The C2 Domain of the Ca^{2+}-Independent Protein Kinase C Apl II Inhibits Phorbol Ester Binding to the C1 Domain in a Phosphatidic Acid-Sensitive Manner†

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ABSTRACT: There are two protein kinase Cs (PKCs) in the *Aplysia* nervous system, PKC Apl I, which is homologous to the Ca^{2+}-activated PKC family, and PKC Apl II, which is homologous to the Ca^{2+}-independent PKCs ϵ and η. Purified PKC Apl I requires much less phosphatidylserine for activation than does purified PKC Apl II, and this may explain why the neurotransmitter serotonin activates PKC Apl I but not PKC Apl II in the intact nervous system [Sossin, W. S., Fan, X., and Baseri, F. (1996) *J. Neurosci. 16*, 10−18]. PKC Apl II's requirement for high levels of phosphatidylserine may be mediated by its C2 domain, since removal of this domain allows PKC Apl II to be activated at lower concentrations of phosphatidylserine. To begin to understand how this inhibition is mediated, we generated fusion proteins containing the C1 and C2 domains from PKC Apl II and determined their lipid dependence for phorbol ester binding. Our results indicate that the presence of the C2 domain lowers the affinity of protein kinase C activators for the C1 domains and this inhibition can be removed by phosphatidylserine. Phosphatidic acid, however, is much more potent than phosphatidylserine in reducing C2 domain-mediated inhibition, suggesting that phosphatidic acid may be a required cofactor for the activation of PKC Apl II.

The C1 and C2 domains are conserved amino acid modules first identified in the protein kinase C (PKC)[1] family (*1*, *2*). This family contains both Ca^{2+}-activated and Ca^{2+}-independent members, both of which are activated by the combination of diacylglycerol (DAG) and phosphatidylserine (PS). DAG, and its pharmacological analogues phorbol esters, bind to the C1 domain, while PS may act through both C1 and C2 domains (*3*). In Ca^{2+}-activated PKCs, C2 is on the carboxyl-terminal side of C1 and confers Ca^{2+} sensitivity to the enzyme through Ca^{2+}-dependent binding to PS (*3*−*5*). In some Ca^{2+}-independent PKCs, typified by vertebrate PKCϵ and invertebrate PKC Apl II, C2 is on the amino-terminal side of C1 and lacks the critical aspartic acids required for Ca^{2+} binding (*5*, *6*). It has been suggested that the C2 domain of Ca^{2+}-independent PKCs binds to PS constitutively in the absence of Ca^{2+} to help activate these kinases (*5*). However, studies with the *Aplysia* isoform, PKC Apl II, suggest that C2 plays an inhibitory role, since removing this domain lowers the concentration of PS required to activate the kinase (*7*).

A common function for the C2 domain has not yet been elucidated despite the large number of identified C2 domains (*8*) and the considerable amount of structural information about these domains (*9*, *10*). Some C2 domains, like those of synaptotagmin, Ca^{2+}-activated PKCs, and phospholipase A_2, bind to lipid in a Ca^{2+}-dependent manner, suggesting that C2 domains help translocate proteins to an appropriate lipid environment (*4*, *11*, *12*). However, since not all C2 domains bind to Ca^{2+} ions and the lipid-binding sequence of C2 domains is not well-defined, the lipid specificity of most C2 domains is not known. Several C2 domains are involved in protein−protein interactions, but no consensus sequence that these domains bind to has been defined (*13*−*15*). Finally, several C2 domains bind to inositol polyphosphates, but only a few C2 domains contain the consensus sequence for inositol polyphosphate binding (*16*, *17*). Elucidating the role of the C2 domain in Ca^{2+}-independent PKCs, like PKC Apl II, should help define the signal transduction pathway that regulates these enzymes.

In the *Aplysia* nervous system PKC Apl II is activated downstream of receptor tyrosine kinase (RTK) activation but not by stimulation of G protein-activated phospholipase C (*18*−*20*). Vertebrate PKCϵ is also persistently activated by signals acting through RTKs (*21*−*23*), suggesting that this regulation is due to domains conserved between the two proteins. This activation may be mediated by $PI_{3,4}P_2$ produced by RTK-activated PI-3 kinase (*24*, *25*) or by DAG produced through RTK-activated phospholipase D (PLD) and phosphatidic acid phosphohydrolase (*23*, *26*).

In the present study, we constructed fusion proteins containing different domains of the PKC Apl II regulatory region and compared their ability to bind phorbol ester. Our results strongly confirm the inhibitory model of C2 domain function, since fusion proteins that contain the C2 domain have an affinity for phorbol ester that is highly dependent

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[1] Abbreviations: PKC, protein kinase C; PS, phosphatidylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG; phosphatidylglycerol; PI, phosphatidylinositol; DOG, dioctylglycerol; DAG, diacylglycerol; RTK, receptor tyrosine kinase; PDBu, phorbol dibutyrate; GST, glutathione-S-transferase; PLD, phospholipase D; RACK, receptor for activated C kinase.

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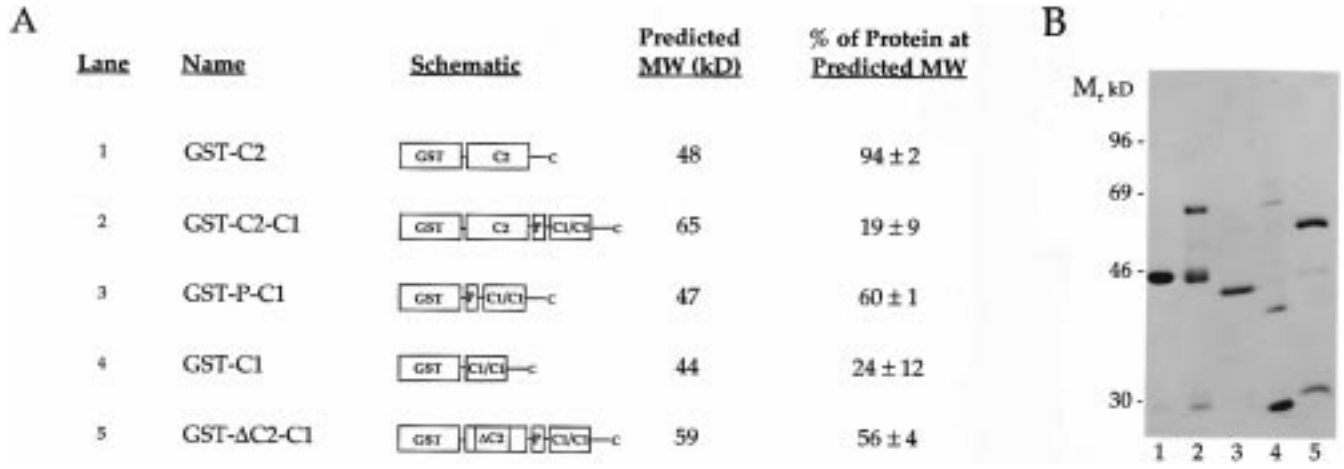

FIGURE 1: Characterization of GST fusion proteins. (A) Schematic of GST fusion proteins along with the expected molecular mass and the percentage of purified protein migrating at the correct molecular mass. (B) GST fusion proteins GST−C2 (lane 1), GST−C2−C1 (lane 2), GST−P−C1 (lane 3), GST−C1 (lane 4), and GST−ΔC2−C1 (lane 5) after separation by SDS−12% PAGE and subsequent staining with Coomassie Blue.

on PS concentration, while fusion proteins that lack this region do not. Furthermore, phosphatidic acid (PA), the product of PLD, removes inhibition mediated by the C2 domain at concentrations far lower than those of PS, suggesting that Ca^{2+}-independent PKCs containing these C2 domains are stimulated by PA.

MATERIALS AND METHODS

Reagents. 4β-Phorbol 12,13-dibutyrate was from LC Services; [^{3}H]-4β-phorbol 12,13-dibutyrate (19.6 Ci/mmol) was from New England Nuclear (Boston, MA); dioleoylphosphatidylserine (PS), dioleoylphosphatidylcholine (PC), distearylphosphatidylglycerol (PG), dioleoylphosphatidylethanolamine (PE), and dioleoylphosphatidic acid (PA) were from Avanti Polar Lipids Inc. (Alabaster, AL); phosphatidylinositol (PI) (mostly linoleic and palmitic acid) was from Sigma (St. Louis, MO); Triton X-100 was from Avanti; Sephacryl S-100 HR was from Pharmacia (Uppsala, Sweden); and prestained molecular weight markers were from Amersham, Oakville (ON). All other reagents were of the highest grade available.

Construction of Plasmids Encoding Fusion Proteins. Apl II cDNA or Apl IIΔC2 cDNA (*7*) was digested with *Sma*I/*Bgl*II, filled in with Klenow, isolated, and ligated into the *Sma*I-digested pGEX-5X-1 plasmid (Pharmacia LKB Biotechnologies Inc., Piscataway, NJ) to generate GST−C2−C1 and GST−ΔC2−C1, respectively. Apl II cDNA was digested with either *Xmn*I/*Bgl*II or *Hin*cII/*Bgl*II, filled in with Klenow, isolated, and ligated into the pGEX-3X plasmid cut with *Eco*RI and filled in with Klenow to generate GST−P−C1 and GST−C1, respectively. Apl II cDNA was digested with *Sma*I/*Xmn*I and ligated into pGEX-3X cut with *Sma*I to generate GST−C2.

Expression of Fusion Proteins. DH5 *Escherichia coli* with plasmids encoding the fusion proteins were grown in LB medium supplemented with 1 μm $ZnSO_4$ at 27 °C to an A_{600} = 0.3−0.4. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.25 mM to induce protein expression and the cultures were grown for an additional 18 h. Cells were pelleted and resuspended in 50 mL of ice-cold phosphate-buffered saline (PBS) with 5 mM 2-mercaptoethanol, 20 μg/mL aprotinin, 5 mM benzamidine, and 0.1 mM leupeptin, sonicated 6 × 10 s with a probe sonicator (Vibracell, Sonics and Materials, Danbury, CT), and incubated in 1% Triton X-100 for 20 min, and the debris was pelleted by centrifugation in a Sorvall RCB2 centrifuge at 12000*g* using an SS34 rotor at 4 °C. The supernatant was loaded onto 1 mL glutathione−Sepharose columns washed with 20−30 volumes of PBS and eluted with reduced glutathione (100 mM Tris, 20 mM glutathione, pH 8.0, and 120 mM NaCl). For each fusion protein preparation, gels were scanned and analysis was performed using the public domain NIH Image 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 (Figure 1A), and these values were used to calculate the stoichiometries for fusion protein binding.

[^{3}H]PDBu-Binding Assay. Fusion proteins were assayed for [^{3}H]PDBu binding by the mixed micelle method (*27, 28*). The 50-μL reaction mixture consisted of 20 μM Tris, pH 7.5, 200 μM $CaCl_2$ or $MgCl_2$, and mixed micelles (0−40 mol %) of phospholipids in a final concentration of 0.6% Triton X-100, [^{3}H]PDBu from 1 to 250 nM, and fusion protein ranging from 0.02 to 1.6 μg. Mixed micelles were prepared by drying the appropriate volume of each lipid under nitrogen, resuspending in 3% Triton X-100, and vortexing for 1 min, followed by incubation at 30 °C for 10 min. Reactions were started by addition of fusion protein and were allowed to proceed for 10 min at room temperature. Reaction tubes were then placed on ice and bound PDBu was separated from free PDBu on Sephacryl S-100 HR gel-filtration columns (2-mL column volume) at 4 °C by washing with equilibration buffer (20 μM Tris, pH 7.5, 0.015% Triton X-100, and 200 μM either $CaCl_2$ or $MgCl_2$). The nonspecific binding component for all experiments was measured in the presence of 10 μM unlabeled PDBu and was subtracted from the total binding to yield the specific binding. No differences were seen in experiments using 200 μM $CaCl_2$ or 200 μM $MgCl_2$.

Quantitation of Data. The dependence of phorbol ester binding on lipid content of micelles was analyzed by a nonlinear least-squares fit, using Systat 5.0, to a modified Hill equation (*28, 29*) $y = a[x^n/(k^n + x^n)]$, where y is the

PDBu binding value, *a* is the maximum PDBu binding value, *x* is the concentration of PS, *k* is the concentration of PS resulting in half-maximal binding, and *n* is the Hill coefficient. K_d and B_{max} values for the Scatchard analysis were calculated with the EBDA binding program (*30*).

RESULTS

Characterization of Fusion Proteins. To determine if the C2 domain of Ca^{2+}-independent PKCs affects phorbol ester binding to the C1 domain, we constructed a number of glutathione-S-transferase (GST) fusion proteins (Figure 1A) containing regulatory regions of PKC Apl II including the C2 domain alone (GST−C2), the C1 domain alone (GST−C1), the pseudosubstrate and the C1 domain (GST−P−C1), the entire regulatory domain (GST−C2−C1), and a deletion of the central core of the C2 domain (GST−ΔC2−C1). Proteins of the correct molecular weight were observed for all constructs (Figure 1A,B), along with several degradation products the size of GST−C2 (lanes 2 and 5) or GST (all lanes) (Figure 1B). Since neither GST nor GST−C2 bound to PDBu (data not shown), these proteins should not interfere with the PDBu binding assays.

Dependence of PDBu Binding on Phosphatidylserine Concentration. All experiments used the mixed micelle PDBu binding assay, since this assay allows for the measurement of PDBu binding over a wide range of lipid types and concentrations (*27*). In this assay bound ^{3}H-PDBu is separated from free ^{3}H-PDBu by gel filtration since micelles bound to protein and PDBu are larger than the micelles bound to PDBu alone. Nonspecific association of protein and PDBu is determined in the presence of an excess of cold PDBu. This assay will only detect protein bound to PDBu that is also bound to micelles and thus will not detect PDBu binding that is independent of lipids (*31*). This assay has been used extensively to determine the lipid dependence for phorbol ester binding of Ca^{2+}-activated PKCs and of GST regulatory domain fusion proteins (*28, 29, 32−34*). Even at saturating concentrations of PS and PDBu, much less than one molecule of PDBu binds to each molecule of fusion protein (Tables 1 and 2), suggesting that most of the fusion protein is not correctly folded and thus cannot bind PDBu. Similar results are seen with all other PKC regulatory region fusion proteins, and since the unfolded fusion proteins do not bind to PDBu, they should not interfere with this assay (*31, 33*).

As expected from previous studies with Ca^{2+}-activated PKCs, PDBu bound to both GST−C1 and GST−C2−C1 in a PS-dependent manner (*27, 29, 32, 33, 35*) (Figure 2A,B). The $K_{1/2}$ values for PS were similar for PDBu binding to GST−C1 and GST−C2−C1 (Figure 2A,B; Table 1). However, GST−C2−C1 bound less PDBu when the concentration of PDBu was low (20 nM compared to 150 nM), while GST−C1 bound similar amounts of PDBu at the two different concentrations (Figure 2A,B; Table 1). This suggests that the C2 domain affects the affinity of the fusion protein for PDBu. Moreover, the fusion protein containing the C2 domain showed an increase in the cooperativity of PS needed for PDBu binding, indicating a requirement for more molecules of PS (Figure 2C; Table 1). Both of these findings are consistent with an inhibitory role for the C2 domain (*7*). However, since the amount of PDBu bound to GST−C1 alone was also highly dependent on PS, the specific

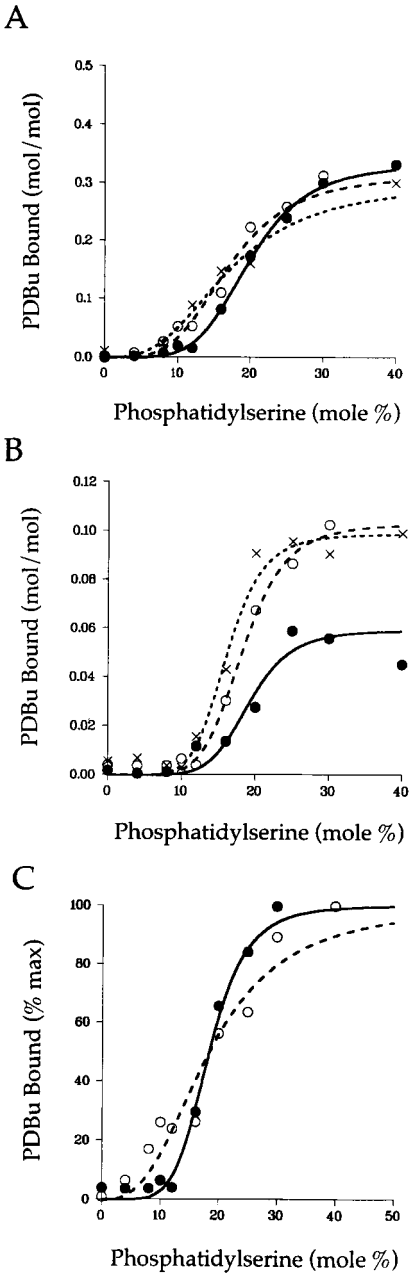

FIGURE 2: Dependence of PDBu binding on PS. The amount of PDBu bound to GST−C1 (A) or GST−C2−C1 (B) was measured at various concentrations of PS using either 20 nM (●, —), 150 nM (○, − − −), or 500 nM (×, - - -) PDBu. The lines drawn represent fits to a modified version of the Hill equation (see Materials and Methods) using Hill coefficients and $K_{1/2}$ values calculated by nonlinear regression using Systat 5.0 (Table 2). The moles of PDBu bound was standardized to the moles of fusion protein migrating at the correct molecular mass added to the reaction (see Figure 1). (C) Comparison of PDBu binding to GST−C1 (○, − − −) or GST−C2−C1 (●, —) at 150 nM PDBu standardized to 100% binding in order to compare the activation curves between the two constructs.

effect of PS on the construct containing the C2 domain was difficult to ascertain in these experiments.

In an effort to determine the specific role of the C2 domain, we performed Scatchard analysis of PDBu binding to GST−C1 and GST−C2−C1 at different concentrations of PS. In these experiments we were able to differentiate the effect of PS on the amount of PDBu bound at saturating concentrations of PDBu (B_{max}) and the effect of PS on the

Table 1: Cooperativity of Lipid Activation Is Dependent on the C2 Domain[a]

fusion protein	PDBu (nM)	lipid	Hill number (n) ± SD	lipid for half-maximal PDBu binding (mol % ± SD)	maximal PDBu binding (mol/mol ± SD)	N
GST−C2−C1	20	PS	6.2 ± 2	22 ± 3	0.063 ± 0.005	3
	150	PS	7.0 ± 2	19 ± 1	0.089 ± 0.02	3
	150	**PA**	**2.8 ± 0.6**	**10 ± 2**	**0.14 ± 0.04**	2
	500	PS	4.2 ± 2	15 ± 3	0.11 ± 0.05	4
GST−C1	20	PS	5.0 ± 0.5	20 ± 0.6	0.29 ± 0.03	3
	150	PS	3.4 ± 0.6	18 ± 1	0.24 ± 0.07	3
	150	**PA**	**2.8 ± 0.3**	**8.5 ± 2**	**0.25 ± 0.09**	2
	500	PS	3.1 ± 0.2	17 ± 3	0.37 ± 0.07	3

[a] Values for the Hill coefficient and for half-maximal binding were calculated by nonlinear regression (see Materials and Methods). *N* refers to the number of independent experiments.

Table 2: Affinity for C1 Activators Is Dependent on the C2 Domain[a]

fusion protein	lipid	mol %	affinity for PDBu, K_d (nM) ± SD	B_{max} (mol/mol) ± SD	N
GST−C2−C1	PS	30	8.7 ± 4	0.053 ± 0.02	3
	PS	20	27 ± 8	0.039 ± 0.02	3
	PS	12	227 ± 33	0.018 ± 0.008	5
	PA	**5**	**13 ± 0.6**	**0.014 ± 0.007**	3
	PS/PA	**12/0.5**	**35 ± 4**	**0.02 ± 0.003**	2
GST−C1	PS	20	5.9 ± 3	0.23 ± 0.1	3
	PS	8	14 ± 5	0.044 ± 0.01	3
	PA	**5**	**11 ± 6**	**0.032 ± 0.02**	3

[a] B_{max} and K_d values for PDBu binding to GST−C2−C1 and GST−C1 were calculated with EBDA (*30*). *N* refers to the number of independent experiments.

affinity of the fusion protein for PDBu (K_d). The B_{max} for both fusion proteins showed a strong dependence on PS (Table 2), suggesting that the level of PS determines the amount of fusion protein available to bind to PDBu independently of the C2 domain. In contrast, the presence of the C2 domain had a significant effect on the affinity for PDBu (K_d). At low concentrations of PS, the construct containing the C2 domain had a much lower affinity than the construct without this domain (Figure 3A,B; Table 2). At higher concentrations of PS this C2-mediated inhibition was reduced, suggesting that the C2 domain affects K_d in a PS-dependent manner (Figure 3A; Table 2). This was not due to low-affinity binding of PDBu to the C2 domain itself, since a fusion protein containing only the C2 domain (GST−C2) (Figure 1) bound no PDBu at any concentration tested. Furthermore, since the pseudosubstrate of Apl II is positioned between the C2 and C1 domain, we tested a fusion protein containing only the pseudosubstrate and C1 domain (GST−P−C1; Figure 1). This protein behaved similarly to GST−C1 (data not shown), demonstrating that the low binding affinity of GST−C2−C1 for PDBu is not due to the pseudosubstrate. These results indicate that the presence of the C2 domain lowers the affinity of PDBu binding to C1 and that this inhibition can be reduced by high concentrations of PS. However, the level of PS required for this function is quite high, and thus PS may not be the physiological effector of this function. Therefore, we tested the ability of PA, the product of RTK-activated PLD, to facilitate PDBu binding.

PA Is the Most Effective Lipid for Reducing C2 Domain-Mediated Inhibition. PA was more effective than PS in increasing PDBu binding to GST−C1 (Figure 4A). This

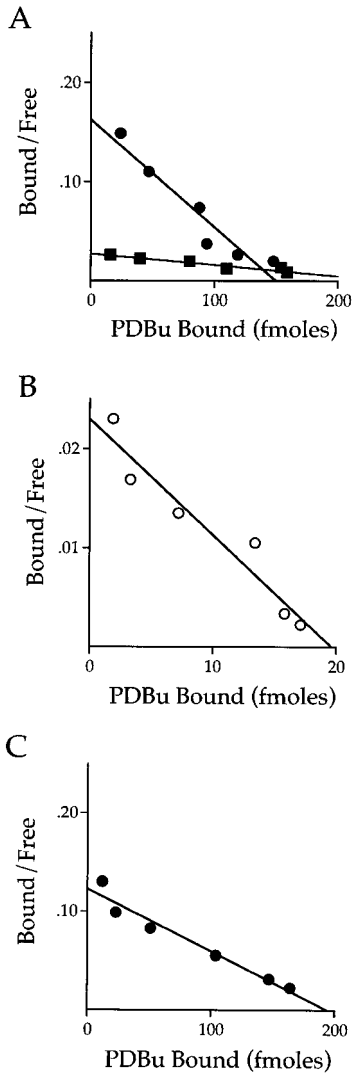

FIGURE 3: PS and PA reduced C2 domain-mediated inhibition of PDBu binding to C1. The scales for all Scatchard plots are standardized so the slopes of the line (or $-1/K_d$) can be directly compared between experiments. Different amounts of protein are used in each experiment and thus the number of femtomoles bound does not reflect the B_{max} for each protein. Values for B_{max} are shown in Table 2. Scatchard plots are shown for PDBu binding to (A) GST−C2−C1 at 12 mol % PS (■) and at 20 mol % PS (●), (B) GST−C1 at 8 mol % PS, and (C) GST−C2−C1 at 12 mol % PS + 0.5 mol % PA. In panel A, 0.57 μg (12 mol % PS) and 0.18 μg (20 mol % PS) of GST−C2−C1 (6.4 or 0.9 μg of total protein) were used; in panel B, 0.03 μg of GST−C1 (0.15 μg of total protein) was used; and in panel C, 0.18 μg of GST−C2−C1 (1.8 μg of total protein) was used.

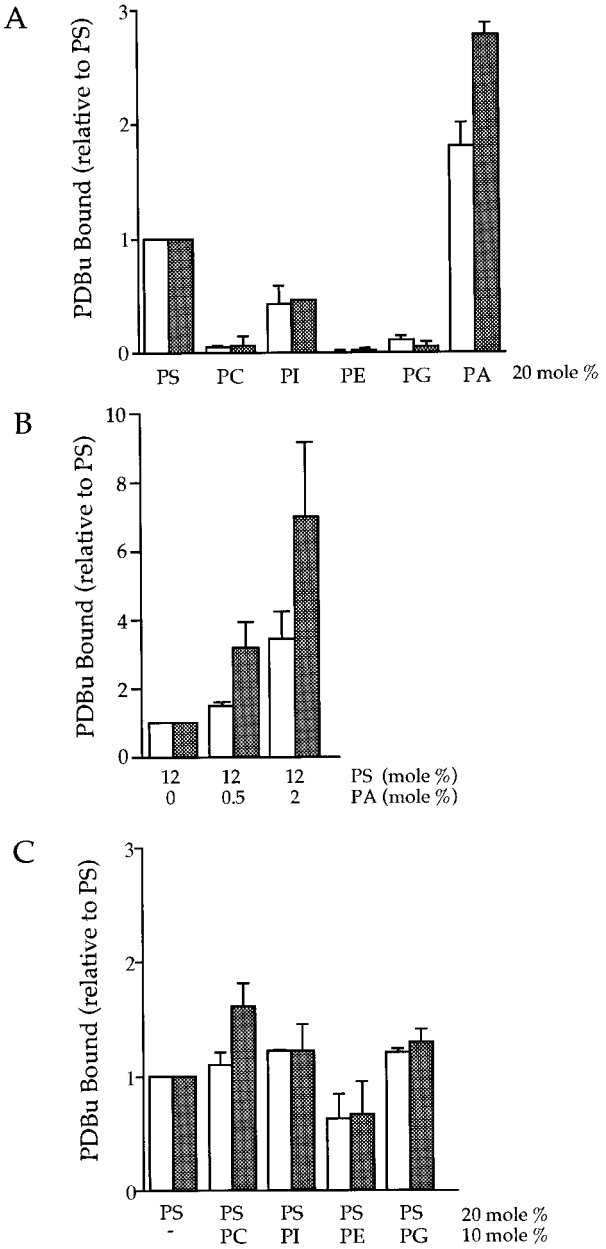

FIGURE 4: PA is the most effective phospholipid at removing C2 domain-mediated inhibition. (A) Fold increase in PDBu binding to GST−C1 (open bars) or GST−C2−C1 (shaded bars) at 20 mol % of various phospholipids and 150 nM of PDBu compared to 20 mol % PS (set to 1). Values are averages ± SD for $n = 2$ independent experiments. (B) Fold increase in PDBu binding to GST−C1 (open bars) or GST−C2−C1 (shaded bars) by 0.5 mol % PA or 2 mol % PA in the presence of 12 mol % PS and 75 nM PDBu compared to 12 mol % PS (set to 1). Values are averages ± SEM for $n = 4$ independent experiments. (C) Fold increase in PDBu binding to GST−C1 (open bars) or GST−C2−C1 (shaded bars) at 10 mol % of various phospholipids in the presence of 20 mol % PS and 75 nM of PDBu compared to 20 mol % PS (set to 1). Values are averages ± SD for $n = 2$ independent experiments.

effect was specific for PA since other cellular phospholipids were completely ineffective (PC, PE, and PG) or only weakly facilitated PDBu binding (PI) (Figure 4A). The difference between PA and PS was even greater for GST−C2−C1, suggesting that PA also may be more effective than PS in reducing C2 domain-mediated effects on the K_d for PDBu binding (Figure 4A).

To further characterize the effect of PA, we measured PDBu binding at low concentrations of PS (12 mol %) and

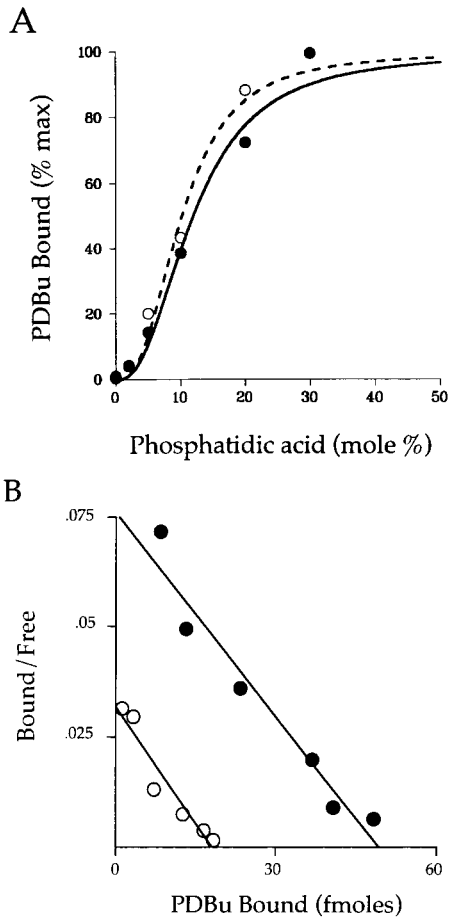

FIGURE 5: Dependence of PDBu binding on PA. (A) The amount of PDBu bound to GST−C1 (○, − − −) or GST−C2−C1 (●, —) was measured at 150 nM PDBu. The lines drawn represent fits to a modified version of the Hill equation (see Materials and Methods) using Hill coefficients and $K_{1/2}$ values calculated by nonlinear regression using Systat 5.0. (B) Scatchard plots measuring PDBu binding to GST−C1 (○) or GST−C2−C1 (●) at 5 mol % PA. GST−C1 (0.03 μg; 0.15 μg of total protein) and GST−C2−C1 (0.18 μg; 1.8 μg of total protein) were used in the experiment shown.

PDBu (75 nM). Figure 4B shows that, under these conditions, 0.5 mol % PA (or approximately 1 molecule of PA/Triton X-100 micelle) increased PDBu binding to GST−C2−C1 more than to GST−C1, indicating that a single PA molecule in the micelle can effectively reduce C2-mediated inhibition of PDBu binding to C1. To demonstrate that addition of PA affected the K_d and not the B_{max} of the GST−C2−C1 fusion protein, we performed Scatchard analysis under these conditions. Indeed, 0.5 mol % PA increased the affinity of GST−C2−C1 for PDBu from 250 to 35 nM but did not significantly change the B_{max} (Figure 3C; Table 2). PC also caused a small increase in PDBu binding to GST−C2−C1 relative to GST−C1 at low concentrations of PDBu, but PE, PG, and PI did not (Figure 4C).

Next, we characterized the ability of PA to directly facilitate PDBu binding to the fusion proteins in the absence of PS. By itself, PA was about twice as potent as PS in stimulating PDBu binding to GST−C1 (Figure 5A; Table 1). To compare the relative ability of PA to affect B_{max} and K_d, we measured the affinity of the fusion proteins for PDBu at 5 mol % PA. At this concentration of PA, the B_{max} values for the fusion proteins are similar to those using 12% PS (Table 2). However, while at 12% PS there was a large

difference in the affinity for PDBu between GST−C2−C1 and GST−C1 (Figure 3; Table 2), the two constructs had identical affinities at 5% PA (Figure 5B; Table 2). Thus, in contrast to PS, PA reduced C2 domain-mediated inhibition at a concentration below that required to increase B_{max}.

DISCUSSION

Two Roles for Lipids in PDBu Binding to the Fusion Proteins. In our experiments, both PS and PA play two roles in PDBu binding to the fusion proteins. First, they affect the amount of fusion protein available to bind PDBu as determined by measuring B_{max}. It is likely that lipids increase B_{max} by facilitating binding of fusion proteins to the mixed micelles in a conformation capable of binding PDBu. PA is 2-fold more potent than PS at this function and this is consistent with reports that show PA (due to its two negative charges) is also 2-fold more potent at binding Ca^{2+}-activated PKCs to mixed micelles (*36*). Second, PS and PA selectively increase the affinity for PDBu (K_d) in constructs that contain the C2 domain. PA was much more potent (>10-fold) than PS at mediating this effect since one molecule of PA in a Triton X-100 micelle (0.5 mol %) had a significant effect in the presence of 24 molecules of PS (12 mol %). The level of PS in the nervous system of *Aplysia* and mammals is between 12 and 18 mol % (*37, 38*). This is in the range where our data suggests that increases in the levels of PA, which has a basal level of 0−1 mol % (*37, 38*), would have a significant ability to activate Ca^{2+}-independent PKCs. Thus, PA may be a physiologically significant mediator of this effect.

Our results suggest that the separate effects of lipids on the B_{max} and K_d of PDBu binding are due to separate lipid binding sites. First, the two effects had different lipid specificities. While PS was equally effective at the two functions, PA increases the affinity of C2 domain containing constructs for PDBu (K_d) at a much lower concentration than it increases the amount of fusion protein available for binding PDBu (B_{max}). Second, more molecules of lipid were required [reflected by increased cooperativity (Table 1)] for binding of fusion proteins to PDBu when lipid affected both K_d and B_{max}. Thus, many molecules of PS were required for binding to GST−C2−C1 at 150 nM PDBu (Figure 2C), since in this case increasing PS increased PDBu binding both by increasing B_{max} and by increasing PDBu affinity (K_d). In contrast, fewer molecules of PS were required for binding of 150 nM PDBu to GST−C1 (Figure 2C) because in this case 150 nM of PDBu was saturating, and thus PS only affected B_{max}. These results suggest that the site where PS or PA binds to the fusion proteins to increase the affinity for PDBu (K_d) is distinct from the site where PS or PA binds to increase B_{max}.

Lipids could increase B_{max} by removing intermolecular interactions that are due to inappropriate folding of the fusion proteins, as opposed to facilitating binding of fusion proteins to the mixed micelles in a conformation capable of binding PDBu. This explanation could account for lipid effects on B_{max} but cannot account for the effects of 0.5 mol % PA on increasing the affinity of GST−C2−C1 (K_d), since in this case there was no significant difference in the amount of fusion protein available (B_{max}) (Table 2), and thus the effect of 0.5 mol % PA was to alter the conformation of fusion proteins that could already bind PDBu. The high level of lipid required for PDBu binding is also unlikely to be an artifact of fusion protein production in bacteria, as we find a similar level of lipid required when we examine kinase activity of full-length PKC Apl II purified from baculovirus (*7*; Xiaotang Fan, Antonio Pepio, and Wayne Sossin, manuscript in preparation). Furthermore, we have found that only a small amount of the purified full-length kinase can bind PDBu at 12 mol % PS and 75 nM PDBu (0.021 ± 0.002 mol of PDBu/mol of PKC, $n = 5$), this amount increases dramatically at 20 mol % PS (0.12 ± 0.01 mol of PDBu/mol of PKC, $n = 4$) or when 0.5 mol % PA is added to 12 mol % PS (0.11 ± 0.01 mol of PDBu/mol of PKC, $n = 3$). Therefore, if lipids are required to remove intermolecular interactions due to inappropriate folding, this is also true for the full-length protein expressed in an insect cell line.

The C2 Domain as a Negative Regulator of PKC. Other reports also suggest a negative regulatory role for the C2 domain. Addition of the C2 domain of PKCγ to a single C1 domain fusion protein decreases the affinity of the C1 domain for PDBu (*28*). Mochly-Rosen and colleagues have suggested that the C2 domain inhibits PKC activity through intraprotein interactions, which mimic interaction with the receptors for activated C-kinase (RACKs) (*39, 40*). Since RACKs bind to the C2 domain of Ca^{2+}-independent PKCs (*41*), removing C2−C1 interactions not only frees the activator binding sites in the C1 domain but also may be important in allowing C2 domains to bind to RACKs. Interestingly, removing the core of the C2 domain from Apl II, but retaining the putative RACK binding site (*41*), resulted in a kinase that was not activated by phorbol esters (*7*). We constructed a C2−C1 fusion protein with an identical mutation (GST−ΔC2−C1). Despite the stability of the fusion protein (56% in correct molecular weight band) it bound very little PDBu (0.04 mol of PDBu/mol of protein) even at high concentrations of PS and PDBu. Furthermore, Scatchard plots were not informative since increasing PDBu did not increase the amount of PDBu bound, suggesting that the affinity for PDBu was too low for the PDBu to remain bound during gel filtration. One interpretation of these results is that in these constructs inhibition of C1 is intact but the ability of lipid to reduce this inhibition is impaired, consistent with a model in which the C2 domain contains separate sequences for the effects of lipid and C1/RACK.

Role of PA in PKC Regulation. A number of reports have examined the ability of PA to stimulate Ca^{2+}-activated PKCs. PA was less effective than PS in stimulating PDBu binding to Ca^{2+}-activated PKCs using a mixed micelle binding assay similar to the one used in this study (*32*), suggesting that the ability of PA to stimulate PDBu binding may be specific for Ca^{2+}-independent PKCs. In some but not all cases PA can assist PS in stimulating kinase activity of Ca^{2+}-activated PKCs (*42*−*45*), but this has not been extensively studied in Ca^{2+}-independent PKCs. It will be important to determine how the ability of PA to stimulate PDBu binding to the fusion proteins translates into PA effects on kinase activity. We have found that low levels of PA (0.5−2 mol %) can significantly increase PKC Apl II activity in the presence of 12 mol % PS (Xiaotang Fan, Antonio Pepio, and Wayne Sossin, manuscript in preparation).

Physiological Role for PA in Activation of PKC Apl II. The importance of PA for the removal of C2 domain-

mediated inhibition suggests that stimulation of PLD leads to Ca^{2+}-independent PKC activation by producing a combination of DAG and PA. In support of this, the time course for the activation of PKCϵ matches the time course of PA production downstream of PLD (*23*, *26*). PLD is stimulated by Ca^{2+}-activated PKCs, PI(4,5)P_2, and the small G proteins ARF, RHO, and RAC (*46*−*49*). Several of these factors are stimulated downstream of RTK activation and provide a pathway underlying activation of Ca^{2+}-independent PKCs by RTKs through activation of PLD (*50*−*52*). In particular, PI 3-kinase has been shown to be important in this pathway (*53*, *54*). Consistent with this, activation of PKC Apl II in bag cell neurons by the insulin RTK is blocked by the PI 3-kinase inhibitor wortmannin (*20*). Wortmannin may also block PLD activation through inhibition of PI 4-kinase and the subsequent decrease of the PLD activator PI(4,5)P_2 (*55*).

RTK activation is implicated in several forms of synaptic plasticity (*56*−*59*). Ca^{2+}-independent PKCs are an excellent candidate to mediate some of the actions of RTKs since they are specifically activated by RTKs (*20*−*23*), are enriched in the nervous system throughout phylogeny (*60*−*62*), and can regulate important synaptic functions (*63*−*65*). In the invertebrate *Aplysia*'s nervous system, the Ca^{2+}-independent PKC Apl II is activated downstream of an insulin-responsive RTK and may mediate the insulin-stimulated increase in Ca^{2+} current (*20*, *66*). It will be important in the future to determine if RTK activation of PLD underlies the activation of PKC Apl II by insulin.

We suggest a novel mechanism for the regulation of PKC Apl II. The C2 domain of this kinase inhibits binding of the PKC activator DAG, and this inhibition can be removed by the actions of phosphatidic acid. This suggests that this kinase is regulated by phospholipase D. Since phospholipase D is activated by RTKs, this suggests a pathway between RTKs and synaptic plasticity.

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