PE in the presence of 1 mM MgCl₂ (−) or 1 mM CaCl₂ (+). Values are mean ± S.E. (n = 3–4). B, binding of the GST-Apl I-C2 domain at different concentrations of Ca²⁺ to the liposomes was measured. Note that in the presence of EGTA (0 Ca²⁺) there is higher background binding to GST and higher binding to GST-Apl I-C2 compared with binding in the presence of 1 mM MgCl₂ as seen in A. Values are mean ± S.E., n = 3. C, binding of GST (open bars), GST-Apl I-C1 (hatched bars), and GST-Apl II-C2 (dark bars) to 3H-labeled liposomes composed of PC only or PC mixed with 20% PS, PA, PI, or PE. There were no differences in binding using 1 mM MgCl₂ or 1 mM CaCl₂ and results were pooled. Values are mean ± S.E. (n = 3).
Role of C2 Domains in PKC Activation

A small amount of PA (0.5 mol %) has a large effect on PDBu binding to both the C1-C2 domain PKC Apl II fusion protein and the purified enzyme in the presence of PS (23). Thus, although adding PA had the same effect as removing the C2 domain, the effect of PA was not occluded by removing the C2 domain.

Another way of testing the hypothesis that lipid binding to the C2 domain in PKC Apl I facilitates the effects of PS acting through the C1 domain is to examine the role of PA, which interacts with the C2 domain of PKC Apl I, but not PKC Apl II, and does not synergize with DOG to stimulate enzyme activity through the C1 domain of either enzyme (Fig. 1). In the presence of saturating levels of DOG and PA, PKC Apl I required less PS for activation than did PKC Apl II or PKC Apl IIΔC2 (Fig. 7). This experiment illustrates both the inhibitory role of the C2 domain in the absence of an effector (PA) at the C2 domain (PKC Apl II versus PKC Apl IIΔC2) and the facilitatory role of the C2 domain in the presence of an effector (PA) at the C2 domain (PKC Apl I versus PKC Apl IIΔC2). In the presence of PA, the $K_{d}$ for PS in PKC Apl I was near 1 mol % (or approximately two molecules in a Triton X-100 micelle) (Table I), suggesting that only a few PS molecules are specifically required to act at the C1 domain in synergy with DOG in the presence of lipids interacting with the C2 domain.

Effect of Ca$^{2+}$ on PKC Apl I—The C2 domain of PKC Apl I contains the aspartic acid residues predicted to confer Ca$^{2+}$ specificity, while the C2 domain of PKC Apl II does not. Indeed, Ca$^{2+}$ activated PKC Apl I, but not PKC Apl II which was actually inhibited at high concentrations of Ca$^{2+}$ ions (Fig. 8, A and B and Ref. 13). Under conditions of limiting PS and DOG, the $K_{d}$ for Ca$^{2+}$ was 390 nm and the Hill coefficient was 2 (Fig. 8A). Although this concentration is high compared with vertebrate PKCs, it is lower than the amount of Ca$^{2+}$ required to increase lipid binding to the isolated C2 domain (Fig. 4B). As another approach to examine the effect of the C2 domain in PKC Apl I, we examined the effects of Ca$^{2+}$ on the levels of PS and DOG required for activity. The effect of Ca$^{2+}$ is to lower the lipid requirement of PKC Apl I at constant DOG, or to decrease the requirement for DOG at constant lipid (Fig. 8, C and D). However, PKC Apl I did not require Ca$^{2+}$ for activity and the presence of Ca$^{2+}$ did not increase the maximal amount of PKC activity at saturating concentrations of PS and DOG (Fig. 8C).

**DISCUSSION**

Lipid Specificity of PKC Apl I—The lipid specificity for stimulation of cPKCs *in vitro* has been well studied (8, 10, 28, 30–34). Both Ca$^{2+}$ and DOG independently act to increase the affinity of the enzyme for PS independently, presumably through C1 domains (DOG) and C2 domains (Ca$^{2+}$) (8). These results led to a model where the C1 domain and C2 domain act independently to translocate the enzyme to the membrane, where specific binding to PS activates the enzyme by inducing a conformational shift of the pseudosubstrate (8). These studies also distinguished binding of the enzyme to membranes, which was specific for acidic lipids and not specific for PS from the synergy with DAG which was specific for PS (10). Our results with PKC Apl I are in general agreement with these results. PS stimulates enzyme activity in the presence of DOG much better than PA despite the ability of PA to bind to both C1 and C2 domains as well as, if not better than, PS. One major difference

**TABLE III**

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<th>Enzyme</th>
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<td></td>
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**Fig. 6. Effect of PA PKC Apl II.** Activation of PKC Apl II (A) or PKC Apl IIΔC2 (B) in the presence of 16 mol % PS and increasing amounts of DOG in the presence (closed circles/squares) or absence (open circles/squares) of 2.5 mol % PA. Values are mean ± S.E., n = 2. C, activation of PKC Apl II (circles) or PKC Apl IIΔC2 (squares) in the presence of 10 mol % DOG in the presence (closed) or absence (open) of 0.5 mol % PA. Activity in the absence of PS was subtracted. Values for PKC Apl II in the absence of PA are the same as in Fig. 1D. Values are mean ± S.E., n = 3.

**Fig. 7. Role of the C2 domain in kinase activation.** Activation of PKC Apl I (open circles), PKC Apl II (closed circles), and PKC Apl IIΔC2 (open squares) in the presence of 10 mol % DOG, 5 mol % PA, and increasing amounts of PS. Activity in the absence of PS was subtracted. Values are mean ± S.E., n = 2.
between PKC Apl I and vertebrate cPKCs in vertebrates is that Ca\textsuperscript{2+} is not necessary for activation of PKC Apl I in the mixed micelle kinase assay. This may be explained by the ability of the C2 domain of PKC Apl I, but not of vertebrate cPKCs (16, 29) to bind to lipids in the absence of Ca\textsuperscript{2+} ions.

**Lipid Specificity of PKC Apl II**—There have been few studies examining the lipid specificity for activation of nPKCs in vitro using the mixed micelle assay or comparing cPKCs and nPKCs in a systematic manner. Our results suggest that the lipid specificity conferred by the C1 domain is similar but not identical to that of cPKCs. Similar to cPKCs, PS is the most effective lipid for stimulating enzyme activation of PKC Apl II and PA is more effective at binding the enzyme than it is at stimulating enzyme activity. In contrast, PI had different effects on PKC Apl II and PKC Apl I. PI was better at stimulating enzyme activity in PKC Apl I than PKC Apl II, but better at inducing PDBu binding to PKC Apl II than PKC Apl I.

**Role of PA in Activation of PKC Apl II**—Previously, we had suggested that PA may specifically activate PKC Apl II by removing inhibition mediated by the C2 domain (23). Our results demonstrate that this is not the case, since effects of PA on enzyme activity are similar in constructs with or without the C2 domain. Previous studies also suggested a role for PA in binding of PDBu to PKC Apl II (23). Our results are consistent with a role for PA in activation of PKC Apl II. First, PA bound to the C1 domain of PKC Apl II better than to the C1 domain of PKC Apl I (Fig. 4). Second, at saturating levels of DOG, 0.5 mol % of PA (approximately 1 molecule in a Triton X-100 micelle) significantly reduced the amount of PS required for PKC activity from a K\textsubscript{m} of 14 to a K\textsubscript{m} of 9 mol % (Table I). There was also not much of an additional effect after raising PA from 0.5 to 5 mol % (Table I). Additionally, PA was very effective at replacing PS in PKC Apl I, but since PA interacts with the C2 domain of PKC Apl I the results are hard to compare.

There is a specific requirement for PS in enzyme activation for both PKC Apl I and PKC Apl II. This agrees with the ability of DOG to increase affinity for PS, but not other lipids in both vertebrate cPKCs and nPKCs (10). This requirement for PS is not conferred solely by the C2 domain since DOG increases affinity for PS even when the C2 domain has been mutated to ablate lipid binding (35) and there is still specificity for activation by PS in PKC Apl II without the C2 domain (data not shown). However, our results suggest that other lipid-binding sites that are not specific for PS are also important for enzyme activation, and that PA can replace PS at these sites. Furthermore, since the PS levels in *Aplysia* are somewhere between 12 and 18 mol % (36), the effect of PA on the C1 domain of PKC Apl II may be important for physiological enzyme activation. At these levels of PS, addition of 0.5 mol % PA can double enzyme activity (Fig. 6C).

**Role of C2 Domains**—The difference in the amount of PS required for stimulation of PKC Apl I compared with PKC Apl II can be explained by lipid binding to the C2 domain of PKC Apl I, but not that of PKC Apl II. Our data are consistent with the idea that lipid binding to the C2 domain increases the ability of lipid binding to the C1 domain to induce the conformational shift required for enzyme activity. Whether binding to the C2 domain is necessary to remove an inhibitory effect of the C2 domain in cPKCs as in nPKCs was difficult to determine from our experiments, since the PS required for binding to the C1 domain of PKC Apl I also bound to the C2 domain. However, the requirement of Ca\textsuperscript{2+} for PKC activity in cPKCs from vertebrates is consistent with an inhibitory effect of the C2 domain in these isoforms. Mutating the Ca\textsuperscript{2+}-binding site in a vertebrate cPKC greatly increases the amount of lipid required for enzyme activity (34). It will be interesting to see if a similar amount of lipid would be required after removal of the C2 domain. Our results with PKC Apl II suggest that less lipid would be required to activate cPKCs when the C2 domain is absent compared with when it is present and cannot bind lipid.

The lipids examined did not bind to the C2 domain of PKC Apl II. While we cannot rule out the possibility that this was due to incorrect folding of the fusion protein, it is unlikely given the stability of this fusion protein (Fig. 3). Furthermore, the pattern of partial trypsin cleavage of the C2 domain is similar regardless of whether the C2 domain is derived from purified...
PKC Apl II or the GST-C2 domain fusion protein (Fig. 9), suggesting that the domain is folded similarly in both proteins. Our results examining kinase activation with and without the C2 domain are also consistent with the lack of lipid binding to this domain. Removing the C2 domain allows PKC Apl II to be activated at lower concentrations of PS, but this difference is not alleviated by increasing DOG concentration or adding PA since these compounds decrease the amount of PS required in PKC Apl II without the C2 domain to a similar extent. These experiments argue against a model where lipids are acting through the C2 domain of PKC Apl II to facilitate enzyme activity. Moreover, there have been no reports of lipid binding to the related C2 domain of PKCe or PKCη, although it has been speculated that they bind lipids constitutively (8), or that they bind to hydrophobic lipids due to the prevalence of hydrophobic residues in the loops corresponding to the calcium-binding regions (CBRs) (37). Other lipid activators of PKC like 
PPIP2, PIP3, or oleic acid stimulate nPKC activity similarly in binding regions (CBRs) (37). Other lipid activators of PKC like phobic residues in the loops corresponding to the calcium-binding sites (38), although it has not yet been shown that the presence of this protein can increase PKC activity.

The Physiological Role of Ca2+ in Stimulating PKC Apl I—Although the amount of Ca2+ required for stimulation of PKC Apl I does not appear to be physiological, it is still possible that under some conditions increases in Ca2+ concentrations are important for activation of PKC Apl I since the amount of Ca2+ required is also dependent on levels of PS and DOG. For example, during pathological events such as axonal damage, Ca2+ concentrations may reach the required level. Short treatments of serotonin activate PKC Apl I (17), but do not appear to raise Ca2+ levels significantly in the absence of action potentials (39). It is interesting that unlike several vertebrate isoforms (27), PKC Apl I does not require Ca2+ for activity in the mixed micelle assay. This parallels the ability of the C2 domain of PKC Apl I to bind to lipid in the absence of Ca2+, while isolated C2 domains from enzymes that require Ca2+ do not bind lipid in the absence of Ca2+ (22). The difference in the C2 domain that confers this change is not clear. There are three regions of the C2 domain that are defined as CBRs from crystallographic studies (7, 37, 40). CBR1 and CBR3 are extremely well conserved between PKC Apl I and vertebrate cPKCs. In contrast, CBR2 is quite different in PKC Apl I compared with vertebrate kinases (Fig. 10). The crystal structure of the cPKC C2 domain has not yet been solved, but CBR2 is important for Ca2+ coordination in cPLA2 and PLCβ1, but not synaptotagmin. Recently, the autophosphorylation of a serine near the carboxyl-terminal of a vertebrate cPKC has been shown to alter the concentration of Ca2+ required for PKC activation, suggesting that interactions between the C2 domain and the carboxyl terminus of the enzyme occur (41). This serine is conserved in PKC Apl I. A role for the carboxyl terminus may explain the large difference in the amount of Ca2+ required for C2 domain binding alone, compared with the amount of Ca2+ required to stimulate enzyme activity.

The Physiological Role of Lipids in Stimulating PKC Apl II—In Aplysia activation of PKC by serotonin leads to the reversal of synaptic depression, but does not play a role in the augmentation of synaptic responses also mediated by serotonin (42). In contrast, PDBu causes both augmentation and the reversal of synaptic depression (43). Short applications of serotonin activate PKC Apl I, but not PKC Apl II leading to a model whereby PKC Apl II may lead to augmentation stimulated by serotonin (31). Moreover, addition of serotonin to the bag cell neurons of Aplysia transiently activates PKC Apl I, but then later activates PKC Apl II (18). These results suggest that a transient increase in DAG, either through G-protein-activated phospholipase C-β or tyrosine kinase-activated phospholipase C-γ is sufficient to activate PKC Apl I, but not PKC Apl II which appears to require an additional stimulus. The activation of PKC Apl II by insulin is blocked by wortmannin which implies a role for either lipids downstream of PI3-kinase or PA downstream of phospholipase D (18) in activation of PKC Apl II. Either lipid may help activate PKC Apl II through binding to the C1 domain, but perhaps more likely, these stimuli may lead to the production of a signaling molecule that can interact with the C2 domain of PKC Apl II and activate the enzyme. Identifying this molecule will be important in the study of PKC regulation.

REFERENCES


Fig. 9. Trypsin digests of PKC Apl II and GST-C2. PKC Apl II (200 ng), or GST-Apl II-C2 (50 ng) were cleaved with increasing concentrations of trypsin (0, 0.4, 1, 2, 5, and 10 μg/ml), separated on a 17% SDS-polyacrylamide gel electrophoresis gel, and immunoblotted with an antibody specific for the C2 domain of PKC Apl II. 0.4 μg/ml trypsin was sufficient to cleave the C2 domain from both PKC Apl II and the GST-C2 domain fusion protein (arrowheads). Increasing concentrations of trypsin show a similar pattern of fragmentation whether the C2 domain is derived from PKC or the GST fusion protein (arrowheads). The predicted size of the C2 domain core is 17.5 kDa. The antibody exhibited a cross-reaction with trypsin seen at the highest concentrations of trypsin, but did not cross-react with GST.

Fig. 10. Comparison of the C2 domain of PKC Apl I and vertebrate C2 domains. Strand positions and CBRs are as described (7, 37). Shaded residues are conserved in all isoforms. D represents conserved aspartic acids involved in Ca2+ binding. Asterisks emphasize the difference in CBR2 between PKC Apl I and vertebrate cPKCs.

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Mr (kD) | \( \text{PKC Apl II} \) | \( \text{GST} \) | \( \text{Trypsin} \) | \( \text{Increasing Trypsin} \) | \( \text{PKC Apl II} \) | \( \text{PKC Apl II-C2} \)
---
96 | 66 | 46 | 21 | 14

PKCa
PGRDKLILIGAVKG-SKVLGEILENCAGLPRCT
PKKb
PGRDKLILIGAVKG-SKVLGEILENCAGLPRCT
PKCb
PGRDKLILIGAVKG-SKVLGEILENCAGLPRCT
PKCe
PGRDKLILIGAVKG-SKVLGEILENCAGLPRCT
APL
PGRDKLILIGAVKG-SKVLGEILENCAGLPRCT

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Strand 1 | Strain 2 | Strain 3 | Strain 4 | Strain 5 | Strain 6
---

PKCa
GRLGLLEIRPTADEIN-YEVCG-RRCFQFVTVK
PKKb
GRLGLLEIRPTADEIN-YEVCG-RRCFQFVTVK
PKCb
GRLGLLEIRPTADEIN-YEVCG-RRCFQFVTVK
PKCe
GRLGLLEIRPTADEIN-YEVCG-RRCFQFVTVK
APL
GRLGLLEIRPTADEIN-YEVCG-RRCFQFVTVK

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CBR1
HydAHKL-GLYVEFOXAVK-PYV
CBR2
HydAHKL-GLYVEFOXAVK-PYV
CBR3
HydAHKL-GLYVEFOXAVK-PYV
CBR4
HydAHKL-GLYVEFOXAVK-PYV
CBR5
HydAHKL-GLYVEFOXAVK-PYV
CBR6
HydAHKL-GLYVEFOXAVK-PYV

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Role of C2 Domains in PKC Activation

Additions and Corrections


The role of C2 domains in Ca\(^{2+}\)-activated and Ca\(^{2+}\)-independent protein kinase Cs in aplysia.

Antonio M. Pepio, Xiaotang Fan, and Wayne S. Sossin

Page 19047, Fig. 10: CBR2 was incorrectly assigned to the region between strand 3 and strand 4. There is, thus, not a large difference between the CBR2 domain of APL1 and those of the other PKCs, contrary to the original statement. The correct Fig. 10 is shown below.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
The Role of C2 Domains in Ca\textsuperscript{2+}-activated and Ca\textsuperscript{2+}-independent Protein Kinase Cs in Aplysia

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