Expression and Characterization of *Aplysia* Protein Kinase C: A Negative Regulatory Role for the E Region

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The *Aplysia* nervous system contains two phorbol ester-activated protein kinase C isoforms, the Ca^{2+}-activated Apl I and the Ca^{2+}-independent Apl II. Short-term applications of the facilitatory transmitter serotonin (5-HT) activates Apl I, but not Apl II. In contrast, Apl II, but not Apl I, can form an autonomous kinase. To investigate the biochemical characteristics of the *Aplysia* kinases that might underlie their differential activation, we expressed Apl I, Apl II, and two derivatives of Apl II with deletions in the amino-terminal 150 amino acid E region in insect cells using the baculovirus system. Similar to nervous system extracts, expressed Apl II has more autonomous activity than Apl I. Removal of the E region lowered the amount of phosphatidylserine required for activation of Apl II, but did not remove the autonomous kinase activity. In addition, phosphatidylserine vesicles could sediment fusion proteins containing the E region, consistent with a role for the E region in lipid interactions. A partial deletion of the E region modifies activation of Apl II by phorbol esters and oleic acid, suggesting that in the intact enzyme the E region interacts with the phorbol ester-binding domain of the kinase. These results introduce a model whereby the E region acts as a negative regulator of Apl II activation and suggest that this inhibition may explain the inability of short-term applications of 5-HT to activate Apl II.

Key words: protein kinase C; Aplysia*; learning and memory; synaptic plasticity; autophosphorylation; autonomous kinase*

The protein kinase C family (PKC) is involved in multiple signal transduction pathways (Nishizuka, 1992). In the nervous system PKCs are associated with short- and long-term modulation of synaptic efficacy (Olds and Alkon, 1991; Schwartz, 1993). These isoforms can be subdivided into three types: Ca^{2+}-activated PKCs (α, β, γ), Ca^{2+}-independent PKCs (δ, ϵ, η, θ), and atypical PKCs that are not activated by diacylglycerol (ζ, ι/λ). All Ca^{2+}-activated PKCs contain a domain, called CalB, that binds phosphatidylserine in a Ca^{2+}-dependent manner (Brose et al., 1992) and confers Ca^{2+}-dependent activation to these isoforms (Kaibuchi et al., 1989). Ca^{2+}-independent PKCs lack a CalB domain, but do contain a conserved region at the amino-terminal end of the protein named the E region in PKCϵ and PKCη (Osada et al., 1990; Kruger et al., 1991; Land et al., 1994). A domain of the E region (E2) shows homology to the CalB domain (Sossin and Schwartz, 1993), suggesting that the E region may have a function analogous to the regulatory role of CalB in Ca^{2+}-activated PKCs.

In the marine mollusk *Aplysia californica*, the nervous system has only two phorbol ester-activated PKC isoforms, the Ca^{2+}-activated Apl I, which contains a CalB domain, and the Ca^{2+}-independent Apl II, which contains an E region (Kruger et al., 1991; Sossin et al., 1993). The simplicity of isoform distribution, coupled to physiological information about the role of protein kinase C in regulating synaptic transmission in *Aplysia* (Braha et al., 1990, 1993; Sacktor and Schwartz, 1990; Ghirardi et al., 1992; Sugita et al., 1992, 1994), makes this an ideal system for determining if different isoforms of PKC play unique physiological roles in regulating synaptic transmission.

There are isoform-specific differences in the activation of Apl I and Apl II. First, short-term applications of serotonin, a facilitating transmitter, activate Apl I, but not Apl II (Sossin and Schwartz, 1992; Sossin et al., 1994). Second, in *Aplysia* nervous system extracts, autonomous kinase activity (measured as the amount of phosphorylation of a substrate peptide in the absence of the PKC activators, phosphatidylserine and phorbol ester) can be immunoprecipitated largely by an antibody against Apl II, but not by an antibody against Apl I (Sossin et al., 1994), suggesting that a fraction of Apl II molecules are active in the absence of PKC activators. Thus, in the *Aplysia* nervous system, Apl II is both more resistant to stimulation and more able to form an autonomous kinase than is Apl I. To investigate further the biochemical properties of Apl I and Apl II, we have expressed the kinases in SF9 cells using the baculovirus system. Many of the properties of these kinases observed in nervous system extracts are maintained in partially purified preparations from SF9 cells. Furthermore, results from expression of derivatives of Apl II with full or partial deletions of the E region suggest that the E region acts to negatively regulate Apl II by interacting with its phorbol ester-binding domain.

MATERIALS AND METHODS

Construction of plasmids. A complete Apl I cDNA was constructed in Bluescript KS^- (Stratagene, La Jolla, CA) by ligating two cDNA clones (2-1 encoding the N-terminal region of Apl I and 15-1 encoding the C-terminal region of Apl I; (Kruger et al., 1991)) at their common *Bgl*II site. A complete Apl II cDNA was constructed in Bluescript SK^+ (Stratagene) by ligating two cDNA clones (9-1 encoding the C-terminal region and eps-5 containing the N-terminal region (Kruger et al., 1991)) at their common *Bsp*HI site.

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To construct baculovirus transfer vectors, the Apl I cDNA was removed from SK by partially digesting with *Bam*HI and inserted into the *Bam*HI site of the pBlueBacIII vector (Invitrogen, San Diego, CA). In this construct, 400 bp of 3′-untranslated region from the Apl I cDNA were removed. The Apl II cDNA was removed from SK by digesting with *Eco*RI and inserted into pBlueBacIII after partial digestion of pBlueBacIII at the *Eco*RI site in the polylinker of the construct. The Apl IIΔE deletion was constructed by ligating an *Xmn*I-*Eco*RV fragment from the Apl II cDNA to the fusion vector pAC360 (Invitrogen) after filling in the *Bam*HI site with Klenow. The junction was confirmed by sequencing and results in the replacement of the E region by amino acids 1–10 of the baculovirus polyhedron protein. The Apl IIΔE2 construct was generated with a two-step mutagenic procedure using the polymerase chain reaction (PCR). First-round PCR used the Apl II cDNA as a template and either the outside 5′ primer 5′-TCTAGAACTAGTGGATC and the inside 3′ primer 5′-GGCCGCCATCTGCGAGGCTTTTTCTTTCGT or the inside 5′ primer 5′-TCGCAGATGGCGGCCGCAATTCCACCCGACGAG and the outside 3′ primer 5′-CATTTGGTGACTATATGCTGGTGA. The products from the first-round synthesis were combined and used as the template for the second-round synthesis using the two outside primers. The resultant product was then cut with *Eco*RI and *Kpn*I and reinserted into the full length cDNA. This resulted in the joining of residue 292 to residue 445 or amino acids SQM to amino acids AAI with an intervening alanine to insert a *Not*I site to mark the deletion. The deletion was sequenced over the entire amplified region, and the mutated Apl II was inserted into the pBlueBacIII baculovirus transfer vector as described above.

To construct fusion protein vectors, the eps-5 Apl II cDNA (Kruger et al., 1991) was excised with *Stu*I and *Pst*I and ligated to the pRIT2T vector (Pharmacia, Piscataway, NJ) cut with *Sma*I and *Pst*I. The amino-terminal fragment of Apl II, which ends at amino acids DDV (residue 502), was removed from pRIT2T with *Eco*RI, and the ends were filled in with Klenow and ligated into the pMALC-R2* vector cut with *Xmn*I to generate the construct pMALC-E1-E3. The pMALC-R2* vector is the pMALC-R2 vector (New England Biolabs, Beverly, MA) cut with *Bam*HI, filled in with Klenow, and religated to create a stop codon. The pMALC-E1-E3 construct was digested with *Nla*IV and *Ssp*I and religated to form pMALC-E1-E3ΔPY. pMALC-E1-E3ΔE2 was constructed using the same mutagenesis strategy described above, except with outside primers from the pMALC vector 5′-GGTCGTCAGACTGTCGA and 5′-CGCCAGGGTTTTCCCAGTCACGAC. A *Sma*I-*Xmn*I fragment from the Apl II full length cDNA was ligated into the pMALC-R2 *Xmn*I site to make the pMALC-E1-E4 construct, and a *Ssp*I-*Xmn*I fragment was ligated into the pMALC-R2 *Xmn*I site to make the pMALC-E2-E4 vector.

Baculovirus expression. Spodoptera frugiperda (SF9; Invitrogen) cells were grown in suspension cultures with supplemented Grace's media (Life Technologies, Inc., Gaithersburg, MD) and 10% fetal bovine serum (Life Technologies). The baculovirus transfer vectors were cotransfected with linearized baculovirus (Invitrogen) into SF9 cells using cationic liposomes following standard procedures (Invitrogen), and the resultant blue colonies were plaque-purified. For Apl IIΔE, positive plaques were isolated by serial dilution. Positive colonies were confirmed by PCR and by immunoblotting with anti-Apl I or anti-Apl II antibodies (Kruger et al., 1991; Sossin et al., 1993). High titer viral stocks (10^8–10^9 plaque-forming units [PFU]/ml) were generated as described (Summers and Smith, 1988).

Purification of PKCs. Typically 100 ml of SF9 cells (2×10^6 cells/ml) were infected with 1×10^9 PFUs of baculovirus and incubated for 64 hr for optimal expression of PKCs. Cells were then pelleted and resuspended in 20 ml of ice-cold homogenization buffer (10 mM Tris 8.0, 1 mM EGTA, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 20 μg/ml aprotinin, 5 mM benzamidine, and 0.1 mM leupeptin). All further steps were performed at 4°C. Cells were sonicated 6×10 sec with a probe sonicator (Vibracell, Sonics and Materials, Danbury, CT) and debris pelleted by centrifugation in a Sorvall RCB2 centrifuge at $12{,}000 \times g$ in an SS34 rotor. The supernatant was filtered through a 0.22 μM filter, diluted to 50 ml with Buffer A (20 mM Tris-HCl, pH 8.0, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol), and loaded onto a MemSep DEAE 1000 cartridge previously equilibrated in Buffer A at a flow rate of 3 ml/min using a ConSepLC100 system (Millipore, Milford, MA). After 50 ml of wash with Buffer A, PKCs were eluted with a linear gradient from 0 to 300 mM NaCl. Fractions positive for PKC activity as detected by a kinase assay were pooled, and ammonium sulfate was added to a final concentration of 2.4 M. The sample was then diluted to 50 ml with Buffer B (100 mM $NaHPO_4$, pH 6.8, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, 2.4 M $(NH_4)_2SO_4$) and loaded onto a 5 ml hydrophobic interaction cartridge (HIC; Bio-Rad, Richmond, CA), washed with 20 ml of Buffer B and eluted with a linear gradient from 2.4 to 0 M $(NH_4)_2SO_4$ for 1 hr at a flow rate of 1 ml/min. The positive fractions were pooled and either dialyzed against Buffer C (20 mM $KHPO_4$, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 10% glycerol) overnight or diluted into 50 ml of Buffer C. The sample was then loaded onto a 5 ml hydroxyapatite column cartridge (HAP, Bio-Rad) previously equilibrated with Buffer C. Proteins were eluted with a linear gradient from 20 to 500 mM $KHPO_4$ for 40 min at a flow rate of 1 ml/min. Positive fractions were pooled, concentrated 2–4 times by centrifugation in a Centricon-10 microconcentrator (Amicon, Beverly, MA), diluted in PKC storage buffer (50 mM Tris 8.0, 100 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM β-mercaptoethanol, 10% glycerol), and concentrated again by centrifugation using Centricon-10. Samples were aliquoted and stored frozen at −70°C or diluted with 50% glycerol and stored at −20°C. Kinase activity was stable for up to 6 months, but there was an approximately twofold decrease in stimulation by phorbol esters after freeze-thaw.

PKC assays. Assays were done as described (Sossin and Schwartz, 1992; Sossin et al., 1993). The reaction mixture (30 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, and 5 μM Aε-pep (LNRRRGSMRRRVHQVNGH) in the presence or absence of 50 μg/ml of dioleol phosphatidylserine (Avanti, Alabaster, AL), and 20 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Aε-pep is a synthetic peptide based on the pseudosubstrate peptide of Apl II, which is phosphorylated well by both Apl I and Apl II (Sossin and Schwartz, 1992; Sossin et al., 1993). After addition of 10 μl of purified PKCs, diluted to remain in the linear range of the assay (Sossin and Schwartz, 1992), the reaction was started with 10 μl of [γ-^{32}P]ATP (DuPont NEN, Boston, MA; 1 μCi; 50 μM final concentration). After 30 min at 20°C, 40 μl of the 50 μl reaction mixture was spotted onto a Whatman phosphocellulose paper disk, which was washed in 100 ml of 2% (w/v) ATP. The disks were then rinsed four times for 5 min with 0.425% (v/v) phosphoric acid, and radioactivity was counted by scintillation. Each value is the average of duplicate assays.

For experiments to determine activation by oleic acid or TPA, a concentrated reaction mixture (20 μl) was added to 10 μl of the appropriate concentration of TPA or oleic acid. Stock concentrations of oleic acid (Nu Chek Prep, Elysian, MN) and phosphatidylserine were stored in small batches in chloroform. Before each experiment they were dried under N_2 gas, suspended in water, vortexed vigorously, and sonicated either in a cup sonicator (FS3; Fisher Scientific, Ottawa, Ontario) for phosphatidylserine or with a probe sonicator (2×10 sec) for oleic acid. In some experiments oleic acid was resuspended in ethanol after drying and then diluted from the ethanol stock into water. The mixed micelle assay to determine activation by phosphatidylserine was performed as described (Hannun and Bell, 1987) with 2 mole percent dioctylglycerol (dIC_8) and 0.3% Triton X-100.

Autophosphorylation was done using the same procedure but with a higher specific activity of ATP (4 μCi in 50 μM ATP) and a higher concentration of phosphatidylserine (150 μg/ml). Autophosphorylations were carried out for 10 min, at which point the incorporation rate was linear with time (data not shown).

Sedimentation assay. Fusion proteins were purified by affinity chromatography on amylose columns (New England Biolabs). For the sedimentation assay, fusion protein (2 μM) was incubated in 100 μl of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol in the presence or absence of 100 μg/ml 90% dioleol phosphatidylserine/10% dioleol phosphatidylcholine vesicles (Princeton Lipids, Princeton, NJ) (9:1 w/w). After 20 min at 20°C, the vesicles were sedimented at $100{,}000 \times g$ in a TL-100 centrifuge (Beckman, Palo Alto, CA) for 30 min, and the supernatants and pellets separated on 10% sodium dodecyl sulfate (SDS)-Laemmli gels and stained with Coomassie. Experiments using [^{14}C]phosphatidylcholine as a marker demonstrated >90% sedimentation of vesicles using this approach (Quest et al., 1994) (data not shown). Gels were quantified using a Java imaging system (Jandel Scientific, San Rafael, CA).

Other procedures. SDS-polyacrylamide gels (8.5%) and immunoblotting were carried out as described (Sossin et al., 1993). The antibody to Apl II was raised against a carboxyl-terminal peptide (Kruger et al., 1991) and thus should recognize all constructs equally.

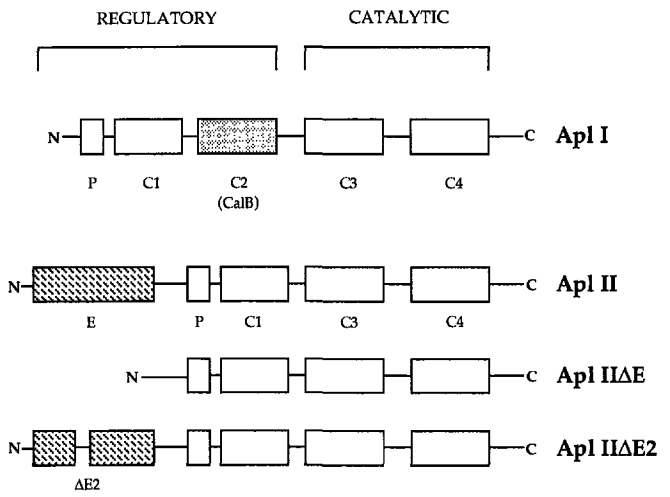

Figure 1. Domain structure of PKCs. PKCs are made up of conserved domains (C regions) that are separated by variable domains which are distinct for each isoform. The catalytic domain that phosphorylates substrates is made up of the C3 and C4 domain and is similar among all PKCs. The regulatory portion of the molecule contains the P (pseudosubstrate) domain, which interacts with the catalytic domain to inhibit kinase activity, and the C1 phorbol ester/diacylglycerol binding domain, which regulates enzyme activity. Ca^{2+}-activated PKCs such as Apl I contain a C2 (or CalB) domain that confers the ability of the enzymes to be activated by Ca^{2+}. Ca^{2+}-independent PKCs contain a conserved amino-terminal region, which in Apl II is named the E region because of its conservation to vertebrate *E*psilon and *E*ta PKCs. In Apl IIΔE, the E region is replaced by amino acids 1–10 of the baculovirus polyhedrin protein. In Apl IIΔE2, 51 amino acids from the middle of the E domain with sequence homology to the CalB domain are deleted.

RESULTS

Expression and purification of *Aplysia* PKCs

We constructed baculovirus transfer vectors for Apl I, Apl II, and two derivatives of Apl II: (1) Apl IIΔE, which encodes a protein that has the E region deleted, and (2) Apl IIΔE2, which encodes a protein that has a partial deletion in the E region removing the domain that shows homology to CalB (Fig. 1). The transfer vectors were used to produce recombinant baculovirus for expression of the PKCs in SF9 cells, because SF9 cells have little or no detectable PKC activity that would interfere with these experiments (Patel and Stabel, 1989) (data not shown). Expression of the *Aplysia* kinases was confirmed by immunoblotting (data not shown), and the kinases were partially purified over DEAE, hydrophobic interaction, and hydroxyapatite columns (Table 1, Fig. 2). After purification, the expressed kinases are the major Coomassie-stained band in the preparation, but are not homogeneous in the final preparation (Fig. 2).

In extracts of *Aplysia* nervous system, the majority of autonomous kinase activity (measured as the amount of phosphorylation of a substrate peptide in the absence of the PKC activators, phosphatidylserine and phorbol ester) can be immunoprecipitated by an antibody to Apl II (Sossin et al., 1994). Consistent with this finding, the percentage of autonomous activity in purified preparations from SF9 cells was fivefold greater for Apl II than for Apl I (Table 2). Apl IIΔE and Apl IIΔE2 preparations had a similar amount of autonomous activity when measured as activity per unit of immunoreactive protein, demonstrating that the E region is not responsible for the autonomous activity of Apl II (Table 2). The autonomous activity did not result from proteolysis in the hinge region and formation of a constitutively active fragment of Apl II (Schaap and Parker, 1990; Baxter et al., 1992), because this product was separated from intact PKCs during purification (data not shown). The autonomous activity was not attributable to activation of the enzyme by using the pseudosubstrate-derived peptide as a substrate, because a fivefold difference in autonomous activity between Apl I and Apl II also was observed when a peptide from the myelin basic protein was used as a substrate. It is possible that the regulatory region of Apl II is more easily denatured than Apl I, and that this accounts for a higher basal activity. If this were true, one might expect that after storage at −70°C, the autonomous activity would increase. Although we did see an increase in the percentage of autonomous activity after thawing, this was the same for both Apl I and Apl II and appears to be because of a reduction in the amount of activation by phorbol esters rather than an increase in the amount of autonomous enzyme. These results demonstrate that the autonomous activity of Apl II in *Aplysia* nervous system extracts can also be observed in the expressed kinase.

Activation of expressed proteins by phosphatidylserine

A major difference between vertebrate Ca^{2+}-activated and Ca^{2+}-independent PKCs is that Ca^{2+}-independent PKCs require higher levels of phosphatidylserine for activation in the mixed micelle assay, an assay that is commonly used to determine the level of lipids required for PKC activation (Hannun et al., 1986; Hannun and Bell, 1987; Schaap and Parker, 1990). Similar to vertebrate PKCs, the Ca^{2+}-independent Apl II requires larger amounts of phosphatidylserine in this assay than does the Ca^{2+}-activated Apl I (Fig. 3*A*). Unlike vertebrate PKCs, Apl I is Ca^{2+}-activated but not completely Ca^{2+}-dependent in the mixed micelle assay, because Apl I can be fully activated even in the presence of 5 mM EGTA. Ca^{2+} ions do decrease the amount of phosphatidylserine required for Apl I activation, but not for Apl II (Fig. 3*A*) (data not shown) confirming their identities as Ca^{2+}-activated and Ca^{2+}-independent isoforms.

Removal of the E region reduces the requirement of Apl II for phosphatidylserine. Using the mixed micelle assay, Apl IIΔE and Apl IIΔE2 were both fully activated at lower levels of phosphatidylserine than Apl II (Fig. 3*B*). Moreover, the level of phosphatidylserine required for activation of Apl II when the E region is removed is similar to that required for Apl I in the absence of Ca^{2+} ions. Phosphatidylserine activates PKC mainly by binding to the C1 region and increasing the affinity of C1 for diacylglycerol (Quest et al., 1994). This result suggests that the requirement for phosphatidylserine binding to C1 is similar in Apl I and Apl II and that the difference in required phosphatidylserine levels between Apl I and Apl II can be explained by an additional requirement in Apl II for phosphatidylserine binding to the E region.

To determine whether the E region directly interacts with phosphatidylserine, we constructed maltose-binding protein (MBP)-E region fusion proteins, affinity-purified the proteins from bacteria, and tested their ability to bind to phosphatidylserine using a sedimentation assay (Sossin and Schwartz, 1992; Quest et al., 1994). In this assay, vesicles made of 90% phosphatidylserine and 10% phosphatidylcholine are mixed with the test protein, and association with the vesicles is measured by the ability of the protein to cosediment with the vesicles after centrifugation at 100,000 × *g*. A fusion protein containing regions E1–E3 was sedimented by the vesicles, whereas maltose-binding protein alone was not sedimented (Figs. 4, 5). The amount of protein sedimented (40%) is similar to that reported for the known phosphatidylserine-interacting C1 region using a similar assay

Table 1. Purification of PKCs

Enzyme	Column fractions	PKC activity (pmol · min^{-1} · ml^{-1})	Specific activity (nmol · min^{-1} · mg^{-1})	Total protein (mg)	Yield (% start)
Apl I	Start	1.3	1.0	32	100
	DEAE (75–150 mM NaCl)	4.1	3.0	12	110
	HIC (600–200 mM NH_4SO_4)	2.5	6.4	2.4	48
	HAP (100–150 mM $KHPO_4$)	5.4	22	0.2	15
Apl II	Start	5.7	5.2	55	100
	DEAE (120–175 mM NaCl)	6.1	7.2	13	32
	HIC (600–400 mM NH_4SO_4)	4.2	20	1.6	11
	HAP (150–200 mM $KHPO_4$)	1.1	48	0.4	6
Apl IIΔE	Start	0.5	0.4	17.4	100
	DEAE (100–200 mM NaCl)	0.8	0.6	5.2	43
	HIC (500–200 mM NH_4SO_4)	0.4	1.2	3.5	64
	HAP (150–200 mM $KHPO_4$)	1.8	18	0.3	9
Apl IIΔE2	Start	1.1	0.6	86	100
	DEAE (100–200 mM NaCl)	0.3	0.2	26	10
	HIC (500–200 mM NH_4SO_4)	0.4	1.2	2.6	6
	HAP (175–225 mM $KHPO_4$)	0.1	2.6	0.8	4

Summary of individual purifications for each of the expressed isoforms. No activity was detected after a mock infection with wild-type virus.

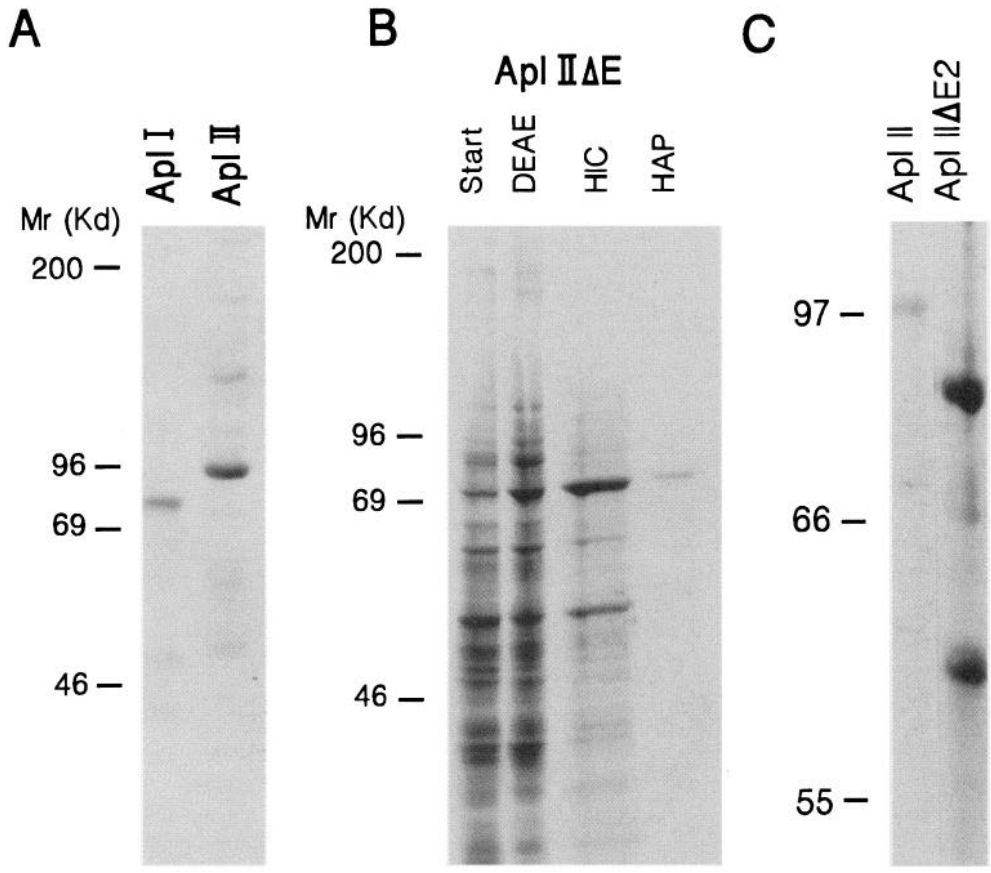

Figure 2. Purification of PKCs expressed in SF9 cells. *A*, Five micrograms of Apl I and Apl II after the final purification step were separated on 8.5% SDS-polyacrylamide gels and stained with Coomassie. The major bands seen correspond to the expected sizes of Apl I and Apl II, respectively. *B*, Twenty micrograms of the *Start*, *DEAE* peak, *HIC* peak, and 5 μg of the *HAP* peak from a purification of Apl IIΔE were separated on 8.5% SDS-polyacrylamide gels and stained with Coomassie. The major band seen in the HAP purification corresponds to the expected size of Apl IIΔE. *C*, Two micrograms of Apl II and 5 μg of Apl IIΔE2 after the final purification step were separated on an 8% polyacrylamide gel and stained with Coomassie. The major bands seen correspond to the expected sizes of Apl II, and Apl IIΔE2, respectively. The Apl II preparation shown in *C* is different from that shown in *A*.

(Quest et al., 1994). Sedimentation in the assay was not dependent on the fusion partner because a separate fusion protein consisting of regions E1–E3 attached to Protein A also was sedimented (data not shown). Lowering the phosphatidylserine content from 90 to 20% did not significantly reduce sedimentation, showing that the interaction can occur at physiological levels of phosphatidylserine (data not shown). Surprisingly, a fusion protein that also contained E4 did not significantly bind to phosphatidylserine (Figs. 4, 5), suggesting that the conformation of the E region may be important for interactions with phosphatidylserine. This is consis-

Table 2. Autonomous PKC activity of expressed PKCs

Enzyme	Autonomous activity (nmol · min^{-1} · mg^{-1})	Autonomous activity (% TPA stimulated activity)	Autonomous activity (per immunoreactive protein)
Apl I	0.8 ± 0.2	3 ± 1	N.A.[a]
Apl II	3.8 ± 1.4	16 ± 5	1
Apl IIΔE	5.0 ± 2.3	30 ± 3	1.5 ± 0.7
Apl IIΔE2	1.2 ± 1.1	43 ± 4	0.9 ± 0.1

The comparison of autonomous activities of purified PKCs was done immediately after purification, before storage. Autonomous activities are measured as the amount of substrate phosphorylation in the presence of enzyme and 5 mM EGTA. The activity stimulated by TPA was measured using 50 μg/ml phosphatidylserine and 20 nM TPA. This amount of TPA is not saturating for Apl IIΔE2 (see Fig. 6*A*) and partly explains its high percentage of autonomous activity. Because the PKCs are not homogenous in the final purification and purity differs between preparations, the specific activity (nmol · min^{-1} · mg^{-1}) is only a lower limit to the true amount of autonomous activity. Results are from two or three independent purifications of enzyme, and the errors are SEM. Thawed enzyme was used for the measurement of activity relative to immunoreactive protein. Although freezing does not increase the amount of autonomous PKC, it decreases the ability of phorbol esters to activate the enzyme by approximately twofold. Immunoreactive protein was calculated by immunoblotting (three determinations from at least two independent purifications) and was quantitated using JAVA imaging software (Jandel Scientific).

[a] N.A., not applicable.

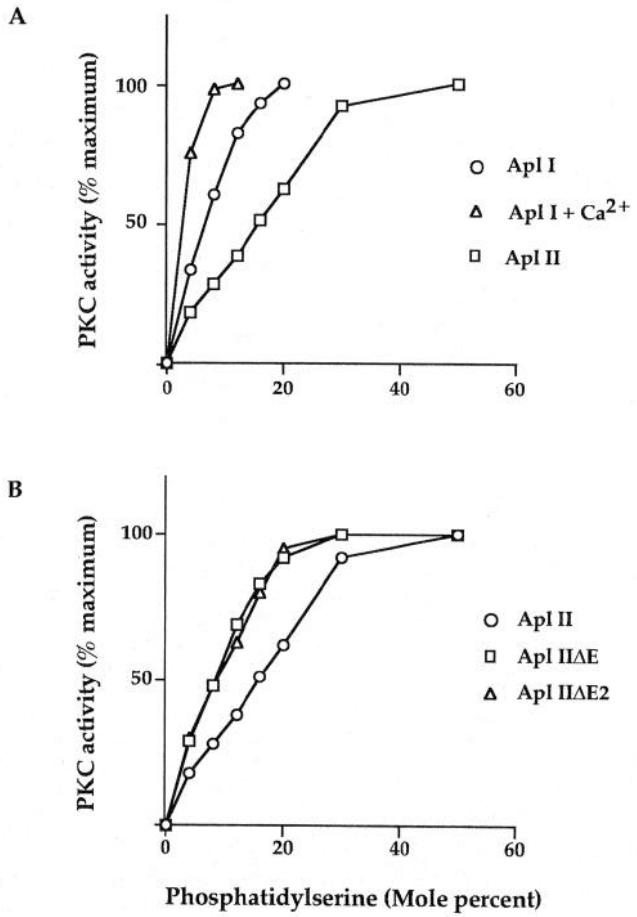

Figure 3. Activation of expressed PKCs by phosphatidylserine. *A*, Mixed micelles of 2 mole percent dioctylglycerol (dIC_8) in Triton X-100 and varied amounts of dioleol phosphatidylserine were used to activate Apl I in the presence of 5 mM EGTA (○), Apl I in the presence of 150 μM Ca^{2+} (△), or Apl II in the presence of 5 mM EGTA. Maximal activity was 15 nmol · min^{-1} · mg^{-1} for Apl I and 10 nmol · min^{-1} · mg^{-1} for Apl II (□). *B*, Mixed micelles of 2 mole percent dIC_8 in Triton X-100 and varied amounts of dioleol phosphatidylserine were used to activate Apl II (○), Apl IIΔE (□), or Apl IIΔE2 (△). Maximal activity was 10 nmol · min^{-1} · mg^{-1} for Apl II, 6 nmol · min^{-1} · mg^{-1} for Apl IIΔE, and 6 nmol/min/mg for Apl IIΔE2. The data shown are from one experiment; similar results were obtained in three additional experiments.

tent with the fact that Apl II from nervous system extracts does not sediment with phosphatidylserine vesicles (Sossin and Schwartz, 1992).

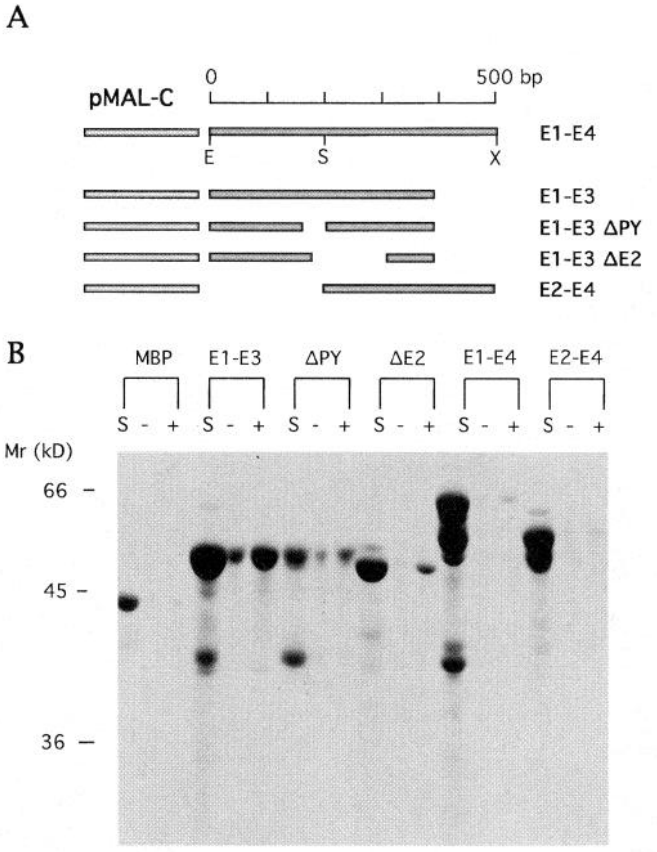

Figure 4. Sedimentation of E region fusion proteins by vesicles. *A*, Schematic of fusion protein constructs. The nomenclature E1–E4 (Sossin and Schwartz, 1993) highlights the blocks of conservation between E regions from different species. All PKC sequences were inserted at the *Xmn*I site of the pMALC-R2 vector. *E*, *Eco*RI; *S*, *Ssp*I; *X*, *Xmn*I. See text for description of deletions. *B*, Affinity-purified pMALC fusion proteins (2 μM) were sedimented in the absence (−) or presence (+) of 100 μg/ml of 90% phosphatidylserine/phosphatidylcholine vesicles, and the starting protein (*S*) and the two pellets (−) and (+) were separated on 10% SDS-polyacrylamide gels and stained with Coomassie. Maltose-binding protein (MBP) is from the original pMALC-R2 vector and includes a β-galactosidase fusion protein. Similar results (quantitated in Fig. 5) are seen with protein from the pMALC-R2* vector (see Materials and Methods), which has an inserted stop codon in the polylinker and does not contain β-galactosidase sequences.

We attempted to localize further the region required for binding to phosphatidylserine. E2 shows homology to the CalB domain that can bind phosphatidylserine (Brose et al., 1992), and

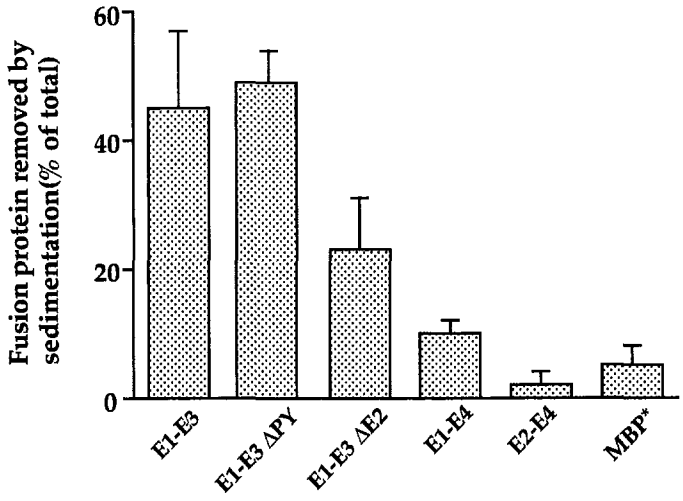

Figure 5. Quantitation of sedimentation of E region fusion proteins by vesicles. Results are the percentage of fusion protein sedimented specifically by the vesicles after subtracting the amount of fusion protein sedimented in the absence of vesicles (presumably because of aggregation). Errors are SEM from five independent experiments. The MBP in this experiment is from protein made with the pMALC-R2* vector (see Materials and Methods).

thus is a good candidate for this region. We therefore tested whether phosphatidylserine binding to the E region is dependent on the E2 domain. Two fusion proteins, one removing a highly conserved PY sequence (Sossin and Schwartz, 1993) and surrounding amino acids (E1–E3ΔPY) and the other removing the entire E2 domain (E1–E3ΔE2), were tested in the assay. Deleting the PY sequence did not affect sedimentation by the vesicles, whereas removing E2 reduced, but did not eliminate, sedimentation in this assay (Figs. 4, 5). These experiments demonstrate a role for E2 in binding phosphatidylserine, but also indicate that additional portions of the E region can bind lipids in this assay.

Activation of expressed PKCs by phorbol esters and oleic acid

Phorbol esters activate PKCs by binding to the cysteine-rich C1 domain (Bell and Burns, 1991). Surprisingly, removal of the E2 domain from full length Apl II modified regulation of Apl II by phorbol esters, which is far removed from the C1 domain. Apl IIΔE2 required higher concentrations of TPA to reach maximal activation and even at high concentrations was activated less than Apl II (Fig. 6*A*; Tables 2 and 3). This result suggests that the E region may interact with the phorbol ester-binding domain, C1. Removal of the entire E region did not affect activation by phorbol esters, suggesting that the interaction between the E region and C1 is inhibitory and therefore not required for high-affinity interaction of phorbol esters with Apl II.

The inability of phorbol esters to activate Apl IIΔE2 is not attributable to a general instability of the kinase, because activation of this deletion by a different PKC activator, oleic acid, was enhanced (Fig. 6*B*; Table 3). Similar to vertebrate PKCs (Koide et al., 1992; Khan et al., 1993), low concentrations of oleic acid activated the Ca^{2+}-independent Apl II more than the Ca^{2+}-activated Apl I (Fig. 6*B*). The E region is not responsible for this enhanced activation, as Apl IIΔE was activated in a similar manner.

Autophosphorylation of the expressed PKCs

Activation of the expressed PKCs also was examined using an assay for autophosphorylation (Fig. 7). In autophosphorylation

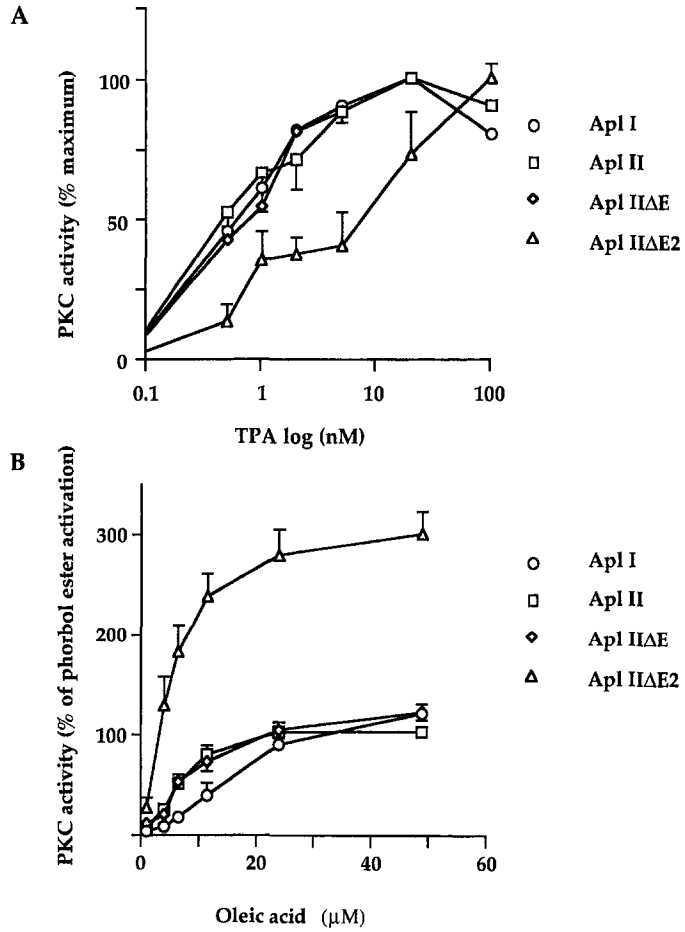

Figure 6. *A*, Activation of expressed PKCs by TPA and oleic acid. Activation of Apl I (○), Apl II (□), Apl IIΔE (◇), and Apl IIΔE2 (△) by various concentrations of TPA in the presence of 20 μg/ml dioleol phosphatidylserine and 5 mM EGTA. Data are the average of four independent experiments. Maximal activities (in nM · min^{-1} · mg^{-1}) were 10 ± 4 for Apl I, 12 ± 2 for Apl II, 8 ± 1 for Apl IIΔE, and 6 ± 1 for Apl IIΔE2. Error bars are SEM; errors for Apl I and Apl IIΔE are not shown, but are smaller than the errors shown for Apl II. No further activation of Apl IIΔE2 is seen at 400 nM TPA. *B*, Activation of Apl I (○), Apl II (□), Apl IIΔE (◇), and Apl IIΔE2 (△) by various concentrations of oleic acid. Values are expressed as the percentage of activity stimulated by 50 μg/ml phosphatidylserine and 20 nM TPA in the same experiment. These activities (in nM · min^{-1} · mg^{-1}) were 11 ± 2 for Apl I, 10 ± 2 for Apl II, 10 ± 2 for Apl IIΔE, and 3 ± 1 for Apl IIΔE2. Values are the average of four determinations. Error bars are SEM.

reactions, low concentrations of enzymes favor *cis*-autophosphorylation over *trans*-autophosphorylation, because the rate of *cis*-autophosphorylation varies in a linear manner with concentration of the enzyme, whereas the rate of *trans*-autophosphorylation varies with the square of the concentration. In vertebrates, Ca^{2+}-activated PKCs are *cis*-autophosphorylated after activation by phorbol esters but not after activation by oleic acid (Huang et al., 1986; El Touny et al., 1990). Consistent with this finding, at low concentrations of Apl I (<50 nM), we observed no detectable autophosphorylation after activation by oleic acid even though phorbol esters strongly stimulated autophosphorylation (Fig. 7). When higher concentrations of Apl I were used (>200 nM), we observed a strong autophosphorylation induced by oleic acid (data not shown), consistent with the activation of *trans*-autophosphorylation by oleic acid. In contrast, even at low concentrations of enzyme (<50 nM), autophosphorylation of Apl II was stimulated

Table 3. Comparison of affinities for PKC activators by expressed PKCs

Enzyme	Ka (TPA) (nM)	Ka (oleic acid (μM)	Autophosphorylation (ratio oleic acid/TPA)
Apl I	0.6	52	N.A.[a]
Apl II	0.5	14	1.5 ± 0.4
Apl IIΔE	0.7	17	0.1 ± 0.03
Apl IIΔE2	4	8	4.0 ± 0.7

The Ka for TPA was measured by fitting averaged data (Fig. 6) to double reciprocal plots. All data fit well to the double reciprocal plots ($r > 0.95$) except Apl IIΔE2 ($r = 0.92$). The double reciprocal plots for oleic acid (other than for Apl IIΔE2) showed some positive cooperativity (El Touny et al., 1990), but were still estimated from double reciprocal plots of the average data (Fig. 6). Ratios for autophosphorylation were calculated after first subtracting the amount of phosphorylation seen without activators. Gels from autophosphorylation experiments using low concentrations of the expressed enzymes were either dried down or transferred to nitrocellulose and then quantitated using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Because no autophosphorylation was seen with oleic acid for Apl I, a ratio could not be quantitated. Errors are SEM from four independent experiments.

[a] N.A., not applicable.

by oleic acid at approximately the same rate as by phorbol esters (Fig. 7, Table 3). This result suggests that, in contrast to Apl I, Apl II can *cis*-autophosphorylate in the presence of oleic acid. Autophosphorylation of low concentrations of Apl IIΔE2 is activated to a greater extent with oleic acid than by TPA, consistent with the finding that phosphorylation of substrates by Apl IIΔE2 also is activated more strongly by oleic acid than by TPA (Fig. 6). Surprisingly, although Apl IIΔE is activated equally by oleic acid and TPA for substrate phosphorylation, phorbol esters stimulate autophosphorylation of Apl IIΔE to a much greater extent than does oleic acid. This may reflect that oleic acid-stimulated *cis*-autophosphorylation requires some portion of the E region.

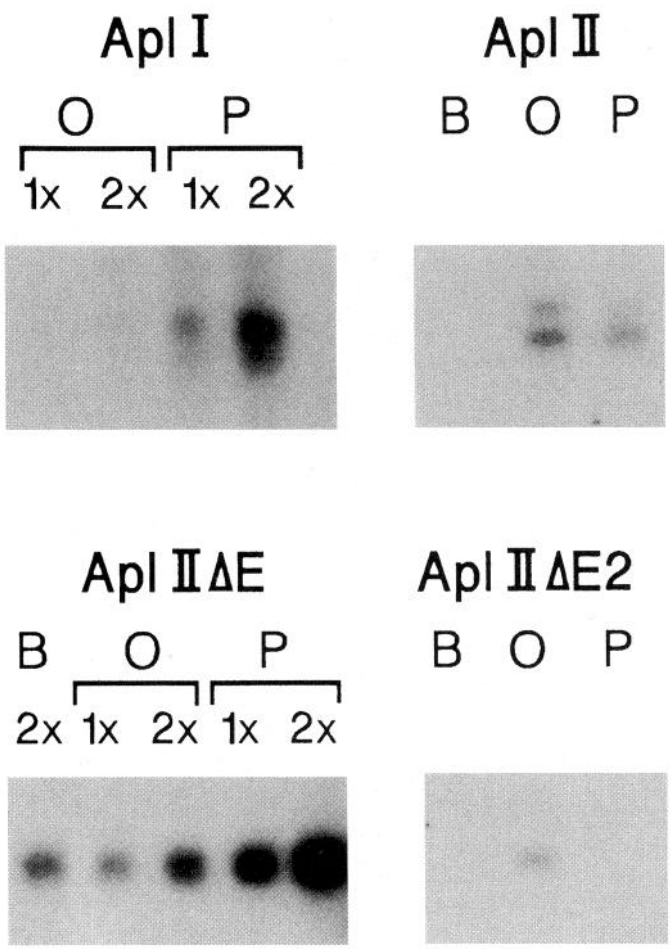

Figure 7. Autophosphorylation of expressed PKCs by oleic acid and TPA. Purified PKCs (Apl I, Apl II, APl IIΔE, and Apl IIΔE2) were activated in the presence of *B* (blank), *O* (50 μM oleic acid), or *P* (150 μg/ml phosphatidylserine and 20 nM TPA) and [γ-^{32}P]ATP followed by separation on 8.5% polyacrylamide gels. The amount of PKCs in the 50 μl reaction were (in ng): Apl I, 10 (*1x*), 20 (*2x*); Apl II, 20; Apl IIΔE, 40 (*1x*), 80 (*2x*); Apl IIΔE2, 100. Assuming homogeneity (which is an overestimate, see Fig. 2), the final concentrations of the *1x* PKCs in the assay were (in nM): Apl I, 3; Apl II, 4.5; Apl IIΔE, 12; and Apl IIΔE2, 24.

DISCUSSION

Role of the E region in the regulation of Apl II

Expression of *Aplysia* PKCs in the baculovirus system has confirmed the properties of the kinases inferred from nervous system extracts. Furthermore, our results indicate that the E region serves to negatively regulate Apl II, possibly through interaction with the phorbol ester-binding domain, C1.

Few previous studies have evaluated the role of the E region in the regulation of PKCs. For PKCη, removal of the E region did not affect substrate selectivity or fold activation by phorbol esters (Dekker et al., 1993b). Further removal or alteration of the pseudosubstrate did modulate substrate selectivity (Dekker et al., 1993a,b). Our results are consistent with this, because we did not observe large changes in the fold activation by phorbol esters after removal of the entire E region (Fig. 6*A*, Table 2), and also did not observe any differences in substrate specificity with Apl IIΔE and Apl IIΔE2 (data not shown).

Our results show that in *Aplysia*, as reported previously for vertebrate PKCs (Schaap and Parker, 1990), a Ca^{2+}-independent kinase, Apl II, requires higher levels of phosphatidylserine in the mixed micelle assay than does a Ca^{2+}-activated kinase, Apl I. Our findings also suggest that this higher requirement is attributable to the presence of the E region, because deleting the E region reduced the amount of phosphatidylserine required to activate Apl II, and the E region directly interacted with phosphatidylserine in a sedimentation assay. Lipid interaction with the E region may be important in the regulation of Ca^{2+}-independent PKCs. Although we have not addressed the specificity of phosphatidylserine in the interaction with the E region, if other lipids can substitute for phosphatidylserine in binding to the E region, then synergistic interactions in the activation of Ca^{2+}-independent PKCs may be possible. Alternatively, the E region may physiologically interact with a protein, and phosphatidylserine may be interacting nonspecifically with the hydrophobic interface normally used for protein–protein interactions. Our results also suggest that the E region inhibits Apl II activity by interacting with the phorbol ester-binding domain, C1. A deletion of the E2 domain altered the activation by two different PKC activators, TPA and oleic acid. This effect is unlikely to be attributable to a general instability of the kinase, because activation of Apl IIΔE2 by phorbol ester was inhibited, whereas activation by oleic acid was enhanced. Furthermore, the modification of Apl IIΔE2 activation could be seen with both substrate phosphorylation and autophosphorylation. However, complete removal of the E region did not affect activation by oleic acid or TPA. This apparent contradiction can be explained by postulating the following model for the role of the E region in the activation of Apl II (Fig. 8): the E region normally inhibits activation of PKC by diacylglycerol or phorbol esters by binding to C1; binding of phosphatidylserine to

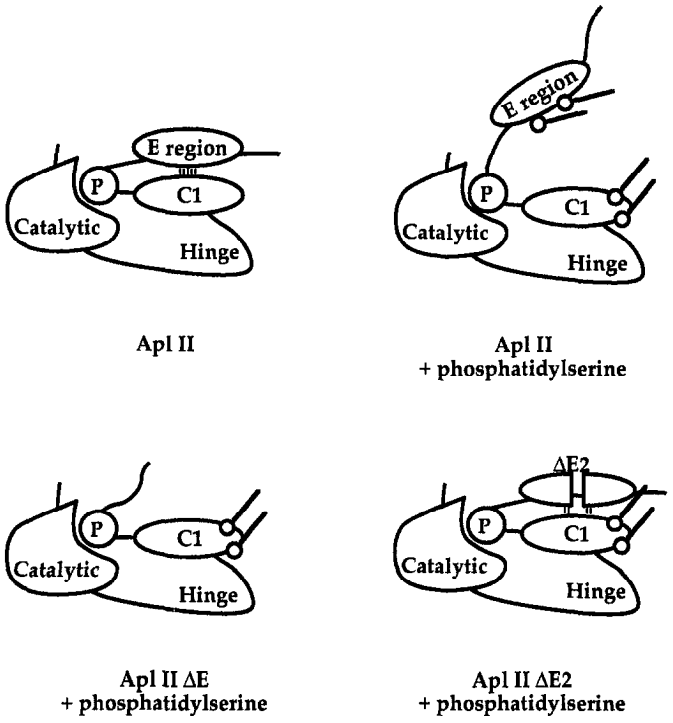

Figure 8. A model for the role of the E region in regulation of Apl II. In inactivated Apl II, where the pseudosubstrate (*P*) is inhibiting the catalytic domain, the E region would interact with the diacylglycerol-binding, phosphatidylserine-binding C1 domain, reducing its affinity for diacylglycerol and inhibiting activation of the enzyme. In the presence of phosphatidylserine (o--) or other lipids that could interact with the E region, the inhibitory interaction of the E region is alleviated and interaction with diacylglycerol can activate the enzyme. In Apl IIΔE, with the E region removed, less phosphatidylserine is required for activation of the kinase, as phosphatidylserine is not required to remove the E region. In Apl IIΔE2, phosphatidylserine binding to the E region is reduced, and its inhibitory role can be seen even in the presence of phosphatidylserine.

the E region disrupts this interaction, thereby relieving the inhibition of PKC activation. In Apl IIΔE2, the E region's effects on regulation would persist even in the presence of phosphatidylserine, either because of reduced binding of phosphatidylserine to the E region in the absence of E2 (Figs. 4,5), or because the confirmation of the E region bound to phosphatidylserine is altered in the absence of E2. This model is also consistent with the reduction in the amount of phosphatidylserine required for activation of Apl IIΔE2 (Fig. 3), because the additional phosphatidylserine usually required for the release of E-region-mediated inhibition has no effect on the activation of Apl IIΔE2.

Our proposed role for the E region is similar to suggestions that have been made for the role of the CalB domain in vertebrate Ca^{2+}-activated PKCs. Studies with fusion proteins generated from Ca^{2+}-activated PKCs show that addition of CalB to a C1 fusion protein confers a Ca^{2+} selectivity to phorbol ester binding (Luo and Weinstein, 1993) and decreases the affinity of a fusion protein containing the C-terminal of C1 for phorbol ester binding (Quest and Bell, 1994). These data are consistent with a model in which the CalB domain interacts with C1 in the native protein. An interaction between sequences in CalB and sequences in C1 also is predicted by the pseudoanchoring hypothesis, whereby the site in PKC that normally binds to a PKC receptor is inhibited by a pseudoanchor located in the CalB domain (Ron and Mochly, 1994). We hypothesize that the E region, which shows homology to CalB (Sossin and Schwartz, 1993), plays an analogous role for Ca^{2+}-independent PKCs.

Relevance to physiological models for PKC activation in *Aplysia*

Expression of the kinases in a heterologous system has allowed the confirmation of several properties of the kinase that previously had been inferred from nervous system extracts. The ability of Apl I to be stimulated in the absence of Ca^{2+} is consistent with data from extracts (Sossin and Schwartz, 1992). The Ca^{2+}-independent stimulation of Apl I may account for the observed activation of Apl I by serotonin, which occurs in the absence of an increase in the steady-state Ca^{2+} levels by serotonin (Eliot et al., 1993). Although Apl I is highly homologous to the vertebrate Ca^{2+}-activated PKCs, differences such as a much shorter amino-terminal V1 region may account for activation of the kinase in the mixed micelle assay in the absence of Ca^{2+}.

Biochemical analyses of the expressed isoforms suggest hypotheses to explain the isoform-specific regulation of PKCs in the *Aplysia* nervous system. Short-term treatment with serotonin activates Apl I, but not Apl II (Sossin and Schwartz, 1992; Sossin et al., 1994). Presumably, serotonin activates Apl I through the production of diacylglycerol. Because the affinity for diacylglycerol is modulated by levels of phosphatidylserine (Hannun et al., 1986; Akita et al., 1990), the difficulty in activating Apl II may be related to the high levels of phosphatidylserine required for Apl II activation. Difficulty in activating vertebrate Ca^{2+}-independent PKCs is not seen in cell lines (Kiley et al., 1990; Pfeffer et al., 1991; Strulovici et al., 1991; Olivier and Parker, 1994) and may be specific for the nervous system because of the presence of increased levels of inhibitors of diacylglycerol-binding (Sossin and Schwartz, 1994). Long-term treatments with serotonin transiently activate both Apl I and Apl II (Sossin et al., 1994); perhaps an additional messenger produced by long-term treatments with serotonin relieves the inhibitory effect of the E region.

Some protein kinases that are normally stimulated by second messengers also have the ability to become autonomous or independent of this signal after activation. The most prominent example of this regulation is the Ca^{2+}-calmodulin-dependent kinase, which no longer requires Ca^{2+}-calmodulin (Hanson and Schulman, 1992) when autophosphorylated. An autonomous kinase activity that phosphorylates a PKC substrate is found in *Aplysia* nervous system extracts, and >60% of this activity is removed by immunoprecipitation with an antibody specific for Apl II, but not by an antibody specific for Apl I (Sossin et al., 1994). This activity increases after long- but not short-term treatment with serotonin in the *Aplysia* nervous system (Sossin et al., 1994). We also observe this activity after expression of Apl II, but not of Apl I, in SF9 cells (Table 2). Autonomous activity of Apl II is not affected by removal of the E region (Table 2), indicating that this region is not required for this activity. The autonomous kinase could be an intrinsic property of Apl II. Alternatively, autonomous activity could be attributable to a post-translational modification such as autophosphorylation, which is present both in *Aplysia* ganglia and in SF9 cells. Because Apl II expressed in SF9 cells retains the ability to become autonomous, elucidation of the mechanism underlying autonomous activation of Apl II should be possible in this system.

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