

Cloning and Characterization of Ca^{2+} -dependent and Ca^{2+} -independent PKCs Expressed in *Aplysia* Sensory Cells

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We isolated cDNA clones from an *Aplysia* sensory-cell library encoding two isoforms of protein kinase C (PKC). Several isozyme-specific regions are conserved in the *Aplysia* kinases, notably the variable regions V5 in the Ca^{2+} -dependent PKC (Apl I) and V1 in the Ca^{2+} -independent PKC (Apl II). Neuronal proteins with the properties expected of these two isoforms can be identified with antibodies raised against peptides synthesized from the amino acid sequences deduced from the clones. Sacktor and Schwartz (1990) measured the proportion of kinase activity that can be translocated to membrane in *Aplysia* sensory neurons and ganglia by stimuli that produce the presynaptic facilitation underlying behavioral sensitization. Much less Apl I and Apl II are translocated, suggesting that still other isoforms of PKC exist in these cells.

Protein kinase C (PKC) has special functions in neurons in addition to its critical role in normal growth and proliferation of many cell types (Nishizuka, 1988). Several neurotransmitters modulate synaptic function by activating this enzyme (Kaczmarek, 1987; Nishizuka, 1988; Rane et al., 1989), and studies in both vertebrates and invertebrates suggest a role for PKC in inducing and maintaining long-term synaptic modifications that are thought to underlie learning and memory (Akers et al., 1986; Farley and Auerbach, 1986; Alkon et al., 1988; Malinow et al., 1988; Olds et al., 1989). In *Aplysia* neurons, activation of PKC has been implicated in several examples of neuronal modulation (Strong et al., 1987; Conn et al., 1989; Sawada et al., 1989; Taussig et al., 1989; Sacktor and Schwartz, 1990). These include presynaptic facilitation of sensory-to-motor neuron synapses, a cellular process underlying behavioral sensitization or dishabituation (Gingrich and Byrne, 1985; Hochner et al., 1986; Braha et al., 1990). Training that produces sensitization in the animal or application of the facilitatory transmitter 5-HT to isolated ganglia causes translocation of kinase activity from cy-

tosol to membrane in sensory neurons, an event linked to the activation of the enzyme (Sacktor and Schwartz, 1990).

Several isoforms of PKC have been described (Nishizuka, 1988). All share conserved regions (C_1 – C_4), which are pieced together with intervening variable regions (V1–V5) specific to each isoform (see Fig. 1). In vertebrates, these enzymes can be placed into two groups, Ca^{2+} -dependent or major (α , β , and γ), and Ca^{2+} -independent or minor (δ , ϵ , ζ , and η). Ca^{2+} -dependent PKCs contain a conserved region, C2, implicated in the Ca^{2+} -dependent translocation of the enzyme to membrane and activation of the kinase (Kaibuchi et al., 1989). Ca^{2+} -independent PKCs lack this region and are not translocated or activated by Ca^{2+} ions (Akita et al., 1990; Kiley et al., 1990; Schaap and Parker, 1990).

We cloned two types of PKC from a sensory-neuron cDNA library and partially characterized their biochemical properties. One isoform (Apl I) is quite similar to other Ca^{2+} -dependent PKCs and is translocated to neuronal membranes by Ca^{2+} . The other isoform (Apl II) is most similar to PKC ϵ , which is Ca^{2+} -independent. The translocation of Apl II is not affected by Ca^{2+} ions, but Apl II can be translocated to phosphatidylserine micelles by phorbol esters. While the reasons for the diversity of kinases are not yet understood, differential affinities for lipid activators, substrate specificity, and susceptibility to inactivation have been offered as explanations for the existence of multiple isoforms of PKC (Sekiguchi et al., 1987; Nishizuka, 1988; Huang et al., 1989; Burns et al., 1990; Huang and Huang, 1990). Characterization of PKCs in *Aplysia* neurons with known physiological actions can lead to an understanding of how specific isoforms function in synaptic plasticity.

Materials and Methods

Aplysia californica weighing 100–200 gm were obtained from the Howard Hughes Mariculture Facility at the Woods Hole Oceanographic Institution (Woods Hole, MA) or from Sea Life Supply (Sand City, CA). All chemicals are from Sigma (St. Louis, MO) unless otherwise stated.

Translocation of PKC. Pleural-pedal ganglia, isolated from animals by dissection, were homogenized in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 0.5 mM EGTA (or 1 mM EDTA), 0.1 mM phenylmethylsulfonylfluoride, 5 mM 2-mercaptoethanol, aprotinin (50 $\mu\text{g}/\text{ml}$), benzamidin (5 mM), and leupeptin (0.1 mM), then centrifuged for 1 min at $1000 \times g$ to remove debris (unhomogenized cells and sheath). Inclusion of MgCl_2 at a concentration physiological for *Aplysia* in the homogenization buffer increases the proportion of cytosolic PKC (Sacktor and Schwartz, 1990). The supernatant was incubated for 20 min at 20°C in the presence or absence of 200 nM phorbol dibutyrate (PDBu), and then centrifuged at $100,000 \times g$ to separate cytosol and membrane fractions. In some experiments, supernatants from extracts not incubated in PDBu were then incubated for 20 min at 20°C with 150 mg/ml phosphatidylserine (Avanti Polar Lipids, Alabaster, AL) in the presence or absence of PDBu.

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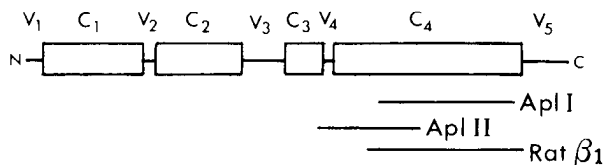


Figure 1. Diagram of a Ca^{2+} -dependent isoform of PKC showing the regions encoded by the cDNA probes used. C_1 – C_4 are conserved domains, and V_1 – V_5 refer to variable regions that differ among isoforms. The C_2 domain is absent in the Ca^{2+} -independent enzymes δ , ϵ , ζ , and η . The Apl I clone shown, the longest cDNA obtained in the screening of the pleural sensory-cell cDNA library, was used in SI protection assays (see Fig. 7) and is shown in the figure beneath the diagram of the major isoforms of PKC. Apl II is the only cDNA for the Ca^{2+} -independent *Aplysia* PKC isolated from the library and was also used in the SI protection assays as well as for *in situ* hybridization (see Fig. 8). The rat $\beta 1$ PKC cDNA was used to screen the library at low stringency.

To test the effect of Ca^{2+} , extracts of pleural-pedal ganglia were diluted into EGTA- Ca^{2+} buffers (Eliot et al., 1989) with 20 mM HEPES (pH, 7.6) and 100 μM leupeptin in the presence or absence of PDBu. Isolated ganglia were exposed to 5-HT and PDBu as described by Sacktor and Schwartz (1990) but with 1 μM PDBu to ensure accessibility of the drug because the connective tissue sheath was not completely removed.

Screening libraries. A pleural sensory-cell library (the gift of Michael Knapp, Philip Goelet, and Vincent Castellucci) containing 3×10^5 phage was screened at low stringency for 16 hr at 42°C in a standard hybridization mixture of 30% formamide, $5 \times \text{SSC}$ ($1 \times \text{SSC}$: 0.15 M NaCl, 0.015 M Na citrate), $10 \times$ Denhardt's solution, 1% SDS, and 20 mM Na phosphate (pH, 7.0) with 250 $\mu\text{g}/\text{ml}$ of denatured salmon sperm DNA, and then washed at 55°C in $2 \times \text{SSC}$ containing 1% SDS. We screened a randomly primed cDNA library of the total *Aplysia* nervous system at high stringency as described by Beushausen et al. (1988).

Anchored PCR cloning of the 5' end of Apl II. Randomly primed *Aplysia* nervous tissue cDNA, tailed at the 5' end with oligo-G by terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Gaithersburg, MD), was amplified with a 3' primer (CACATCAACACGGAATCGTGC) derived from a sequence approximately 100 base pairs (bp) from the end of the longest Apl II cDNA and a 5' primer consisting of polyC. The product was amplified again with an internal 3' primer (GGAATTCCGTCTTCAACGGTTCGGTGAA) and a 5' polyC primer with EcoRI sites at their ends. The amplified DNA was cut with EcoRI and ligated into λ -Zap II (Stratagene, La Jolla, CA).

DNA sequencing. cDNA clones were subcloned into Bluescript phagemids (Stratagene) for sequencing by the dideoxy chain-termination method of Sanger et al. (1977) with reagents and protocols from United States Biochemical Corporation (Cleveland, OH) using ^{35}S -dATP (Amersham, Arlington Heights, IL) as label. Clones were sequenced from their ends using the primers provided by Stratagene. Internal regions were sequenced with primers, 17 nucleotides in length, synthesized by the Protein Chemistry Core Facility at Columbia University using previously determined sequences. All sequencing was done on both strands.

Expression of Apl I and Apl II. Northern blotting, SI nuclease protection experiments, and *in situ* hybridization were done as described by Beushausen et al. (1988).

Production of Apl I and Apl II antibodies. Peptides derived from the C-terminal sequence of Apl I (NH_2 -CSYVNPEFVVTV) and Apl II (NH_2 -CSFANPDYGKLEMEASGQAH) were synthesized by Peninsula Laboratories (Belmont, CA). Cysteine residues were added to the N-termini of the peptides for cross-linking to maleimide-activated bovine serum albumin (BSA) using the protocol provided by the manufacturer (Pierce Chemical Co., Rockford, IL). The cross-linked peptides (0.5 mg) were emulsified in an equal volume of RAS adjuvant (RIBI, Hamilton, MO) for injection into Hare Marland rabbits four times at monthly intervals. Antisera were purified with a column made by linking the synthesized peptides to Sulfolink beads using instructions provided by Pierce. Antisera were diluted four times in normal phosphate-buffered saline (PBS; 0.15 M NaCl, pH 7.0) and applied to the affinity column (10 ml/hr) at 4°C, which were then washed with three column volumes each of PBS, PBS with 0.65 M NaCl, and then PBS again, and eluted with either 0.1 M glycine (pH 2.3) or 0.1 M triethylamine (pH 11.5). Fractions (1 ml) were collected and neutralized rapidly with either 0.1 ml of 1 M Tris base (pH 9.5) or 0.1 ml of 1 M triethanolamine (pH 6.0).

Western blotting. Protein samples were electrophoresed on 8–10% SDS-polyacrylamide gels (Laemmli, 1970) and blotted onto nitrocellulose filters (BioRad, Rockville Center, NY). The filters were then blocked with 5% BSA in TSN (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05–0.2% NP-40) for 30 min at room temperature, and then treated with either a 1:1000 dilution of affinity-purified Apl II antiserum, a 1:1000 dilution of anti-rat $\beta 1$ (the gift of Drs. K. Ase and Y. Nishizuka, Kobe University School of Medicine; Ase et al., 1988), or a 1:500 dilution of affinity-purified Apl I antiserum in TSN. Because the Apl I and Apl II proteins are well separated by gel electrophoresis, we often used the two antisera together on the same blot. Blots were washed six times each for 5 min with TSN and then incubated in TSN with a 1:5000 dilution of secondary antibody linked to alkaline phosphatase and developed according to the manufacturer's instructions (Promega, Madison, MI). Results were normalized to total membrane protein as measured by a modification of the Bradford assay (Sacktor and Schwartz, 1990) and were in the linear range (20 μg protein) for detection by the phosphatase reaction. For the experiments presented in Table 1, two or three pairs of pleural-pedal ganglia were needed for each determination. Proteins on Western blots were quantitated with a scanning densitometer (model GS300, Hoefer, San Francisco, CA).

Results

Two isoforms of PKC cloned from the *Aplysia* nervous system

We obtained four hybridizing cDNAs by screening 3.6×10^5 phage from a pleural sensory cell cDNA library at low stringency with a rat $\beta 1$ PKC cDNA probe (the gift of G. M. Housey and I. B. Weinstein, Columbia University; Housey et al., 1987). This heterologous probe encodes C_4 , a conserved segment in the catalytic domain (Fig. 1). Hybridization analyses and sequencing of the *Aplysia* cDNAs indicated that we had isolated two types of clones, Apl I (three of the clones) and Apl II.

The sequence of Apl I PKC

To obtain the complete cloning sequence of Apl I, we screened a randomly primed cDNA library of the total nervous system at high stringency with the longest of the three Apl I cDNAs (Fig. 1). The coding sequence of Apl I cDNA and its deduced amino acid sequence are shown in Figure 2.

Apl I is similar to all of the Ca^{2+} -dependent isoforms described (65–73% amino acid identity; Parker et al., 1986; Kikkawa et al., 1987; Fig. 3); it is closest to the PKC of *Drosophila* brain (Rosenthal et al., 1987). The nucleotide sequence around the codon putatively identified as the initiator for Apl I fulfills some of the criteria for a eukaryotic initiator methionine residue (Kozak, 1981). The five variable (V_1 – V_5) and four constant (C_1 – C_4) regions align with those of the vertebrate Ca^{2+} -dependent kinases. With the exception of V_1 , the length of each region is also well conserved.

All but one of the variable regions in Apl I are not characteristic of any vertebrate homolog. The only residues conserved in the variable regions (V_1 – V_4) are those that are also conserved between the α -, β -, and γ -isoforms. The exception is in V_5 , which contains several amino acids that are identical to the mammalian $\beta 1$ kinase but not to any of the other isoforms (asterisks in Fig. 3). Most of these amino acids are also conserved in the *Drosophila* Ca^{2+} -dependent PKCs, though the published dPKC53G (br) sequence is frame-shifted at bp 2758 just before the strong similarity to $\beta 1$ (Rosenthal et al., 1987). Glu-611, Ser-606, and Asp-604 are also conserved in the yeast enzyme (Levin et al., 1990).

The sequence of Apl II PKC

The sequence of Apl II is shown in Figure 4. This isoform is most similar to mammalian PKC ϵ and the 98F kinase of *Dro*-

sophila (65% identity; Ohno et al., 1988; Ono et al., 1988; Schaefer et al., 1989). The absence of a C2 region is an indication that Apl II is Ca^{2+} -independent. Unlike Apl I, Apl II contains sequences in variable regions throughout the molecule that are highly conserved in all ϵ -isoforms thus far cloned, but not present in other PKCs (asterisks in Fig. 5).

did not notice that the V1 region is conserved throughout phylogeny. The third domain, the pseudosubstrate sequence and the region immediately C-terminal to it, is also peculiar to ϵ -isoforms. The fourth, which is an insert in C1, is similar in both the δ - and ϵ -isoforms. Finally, there are sequences in the C4 region that are common to all PKC ϵ s.

Northern blot analysis demonstrates that both Apl I and Apl II are expressed in nervous tissue as transcripts much longer than 5 kilobases (kb; Fig. 6). A large size is not unusual for *Aplysia* transcripts; for example, both regulatory and catalytic subunits of the cAMP-dependent protein kinase are also encoded by large transcripts (Beushausen et al., 1988; Bergold et al., 1990).

Apl I	RVARRGALRQKNVHEVKNHKFLARFFKQPTFCSHCKDFIWGFGKQGFQCCVCSLVVHKRCHEFV	65
Rβ1	RFARKGPLRQKNVHEVKNHKFTARFFKQPTFCSHCTDFIWGFGKQGFQCCVFCVVKRCHEFV	80
Apl I	CFICPGADKGPDSAT-NLHKFKLHSGSPTFCDHCGSLLYGLLHQGLKCDSCDMNVHKRCCKN	129
Rβ1	TFSCPGADKGPASDDPRSKHKFKIHTYSSPTFCDHCGSLLYGLIHQGMKCDTCMMNVHKRCVMN	144
Apl I	VPLLCGTDHTERRGRILIKGAVKGSKVLVEILEAKNLCMPDNGLADPYVKVLIPYDAHKLKL	192
Rβ1	VPSLCGTDHTERRGRIYQAHIDREVLIVVVRDAKNLVMPDNGLSDFYVKLKLIPDPKSESKQ	208
Apl I	KTKTIKASLNPVWNESTVDIGPEDNSKRLSLEVWDWDRSRNDFMGSLSFSGISELIKSPVEGW	256
Rβ1	KTKTIKCSLNPWNENETFRFQLKESDKDRRLSVEIWDWDLTSRNDFMGSLSFSGISELQKAGVDGW	272
Apl I	FKLLNQEEGEFYGVPTDDITESIQEIKSKMHRSSISSEKRYPEPD-----KVQNMSKQDIVR	320
Rβ1	FKLLSQEEGEFYFNPVPPPEESEGNEELRQKFERAKIGQGTAKPEEKTANTISKFDNNGNRDRMK	336
Apl I	ASDFNFLTVLGKGSFGKVVLAERKGTDELYAIKILKDVIIQDDDVECTMIEKRVLALPKPPF	378
Rβ1	LTDFNFLMVLGKGSFGKVMLSERKGTDELYAVKILKDVVVIQDDDVECTMVEKRVLALPKPPF	400
Apl I	LVQLHSCFQTMDRLYFVMEYVNGGDLMYRIQQEGKFKEPVAAFYAAEIAIGLFYLHTQGIVYRD	442
Rβ1	LTQLHSCFQTMDRLYFVMEYVNGGDLMYHIQQVGRFKEPFAVFAEIAIGLFFLQSKGIIYRD	464
Apl I	LKLDNVMLDAEGHIKIADFGMCKENIMGDKTTRTFCGTPDYIAPEIVLYQPYGRSVDWWAYGVL	508
Rβ1	LKLDNVMLDSEGHIKIADFGMCKENIWDGVTTKTCGTPDYIAPEIIAYQPYGKSVDWWAFGVL	530
Apl I	LYEMLAGQPPFDGEDEEELFTSITDHNVSYPKALSREAVSLCKGLLTKTPAKRLGCGPNERDI	572
Rβ1	LYEMLAGQAPFGEDEDELFSQIMEHNVAYPKMSKEAVAICKGLMTKHPGKRLGCGPEGERDI	594
Apl I	KDRAFFRPIQWERIELREVQPPYKPRIKSRKDVSNFDREFTSEAPNVTPTDKLFIMNLDQCEFS	636
Rβ1	KEHAFFRYIDWEKLERKEIQPPYKPKARDKRDTSNFDKEFTROPVELTPTDKLFIMNLDQNEFA	658
Apl I	GFSYVNPEFVTV	649
Rβ1	GFSYTNPEFVINV	671

Figure 3. Comparison of Apl I with rat βI PKC. Output by the Bestfit algorithm (Devereaux et al., 1984). |, identity; :, a conservative substitution. Gaps are represented by -. Asterisks above the sequence indicate residues that are identical between Apl I and βI PKCs but that are not present in the mammalian βII, the α-, or the γ-isoforms. The diamond points to the splice junction in the mammalian enzyme (Coussens et al., 1987).

Amounts of the *Aplysia* PKC transcripts were assessed in nervous tissue and in several other organs by nuclease S1 protection experiments (Fig. 7). In RNA from nervous tissue, Apl II is approximately three times more abundant than Apl I (Fig. 7A). To survey the expression of the two isoforms in other tissues (Fig. 7B), we hybridized labeled antisense probes to total RNA isolated from buccal muscle (Fig. 7B, lanes 5, 6), gut (lanes 7, 8), salivary gland (lanes 9, 10), and ovotestis (lanes 11, 12), in addition to nervous tissue (lanes 1, 3). While Apl I RNA is much more plentiful in the nervous system, longer exposure of the autoradiograms shows that small amounts are also present in the other tissues. Apl II mRNA, the more abundant transcript in nervous tissue, is present in substantial amounts in ovotestis and gut, but is not plentiful in the other tissues.

The Apl I transcript is probably alternatively spliced. Longer exposure of the autoradiograph also reveals a protected fragment of the Apl I mRNA that is much more abundant in gut and ovotestis than in nervous tissue (arrows in Fig. 7B). The predicted splice junction corresponds to the splice site reported for

the mammalian βI/βII PKC gene (diamond in Fig. 3). Alternative splicing at this site results in kinase proteins that differ only in their C-terminal 50 amino acids (Coussens et al., 1987).

Expression of Apl II in identified neurons

In situ hybridization demonstrates that Apl II is expressed in all nerve cells, including sensory neurons (Fig. 8) and bag cells (data not shown). Little hybridization is seen in glia or the connective tissue sheath. Hybridization to the antisense RNA is predominant in the cytoplasm of the sensory neurons and bag cells, but can also be seen in their processes. A similar distribution of hybridization was seen in sensory neurons with the catalytic subunits of the *Aplysia* cAMP-dependent protein kinase (Beushausen et al., 1988). The proximal process of *Aplysia* neurons may actually be considered part of the cell body because it contains ribosomes and other somatic organelles at considerable distances from the perikaryon (e.g., see Schwartz et al., 1986).

1 GTGCTAAAATTCGTATTACAACCTCCCGTGATATTTTTTGGCTGTGATATTCTCAGTTCAACTGGATTAAATTTGATGCCAGGCCTCTAATGCT
-
94 TACCCCGCACGGCAGGATTGGCTGAGTTATCCCCGATCCTCTGCTGAGGCCCTCTGAGCGGGCTTCAATGTGCGCGAGGGCCAAAAATGGTCTTT
M S R R A K M V F

187 CAACGGTTCGGTGAATAAAGGTTTGCAGGCCGTGGATCTCAAACCCACGGACTTCTCGTACGATTGCAGAAGGTTCCACGAAGAAAA
N G S V K I K V C E A V D L K P T D F S L R L Q K G S T K E K

280 AGCCTCCGAGATGATTGAGCCCTACGTGAATATTGATGTGGATGAAGTGTACATAGCTTAAGACCATTACGAAGCCCACCAAAAGTGTTAAACACA
A S Q M I E P Y V N I D V D E V Y I A K T T T K P K S V G K P Q

373 GTGGTTTGGGAACGAGGATTTACGTCGGAAGTTCACAATGGACAGAATGTGAATCTAACGGTGTTCATGACGCGCAATTCCACCCGACGA
W V W N E N D F T C S E V H N G Q N V N L T V F H D A A I P P D E

466 GTTCGTTGCCAACTGCACGATTCGGTTTGATGATGTGAAGAAAAATCTGACTTTTGGATCGATTGGAAACCAATGGAAAACTGCACGTTGT
F V A N C T I P F D D V K G K S D F W I D L E P N G K L H V V

559 TATCGAGCTGTGTGGCTCCGCAACCGAAGCTGCGGAGTCCACACAAAGGAGAAAGTGTCAAAGAAAAGGAAGGTATGCTCAACCGCGCGG
I E L C G S A T E A A E S T P K E K V F K E K E G M L N R R R

652 TGGGGCATCGCTCGTCGGGTTTCATCAGGTCAACGGCCACAAGTTCATGGCAATGTCTTTCAGACAACCCACATCTGCTCGCTGTCGCCGGA
G A M R R R V H Q V N G H K F M A M F F R Q P T F C S L G R D

745 CTTTATCTGGGGTCTTGGCAAGCAAGGTTACGAGTGCCAAGTGTCTACATGCGTGGTTCAACAGCGATGTCACCAGCATATAGTACCAAAATG
F I W L G K Q G Y Q C T V C T C T V H K R C H Q H I V T K C

838 TCCCGTTCCCGGATGTAGCTAATGATGAGGTGACGGGCAAGCGGTTCAACATCAACGTACCACACAGATTCAACGTACACAACATATAGCG
P G S R D V A N D E V T G K R F N I N V P H R F N V H N Y R R

931 GCCAACCTTCTGTGATCATTGTGGCTCTCTGCTCTACGGGCTGGTGAGACGGGACTGCAGTGCAGTGTGTGCAAGATGAACATTCAACAGAG
P T F C D H C G S L L Y G L V R Q G L Q C D V C K M N I H K R

1024 ATGCCAGAAGATGTGGCGAACTGCGGCACGAACCCCGAGACATGGCCAGACTTTACAGGAGATGGGCATATCAGGGGACAACTTAG
C Q K N V A N N C G G T N T R D M A Q I L Q E M G I S G D K C L T

1117 ACCCAAGACCAAAAGCTGTCAATAAGTGAGTCCCATGAGTCCCAAAATGAGTTCCTCCCATGTCATGAGAGATCTTTATCATCCCTATACC
P K T K K L S I S E S H E S P N K S S P M H E R S S L S S P I P

1210 TGTGATCCAAGACTCTGATGAGGCACAAACCCGCGAGATGGGGCTGCCAGCGACAGTCTACCGGTCATGCTAGCAATGAECATCAGGGTTC
V I Q D S D E A Q P G E M G L P S D S L P V N A S N E H Q G S

1303 CCGTACACGGAGCCCCCTCAGTGACCGCTCGCGCTCCCAACAGTCGCGATCAGCTTGCACGACTTCAATTTTATCAAAGTCTGGGCAAGG
R T R S P S S D R S R S H H S R I S L H D F N F I K V L G K G

1396 CAGTTTGGAAAAGTGATGTTAGCAGAGAAGAAAGGGACAGATGAAGTATATGCTATAAAAGTTTGA AAAAGGATGTGATTATACAAGATGA
S F G K V M L A E K K G T D E V Y A I K V L K K D V I I Q D D

1489 TGATGTGGAATGTACAATGACAGAAAAAAGAAATATTAGCATGTGACGCAACATCTTTTCTACTGCATCTCACTCAITCTTCCAGACGAA
D V E C T M T E K R I L A L S A K H P F L T A L H S S F T Q T K

1582 GGAACGATTGTTCTTGTCTGAGGATGATGAAATGAGGAGATCTCATGTTCCAAATTCACGGGCTCGCAAGTTTGTAGAGCTCGGGCAGC
E R L F F V M E Y V N G G D L M F Q I Q R A R K F D E P R A R

1675 GTTTTATCGCGCTGAGGTGACATTAGCTCTTATGTTTTCGATAGGCACGGTATTATATACAGAGATCTGAAATTAGACAACATATTGCTGGA
F Y A A E V T L A L M F L H R H G I I Y R D L K L D N I L L D

1768 TGCTGAAGGACATTGCAAGATTGCTGACTTTGGAATGTGCAAGAAGGCGATGACAGAGAACAATTAACGCGAGACATTCTGTGGCACTCCGGA
A E G H C K I A D F G M C K E G M T E N K L T Q T F C G T P D

1861 CTACATTGACCCGAGATCTGCAAGAACAATAGTATGCGGATGCGGACTGGTGGGCGTGGGCTCTCATGTATGAGATGATGGCAGG
Y I A P E I L Q E L K Y D A S V D W A L G V L M Y E M M A C

1954 ACAGCCCGCGTTTGGGCGGACAAACGAGCACTGTTTGTAGTCTATTTTGCATGACGATGCTCTTTACCTGTCTGGCTCAGCAAAGAGGC
Q P P F E A D N E D D L F E S I L H D D V L Y P V W L S K E A

2047 AGTTTCTATACTCAAGGGGTTTATGACTAAAAACCTGCCAAGCGACTCGGCTGTGTTTACTACCCAAGGTTGCGAAAAAGCAATCTTGTGTA
V S I L K G F M T K N P A K R L G C V T T Q G C E K A I L V H

2140 TCCATTTTCCATGAAAAAATGATTGGGAAGCTTTGGAACAACGCAAGTGAAGCGCCCATTCAGCAAAAAATAAAAACAAAACGGATGC
P F F H E K I D W E A L E Q R K V K P P F K P K I K N K T D A

2233 CAATAACTTCGACCGGACTTTCAGCTCAGAGACCCGTCTGACGCCGTTGGATTCGCGCGGTGATAAAGACCATAAAACCAAGAAGATTCCG
N N F D R D F T S E D P V L T P V D P A I A K T I N Q E E F R

2326 CGGCTTCTCGTTGCAATCCAGACTACGGCAAGTTAGAAATGGAGGCAAGTGCCAGGCGCACTGACCAGCCATCCGGCAGGGGACTGCCCC
G F S F A N P D Y G K L E M E A S G Q A H -

2419 GCCAAGCTCATTCTCCGACCATTCATCTCAAATGATGATGGTGGCTGTGTTCTTCTAGCTCTCTGTGTTGGATTGTTTGTGTTTGTCT

Biochemical properties of Apl I and Apl II PKCs

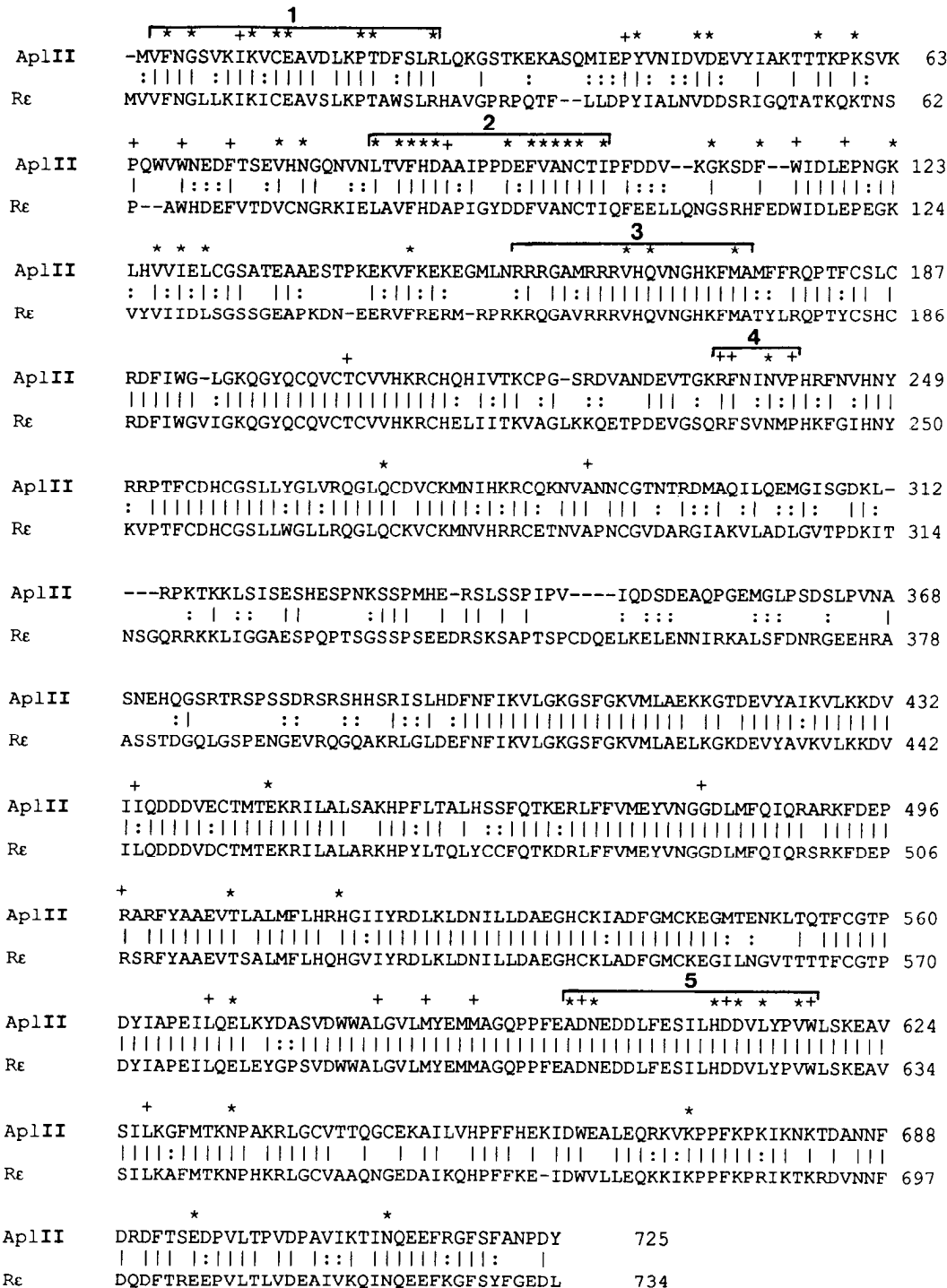


Figure 5. Comparison of Apl II with rabbit PKCε (Ohno et al., 1988). Asterisks above the sequence are residues identical among rabbit PKCε, *Drosophila* 98F, and Apl II, but that are not in any other PKCs (mammalian α, βI, βII, γ, δ, ζ, and *Drosophila* brain or eye-specific kinases). + indicates residues that are conserved in Ca²⁺-independent PKCs (δ or ε) but that are absent in Ca²⁺-dependent PKCs. The regions bracketed are discussed in the Results. Other symbols are as in Figure 3.

low concentration of Ca²⁺ (0.5 mM EGTA) and 10 mM MgCl₂ (Fig. 9A; cf. 1 and 2). In the absence of Ca²⁺, phorbol ester added to the extract before the subcellular fractionation did not result in the translocation of the Apl I antigen to neuronal membranes (Fig. 9A, Table 1). This was surprising because it has previously been shown that PKC activity is translocated under these conditions (Table 1; Sacktor et al., 1988–1989). In contrast, we

found that addition of phorbol ester does translocate the Apl I antigen to phosphatidylserine micelles (Fig. 9A, 3 and 4). This translocation appears to require the presence of some Ca²⁺ ions, because the amount of kinase protein translocated is reduced in extracts prepared with 1 mM EGTA (data not shown).

In the absence of phorbol ester, the Apl I isoform is translocated to neuronal membranes by millimolar concentrations

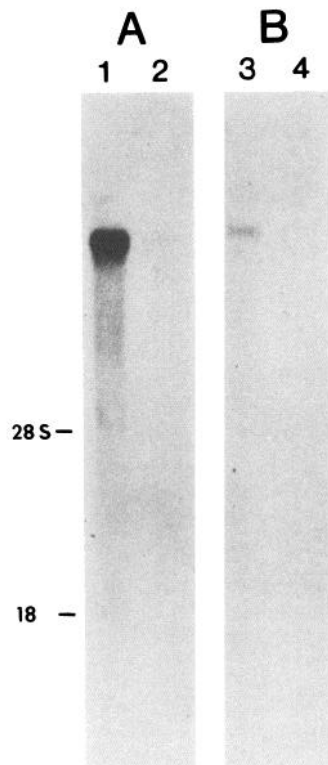


Figure 6. Northern blots of RNA from *Aplysia* nervous tissue with Apl I and Apl II cDNA probes. PolyA⁺ RNA (lanes 1, 3) and total RNA (lanes 2, 4) isolated from nervous tissue (10 μ g each) were electrophoresed and blotted onto nitrocellulose filters that were probed with Apl I (A) or Apl II (B) cDNA (see Fig. 1 for probes). Size is indicated by the location of rat 18 and 28S ribosomal RNA.

Table 1. Isoform-specific translocation of PKC

Experiment	Percent change on membrane		
	Apl I	Apl II	PKC activity
PDBu ^a			
Homogenates	8 \pm 7 (10) ^{NS}	38 \pm 18 (11)	72 \pm 11 (18)
Ganglia	33 \pm 11 (10)	32 \pm 14 (9)	81 \pm 26 (8)
5-HT (20 μ M)			
Ganglia	-9 \pm 8 (8) ^{NS}	-5 \pm 6 (8) ^{NS}	38 \pm 11 (8)

Values (\pm SEs) are expressed as the mean percent difference of PKC on membranes between treated and contralateral control pleural-pedal ganglia determined either by Western blotting or by enzyme assay (Sacktor et al., 1988–1989; Sacktor and Schwartz, 1990). The number of independent determinations is given in parentheses. NS, not significantly different from 0 ($p < 0.05$, one-tailed t test).

^a In homogenates and for the activity measurements in intact ganglia, 200 nM PDBu was used; 1 μ M PDBu was used in the Western blotting experiments with intact ganglia because their connective tissue was not removed completely.

of Ca²⁺. At lower concentrations (100–400 μ M), the translocation is enhanced by phorbol ester (Fig. 9C). These results, which demonstrate that the antigen is a Ca²⁺-dependent PKC, show that the translocation of Apl I to neuronal membranes by PDBu requires more Ca²⁺ than does its binding to phosphatidylserine micelles. The results also suggest that the isoform whose activity is translocated by PDBu at the lower concentrations of Ca²⁺ is not Apl I (Sacktor et al., 1988–1990; Table 1).

Apl II. We also raised antisera to a synthetic peptide with a sequence from the C-terminus of Apl II that recognize an *M*, 87,000 protein (Fig. 9B), approximately the size expected from the cDNA clone (84,000). In this Western blot, the protein appears as a wide band. It is likely that the protein undergoes posttranslational modification, because the band can be resolved into several discrete components (data not shown).

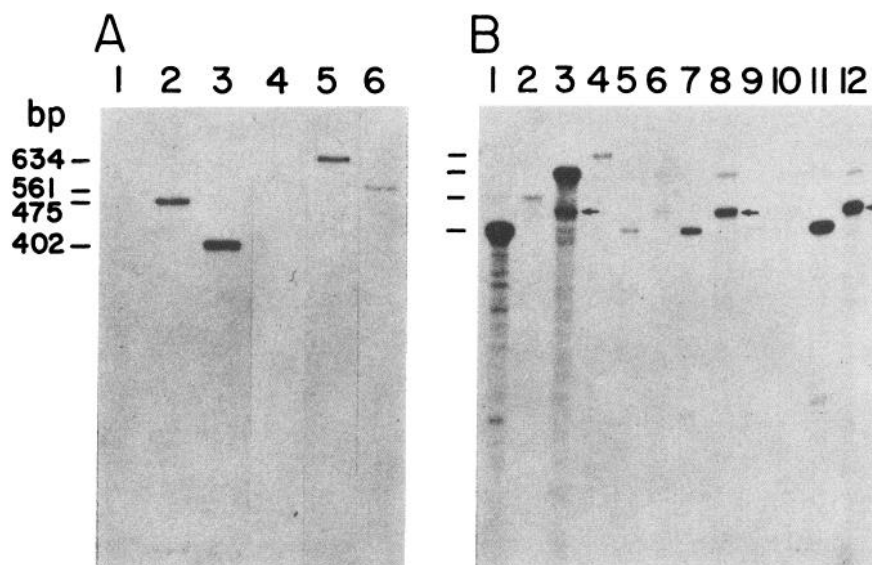


Figure 7. S1 nuclease protection experiments with Apl I and Apl II antisense RNA. **A.** Riboprobes were synthesized from the T3 primer of the cDNA clones described in Figure 1 that are subcloned in Bluescript phagemids. Lanes 2 and 5 show the Apl II and Apl I radiolabeled antisense RNA probes. These probes were completely digested by the nuclease (lanes 1, 4). When mixed with 20 μ g of total nervous tissue RNA, fragments of 400 (lane 3) and 560 (lane 6) bp are protected. Densitometric scanning of the autoradiogram determined that Apl II RNA is three times more abundant than Apl I. The expected sizes (in bp) of protected and unprotected RNA fragments are indicated to the left. **B.** Expression of Apl I and Apl II RNA in *Aplysia* tissues. Total RNA (20 μ g each) from nervous tissue (lanes 1, 3), buccal muscle (lanes 5, 6), gut (lanes 7, 8), salivary gland (lanes 9, 10), and ovotestis (lanes 11, 12) was hybridized with radiolabeled Apl II or Apl I antisense RNA and treated with S1 nuclease. Lanes 2 and 4 are controls: Apl II and Apl I sense RNA hybridized to nervous tissue RNA and digested with the nuclease. The size of the RNA is indicated to the left of the figures. The arrows identify a suspected alternatively spliced form of the Apl I transcript.

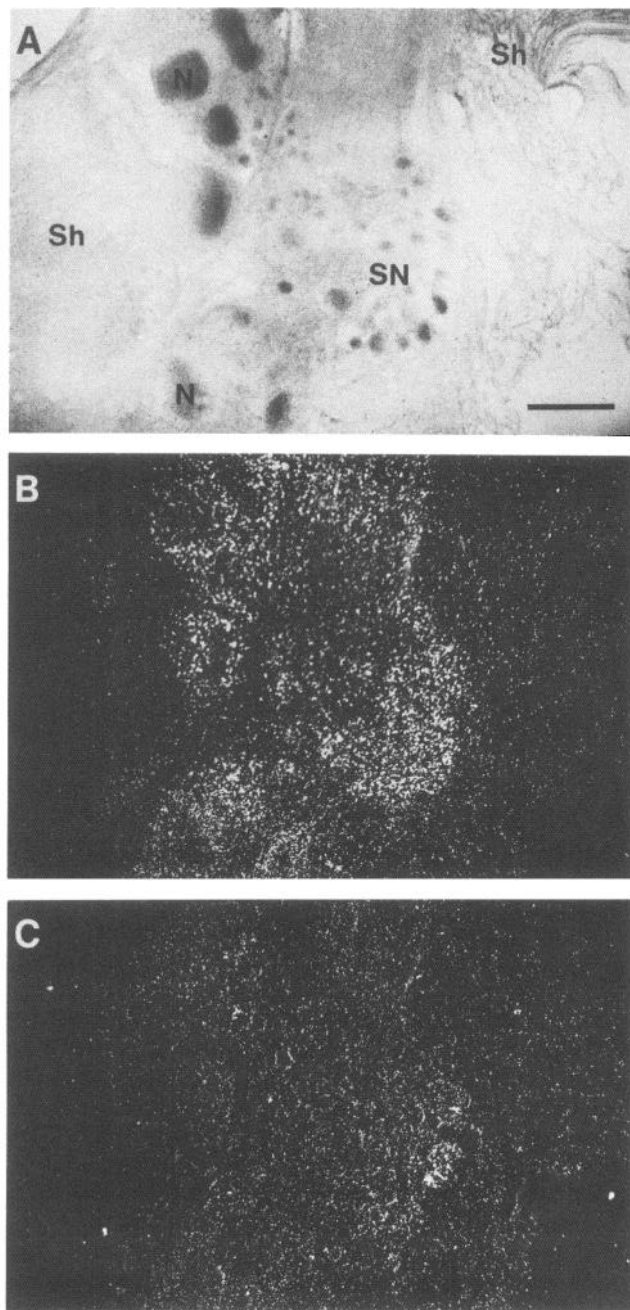


Figure 8. *In situ* hybridization with Apl II antisense RNA. *A*, Bright-field micrograph of a cryostat section (16 μ m) of the pleural ganglion: SN, the cluster of sensory neurons; N, unidentified neurons; Sh, the connective tissue sheath of the ganglion. *B*, Dark-field micrograph of a section through the pleural ganglion hybridized to Apl II antisense RNA. Silver grains are seen over the sensory cells. *C*, Dark-field micrograph of an adjacent section hybridized to Apl II sense RNA (control). Scale bar, 100 μ m.

The *M*, 87,000 protein recognized by Apl II antisera has the biochemical properties expected of a Ca^{2+} -independent PKC. This isoform is translocated both to neuronal membranes and to phosphatidylserine micelles in the presence of phorbol ester (Fig. 9*B*, Table 1). Much more Apl II is translocated to phosphatidylserine micelles, however—as much as 90% in some experiments (data not shown). As expected from the absence of the C2 domain in this isoform, Ca^{2+} ion has no effect on the

translocation of Apl II either to neuronal membranes or to phosphatidylserine micelles (Fig. 9*B*).

Role of Apl I and Apl II in presynaptic facilitation

Phorbol esters have been shown to potentiate synaptic transmission in *Aplysia* (Sawada et al., 1989; Taussig et al., 1989; Braha et al., 1990; Fossier et al., 1990). We found that treatment of isolated ganglia with PDBu results in significant translocation of both PKC isoforms as determined by Western blotting, but the translocation is less than that of PKC enzyme activity (Table 1).

Are the Apl I and Apl II isoforms of PKC translocated during presynaptic facilitation? Application of 5-HT, a transmitter that causes presynaptic facilitation in pleural-pedal ganglia, does not result in the translocation of either isoform (Table 1). This is surprising because sensitizing stimuli delivered to the intact animal and treatment of the isolated ganglia with 5-HT previously have been shown to cause translocation of PKC activity to membrane in sensory neurons and pleural-pedal ganglia (Sacktor and Schwartz, 1990; see also Table 1). These observations suggest that other isoforms of PKC are present in sensory cells, which are selectively translocated at low concentrations of Ca^{2+} by PDBu in homogenates and by 5-HT in intact ganglia.

Discussion

Phylogeny of PKC

Some domains are highly conserved in PKCs, especially the catalytic region (C4) and the region that binds lipid activators (C1; Rosenthal et al., 1987; Schaeffer et al., 1989; Levin et al., 1990). Variable regions that differ among the Ca^{2+} -dependent isoforms must be responsible for differences in the characteristic biochemical properties of these kinases. Because the variable (isoform-specific) regions are not conserved in invertebrates, it is difficult to predict the similarity of the biochemical properties of Apl I to those of the vertebrate major forms. Variable region V5, however, does show isoform-specific conservation throughout phylogeny (Fig. 3). Thus, antisera raised against V5 sequences in the vertebrate β I PKC cross-react with the Apl I protein. This C-terminal region is nearly identical in Apl I, *Drosophila* brain PKC, *Drosophila* 53E eye-specific PKC, and the vertebrate β I PKC. Furthermore, we have some indication that the splice site that results in the two mammalian transcripts, β I and β II, is also conserved in *Aplysia*. V5 is likely to have similar functions in these PKCs.

In contrast, Apl II is similar to mammalian PKC ϵ and to the *Drosophila* 98F kinase throughout the entire molecule, and can be considered a homolog of PKC ϵ . A new member of the vertebrate PKC family (PKC η) expressed in skin and lung also contains most of these homologies (Osada et al., 1990). These enzymes should therefore have similar biochemical characteristics. Consistent with this hypothesis, Apl II does not phosphorylate histone well (W. S. Sossin and J. H. Schwartz, unpublished observations). Even though it is preferred for measuring Ca^{2+} -dependent PKCs, histone is a poor substrate for the vertebrate ϵ -enzyme (Schaap and Parker, 1990). V1 is a highly conserved isoform-specific region of unknown function in PKC ϵ . We suggest that V1 may interact with an as yet unidentified cofactor in a way similar to that by which Ca^{2+} -dependent PKCs are activated by Ca^{2+} ions through C2. It is reasonable to speculate that the Ca^{2+} -independent PKCs also require a factor in addition to a lipid activator that increases their affinity for membrane in order to be enzymatically active.

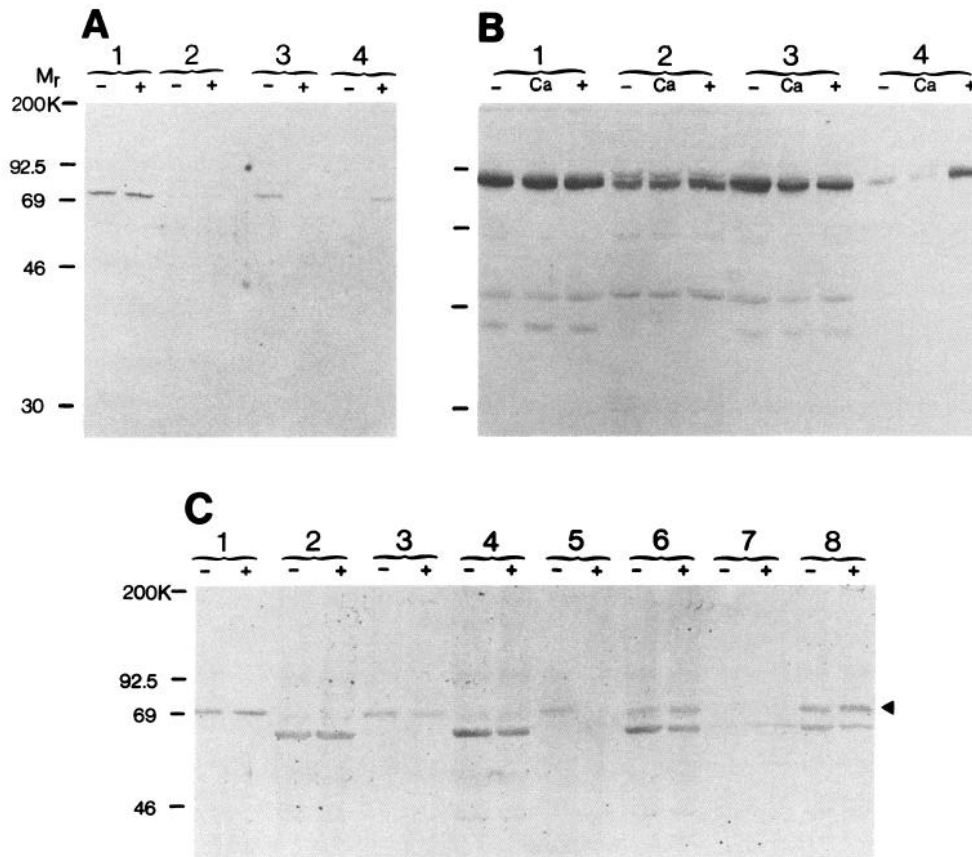


Figure 9. Effects of phorbol ester and Ca^{2+} ion on the subcellular distributions of Apl I and Apl II PKCs. **A**, Apl I: Western blots of supernatant (1) and membrane (2) fractions of extracts of pleural-pedal ganglia (20 μg of protein per lane) incubated in the absence (–) or presence (+) of 200 nM PDBu. The supernatant fraction was incubated with 150 $\mu\text{g}/\text{ml}$ phosphatidylserine without (–) or with (+) 200 nM PDBu and centrifuged again to give a supernatant (3) and a membrane (4) fraction. The rat anti- βI PKC antiserum reacts with an M_r 76,000 protein in the original supernatant. This protein is not translocated to neuronal membranes in the presence of PDBu, but is translocated to phosphatidylserine micelles. **B**, Apl II: Supernatant (1) and membrane (2) fractions incubated in the absence (–) or presence (+) of 200 nM PDBu, or in the presence of 400 μM Ca^{2+} (Ca ; 20 μg of protein per lane). The supernatant fraction was then incubated with 150 $\mu\text{g}/\text{ml}$ phosphatidylserine without (–) or with (+) 200 nM PDBu, or in the presence of 400 μM Ca^{2+} (Ca), and centrifuged again to obtain supernatant (3) and membrane (4) fractions. **C**, Synergistic effect of Ca^{2+} and PDBu on translocation of Apl I. Western blot of supernatant (1, 3, 5, and 7; 15 μg) and membrane fractions (2, 4, 6, and 8; 20 μg) after incubation in Ca^{2+} buffers without (–) or with (+) 200 nM PDBu. Ca^{2+} -ion concentrations are 0 (0.5 EGTA) (1, 2), 120 μM (3, 4), 450 μM (5, 6), and 1 mM (7, 8). As in **A**, Apl I is not translocated to membranes in the absence of Ca^{2+} . Incubation with 120 μM Ca^{2+} reduces the amount of Apl I in the cytoplasm, and the translocation is enhanced by PDBu. In the absence of PDBu, approximately half of the Apl I PKC is translocated in the presence of 450 μM Ca^{2+} . All of it is translocated in the presence of PDBu. Apl I is completely translocated in the presence of 1 mM Ca^{2+} even without PDBu. The arrowhead points to the Apl I antigen. This experiment was repeated three times with similar results. Size is indicated by the migration of protein markers shown to the left.

Biochemical characterization of the two *Aplysia* isoforms

The proteins recognized by antisera for Apl I and Apl II have many of the biochemical properties expected from the sequence similarities to vertebrate enzymes. Apl I can be translocated to membranes by Ca^{2+} , and the translocation is enhanced by phorbol ester. The Apl II isoform is not translocated by Ca^{2+} , but can be translocated by phorbol ester.

There are two biochemical questions that arise from the translocation experiments. First, why are Apl I and Apl II translocated to such a limited extent to neuronal membranes by phorbol ester (Table 1)? In experiments with membranes from other tissues, PKCs have been found to be almost quantitatively translocated by phorbol ester (Kraft and Anderson, 1983; Akita et al., 1990; Godson et al., 1990; Kiley et al., 1990). This difference could be explained if the *Aplysia* membranes are deficient in binding sites for the kinase. We found almost total translocation

of PKC to phosphatidylserine micelles. Neuronal membranes may therefore lack available phosphatidylserine. Perhaps there are phosphatidylserine-binding proteins in neurons that regulate PKC activity.

The second biochemical question is why phorbol ester causes Apl I to be translocated in homogenates lacking Ca^{2+} , but not in intact ganglia (Table 1). How do intact ganglia differ from homogenates? One difference may be higher resting concentrations of Ca^{2+} in intact cells than in homogenates prepared with 0.5 mM EGTA. Another difference may be that Apl I is positioned in cells to facilitate translocation, but that homogenization disrupts this organization.

The role of Apl I and Apl II in synaptic plasticity

PKC activity is translocated to membranes in intact pleural-pedal ganglia exposed to 5-HT and is translocated to membranes in homogenates by phorbol ester (Sacktor et al., 1988–1989;

Sacktor and Schwartz, 1990). It should be pointed out that these measurements, which used histone III-S as substrate, under-represented any contribution from the Ca^{2+} -independent PKCs. Although the translocation of kinase activity observed in these experiments without significant translocation of Apl I and Apl II (Table 1) can be explained in several ways, a likely explanation is that the translocated activity consists of isoforms of PKC other than Apl I and Apl II. We cannot rule out the possibility that the observed translocation of kinase activity results from the activation of enzyme already associated with the membrane, however, because this would not be detected on Western blots. Nevertheless, this is unlikely because cytosolic PKC decreased in the experiments of Sacktor and Schwartz (1990).

Phorbol esters potentiate synaptic transmission in *Aplysia*. Apl I and Apl II are translocated by phorbol ester in intact ganglia (Table 1) and could participate in the potentiation observed in the electrophysiological experiments. It is important to note that there are differences between the pharmacological effect of phorbol esters, which can activate all isoforms of PKC, and neurotransmitters such as 5-HT, which appear to activate specific isoforms selectively.

We found that Apl I needs elevated concentrations of Ca^{2+} to be translocated. Recently, 5-HT has been shown not to cause increased Ca^{2+} influx in sensory cells, though influx of Ca^{2+} is enhanced by exposure to 5-HT if the cell is depolarized (Blumenfeld et al., 1990). Apl I therefore may require both 5-HT and depolarization to be active, while other isoforms of PKC could be active at resting concentrations of Ca^{2+} . Coupling of Ca^{2+} influx and treatment with 5-HT are also the conditions postulated for activity-dependent presynaptic facilitation (Abrams and Kandel, 1988).

Subtle changes in intracellular Ca^{2+} , availability of lipids, and, possibly, protein phosphorylation or the presence of PKC-binding proteins could have profound effects on the translocation and activation of PKC. These regulatory mechanisms differentially operating on the several kinase isoforms might be needed to produce a great variety of actions in the neuron. Further dissection of the biochemical anatomy of the PKCs activated during specific physiological events is necessary for understanding the molecular mechanisms underlying synaptic modulation.

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