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 isofoms, 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

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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 PKCe and PKCη (Osada et al., 1990; Kruger et al., 1991; Lund 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 califomica*, the nervous system has only two phorbol ester-activated PKC isofoms, 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 isofom 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 BgII 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 BglII site.
To construct baculovirus transfer vectors, the Apl I cDNA was removed from SK by partially digesting with BamHI and inserted into the BamHI site of the pBlueBacII 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 EcoRI and inserted into pBlueBacII after partial digestion of pBlueBacII at the EcoRI site in the poly linker of the construct. The Apl IIΔE deletion was constructed by ligating an XmnI-EcoRV fragment from the Apl II cDNA to the fusion vector pC830 (Invitrogen) after filling in the BamHI site in the fusion vector and further confirming by sequence analysis results in the replacement of the E region by amino acids 1-10 of the baculovirus polyhedron protein. The Apl IIΔE construct was generated by 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′-TCTAGAACACTTGGATCC-3′ and the inside 5′ primer 5′-GCCCAAGCTTCCGCAAGAATTCCCAAGCAGACAG-3′ or the outside 5′ primer 5′-TCCAGATGGCCGCGCCAATTTCCCAAGCAGACAG-3′ and the inside 5′ primer 5′-CGCCCAAGGTTTTCCTCCCAGGTA-3′ to make the pMALC-E1-E3 construct was digested with NlaIV and SspI and religated into the pBlueBacII baculovirus transfer vector as described above.

To construct fusion protein vectors, the eps-5 Apl II cDNA (Kruger et al., 1991) was excised with Stul and PstI and ligated to the pRIIT2 vector (Pharmacia, Piscataway, NJ) with Smal and PstI. The amino-terminal fragment of Apl II, which ends at amino acids DDY (residue 502), was removed from pRIIT2 with EcoRI, and the ends were filled in with Klung and ligated into the pMALC-R2 vector (New England Biolabs, Beverly, MA) cut with BamHI, filled in with Klung, and religated to create a stop codon. The pMALC-E1-E3 construct was digested with NdeI and Spel and religated to form pMALC-E1-E3ΔP. pMALC-E1-E3ΔE was constructed using the same mutagenesis strategy described above, except with outside primers 5′-GCGAGCTCATTCTACGCA-3′ and the inside 5′ primer 5′-GGCTTGCACTTGGTCTGGA-3′ to generate the construct pMALC-E1-E3. The pMALC-R2 vector was the pMALC-R2 vector (New England Biolabs, Beverly, MA) cut with BamHI, filled in with Klung, and religated to create a stop codon. The pMALC-E1-E3 construct was digested with NdeI and Spel and religated to form pMALC-E1-E3ΔP. pMALC-E1-E3ΔE was constructed using the same mutagenesis strategy described above, except with outside primers 5′-GCGAGCTCATTCTACGCA-3′ and the inside 5′ primer 5′-GGCTTGCACTTGGTCTGGA-3′ to generate the construct pMALC-E1-E3. The pMALC-R2 vector was cut with XmnI to generate the construct pMALC-E1-E3. The pMALC-R2 vector was cut with XmnI to generate the construct pMALC-E1-E3. The pMALC-R2 vector was cut with XmnI to generate the construct pMALC-E1-E3.

Baculovirus expression. Spodoptera frugiperda (SF9; Invitrogen) cells were grown in suspension cultures with supplemented Grace’s media (Life Technologies, Gaithersburg, MD) and 10% fetal bovine serum. SF9 cells (Invitrogen) cells were grown in suspension cultures with supplemented Grace’s media (Life Technologies, Gaithersburg, MD) and 10% fetal bovine serum (Life Technologies). The baculovirus transfer vectors were cotransfected with linearized baculovirus (Invitrogen) into SF9 cells using calcium liposomes following standard procedures (Invitrogen), and the resultant blue colonies were plaque-purified. For Apl IIΔE, positive plaques were selected on oleic acid (Avanti, Alabaster, AL) and 20 μM 12-O-tetradecanoyl-phosphatidylserine (Avanti, Alabaster, AL) and the resultant white colonies were plaque-purified. For Apl IIΔE, positive plaques were selected on oleic acid (Avanti, Alabaster, AL) and 20 μM 12-O-tetradecanoyl-phosphatidylserine (Avanti, Alabaster, AL) and the resultant white colonies were plaque-purified.

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 phosphorylasein (150 μM/mL). Autophosphorylation experiments 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 amyllose columns (New England Biolabs). For the sedimentation assay, fusion protein (2 μM) was incubated in 100 μM of 50 μM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 10 mM 2-mercaptoethanol in the presence or absence of 100 μM 90% dioleoyl phosphatidylserine/10% dioleoyl phosphatidylcholine vesicles (Princeton Lipids, Princeton, NJ) (9:1 w/w). After 20 min at 20°C, the vesicles were sedimented at 100,000 × 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 [3H]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 carbonyl-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 Cl phosphor ester/diacylglycerol binding domain, which regulates enzyme activity. Ca2+-activated PKCs such as Apl I contain a C2 (or CaB) domain that confers the ability of the enzymes to be activated by Ca2+. Ca2+-independent PKCs contain a conserved amino-terminal region, which in Apl II is named the E region because of its conservation to vertebrate Epsilon and Eta 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 CaB 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 CaB (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 (Petcl 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 Coomasie-stained band in the preparation, but are not homogenous 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, phosphatidylerserine 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 phosphatidyserine

A major difference between vertebrate Ca2+-activated and Ca2+-independent PKCs is that Ca2+-independent PKCs require higher levels of phosphatidyserine 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 Ca2+-independent Apl II requires larger amounts of phosphatidyserine in this assay than does the Ca2+-activated Apl I (Fig. 3A). Unlike vertebrate PKCs, Apl I is Ca2+-activated but not completely Ca2+-dependent in the mixed micelle assay, because Apl I can be fully activated even in the presence of 5 mM EGTA. Ca2+ ions do decrease the amount of phosphatidyserine required for Apl I activation, but not for Apl II (Fig. 3A) (data not shown) confirming their identities as Ca2+-activated and Ca2+-independent isoforms.

Removal of the E region reduces the requirement of Apl II for phosphatidyserine. Using the mixed micelle assay, Apl IIΔE and Apl IIΔE2 were both fully activated at lower levels of phosphatidyserine than Apl II (Fig. 3B). Moreover, the level of phosphatidyserine required for activation of Apl II when the E region is removed is similar to that required for Apl I in the absence of Ca2+ ions. Phosphatidyserine 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 phosphatidyserine binding to C1 is similar in Apl I and Apl II and that the difference in required phosphatidyserine levels between Apl I and Apl II can be explained by an additional requirement in Apl II for phosphatidyserine binding to the E region.

To determine whether the E region directly interacts with phosphatidyserine, we constructed maltose-binding protein (MBP)-E region fusion proteins, affinity-purified the proteins from bacteria, and tested their ability to bind to phosphatidyserine using a sedimentation assay (Sossin and Schwartz, 1992; Quest et al., 1994). In this assay, vesicles made of 90% phosphatidyserine 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 phosphatidyserine-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 NH₄SO₄) | 2.5                           | 6.4                             | 2.4              | 48              |
|        | HAP (100-150 mM KH₂PO₄) | 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 NH₄SO₄) | 4.2                           | 20                              | 1.6              | 11              |
|        | HAP (150-200 mM KH₂PO₄) | 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 NH₄SO₄) | 0.4                           | 1.2                             | 3.5              | 64              |
|         | HAP (150-200 mM KH₂PO₄) | 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 NH₄SO₄) | 0.4                           | 1.2                             | 2.6              | 6               |
|          | HAP (175-225 mM KH₂PO₄) | 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.

(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 consist-
Table 2. Autonomous PKC activity of expressed PKCs

| Enzyme       | Autonomous activity (nmol - min^{-1} * mg^{-1}) | TPA stimulated activity | Autonomous activity (per immunoreactive protein) |
|--------------|-----------------------------------------------|------------------------|-----------------------------------------------|
| Apl I        | 0.8 ± 0.2                                     | 3 ± 1                  | N.A.                                          |
| Apl II       | 3.8 ± 1.4                                     | 16 ± 5                 | 1                                             |
| Apl IIIE     | 5.0 ± 2.3                                     | 30 ± 3                 | 1.5 ± 0.7                                    |
| Apl IIIE2    | 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 μM phosphatidylserine and 20 nM TPA. This amount of TPA is not saturating for Apl IIIE2 (see Fig. 6.4) 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

Figure 3. Activation of expressed PKCs by phosphatidylserine. A, Mixed micelles of 2 mole percent dioctylglycerol (dIOG) 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 dIOG in Triton X-100 and varied amounts of dioleol phosphatidylserine were used to activate Apl II (○), Apl IIIE (□), or Apl IIIE2 (△). Maximal activity was 10 nmol - min^{-1} * mg^{-1} for Apl II, 6 nmol - min^{-1} * mg^{-1} for Apl IIIE, and 6 nmol/min/mg for Apl IIIE2. The data shown are from one experiment; similar results were obtained in three additional experiments.

B

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 XmnI site of the pMALC-R2 vector. E, EcoRI; S, SspI; X, XmnI. See text for description of deletions. B, Affinity-purified pMALC fusion proteins (2 pM) 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
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-E3APY) and the other removing the entire E2 domain (E1-E3AE2), 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. 6A; 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. 6B; Table 3). Similar to vertebrate PKCs (Koide et al., 1992; Khan et al., 1993), low concentrations of oleic acid activated the Ca\textsuperscript{2+}-independent Apl II more than the Ca\textsuperscript{2+}-activated Apl I (Fig. 6B). 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 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\textsuperscript{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       | $K_a$ (TPA) (nM) | $K_a$ (oleic acid) (μM) | Autophosphorylation (ratio oleic acid/TPA) |
|--------------|------------------|------------------------|------------------------------------------|
| Apl I        | 0.6              | 52                     | N.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 $K_a$ 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 ($r = 0.93$). 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 phosphomager (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.

* 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.

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 phosphor ester-binding domain, Cl.

Few previous studies have evaluated the role of the E region in the regulation of PKCs. For PKCα, 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. 6A, 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 Ca2+-independent kinase, Apl II, requires higher levels of phosphatidylserine in the mixed micelle assay than does a Ca2+-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 Ca2+-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 Ca2+-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, Cl. 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 Cl; binding of phosphatidylserine to
which the CalB domain interacts with Cl in the native protein. An protein containing the C-terminal of Cl for phorbol ester binding fusion protein confers a Ca^{2+} selectivity to phorbol ester binding activation of Apl IIAE2 (Fig. 3), because the additional phosphatation of PKC activation. In Apl IIAE2, the E region's effects on Figure 8. inhibition of PKC that normally binds to a PKC receptor is inhibited by a interaction between sequences in CalB and sequences in Cl also from Ca^{2+}-activated PKCs show that addition of CalB to a Cl domain, the E region would interact with the diacylglycerol-binding, phosphatidylserine-binding Cl domain, reducing its affinity for diacylglycerol and inhibiting activation of the enzyme. In the presence of phosphatidylserine (0-) 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 IIAE, 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 IIAE2, 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 IIAE2, 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 IIAE2 (Fig. 3), because the additional phosphatidylserine usually required for the release of E-region-mediated inhibition has no effect on the activation of Apl IIAE2.

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 Cl fusion protein confers a Ca^{2+} selectivity to phorbol ester binding (I no and Weinstein, 1993) and decreases the affinity of a fusion protein containing the C-terminal of Cl for phorbol ester binding (Quest and Bell, 1994). These data are consistent with a model in which the CalB domain interacts with Cl in the native protein. An interaction between sequences in CalB and sequences in Cl 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 Moehly, 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 >90% 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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