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Selective Activation of Ca^{2+}-activated PKCs in *Aplysia* Neurons by 5-HT

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Ca^{2+}-activated and Ca^{2+}-independent protein kinase Cs (PKCs) are present in the nervous system of the marine mollusk *Aplysia californica* (Kruger et al., 1991). Sensitizing stimuli or application of the facilitatory transmitter 5-HT to intact isolated ganglia produces the presynaptic facilitation of sensory-to-motor neuron synapses that underlies behavioral sensitization, which is a simple form of learning. Activation of PKC can also produce this presynaptic facilitation (Braha et al., 1990). To determine which type of PKC is activated, we developed a sensitive and selective assay to measure both Ca^{2+}-activated and Ca^{2+}-independent PKC activities in crude supernatant and membrane fractions of nervous tissue. This assay is based on the specific binding of the Ca^{2+}-activated PKCs to phosphatidylserine vesicles in the presence of Ca^{2+} and makes use of a novel synthetic peptide with sequences conforming to phylogenetically conserved pseudosubstrate regions of the Ca^{2+}-independent kinases. We provide evidence that the presynaptic facilitation is produced by a Ca^{2+}-activated isoform: application of 5-HT increases the amount of the Ca^{2+}-activated PKC activity associated with the membrane. Under these conditions, no increase in Ca^{2+}-independent kinase activity is seen.

In vertebrates, at least eight isoforms of protein kinase C (PKC) have been identified and cloned (Ono et al., 1988; Nishizuka 1988; Ohno et al., 1988; Osada et al., 1990). These can be grouped into two types, Ca^{2+}-activated (α, β, and γ) and Ca^{2+}-independent (δ, ϵ, ζ, and η). All isoforms require lipid activators, but the two types can be distinguished by their dependence on Ca^{2+} ion. The distinguishing molecular feature of the Ca^{2+}-activated enzymes is C2, a conserved region that enables these kinases to respond to Ca^{2+} (Kaibuchi et al., 1989). They are activated by phospholipid and Ca^{2+} in the absence of diacylglycerol or phorbol ester and are translocated to membrane in the presence of Ca^{2+} (Kikkawa et al., 1983; Niedel et al., 1983; Ho et al., 1988; Kiley and Jaken, 1990). The Ca^{2+}-independent kinases, which lack C2, are not regulated by Ca^{2+} (Ono et al., 1988; Ohno et al., 1988; Akita et al., 1990; Schaap and Parker, 1990). The precise functions of the two types are not understood, nor is how different requirements enable particular isoforms to participate in specific physiological mechanisms selectively.

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Information about the the functional significance of the biochemical properties of the two types of kinases would be especially useful in the nervous system. Many functions have been attributed to PKC activity in neurons, including transduction of neurotransmitter signals (Rane et al., 1989; Malhotra et al., 1990), control of ion-channel activity (Kaczmarek, 1989), and the induction of short- and long-term changes in synaptic strength (Akers et al., 1986; Farley and Auerbach, 1986; Malenka et al., 1987; Malinow et al., 1988; Olds et al., 1989). The simple nervous system of *Aplysia* is experimentally convenient for establishing the *physiological* functions of the different types of PKC. Phorbol esters potentiate synaptic transmission at several different identified *Aplysia* synapses including the sensory-to-motor neuron synapses (Strong et al., 1987; Sawada et al., 1989; Taussig et al., 1989; Braha et al., 1990; Fossier et al., 1990). The modulatory transmitter 5-HT produces this presynaptic facilitation, which is thought to underlie behavioral sensitization of several defensive reflexes (Kandel and Schwartz, 1982; Walters et al., 1983). Although the cAMP-dependent protein kinase (PKA) is stimulated by 5-HT and can produce many aspects of sensitization, PKC also plays a major role in the facilitation, especially at depressed synapses (Hochner et al., 1986; Braha et al., 1990). Furthermore, sensitizing stimuli and application of 5-HT cause PKC activity to be translocated in sensory neurons (Sacktor et al., 1988–1989; Sacktor and Schwartz, 1990).

Kruger et al. (1991) cloned a Ca^{2+}-activated isoform (Apl I) and a Ca^{2+}-independent isoform (Apl II) of PKC, both of which are expressed in *Aplysia* neurons. Apl II is a homolog of PKCϵ with extensive conservation, especially in the V1 and pseudosubstrate regions. These regions are also conserved in the *Drosophila* 98F PKC (Schaeffer et al., 1989; Kruger et al., 1991) and vertebrate PKCη (Osada et al., 1990). The similarity in the pseudosubstrate domain promises to be particularly interesting for function since PKCϵ phosphorylates histone much less well than do Ca^{2+}-activated PKCs, indicating that these isoforms have different substrate specificities (Schaap and Parker, 1990).

Recently, PCKϵ has been characterized in heterologous systems expressing the cloned kinase (Ono et al., 1987; Akita et al., 1990; Schaap and Parker, 1990), in cell lines that normally express this isoform (Kiley et al., 1990), in lymphocytes (Strulovichi et al., 1991), and in primary cultured neurons (Heidenreich et al., 1990). These studies demonstrate that PKCϵ is a receptor for phorbol ester and is activated by phospholipids and phorbol esters in the absence of Ca^{2+} ions. In some cell lines, transmitters that activate phospholipase C can cause PKCϵ to be translocated to membranes (Kiley et al., 1990; Strulovichi et al., 1991).

In order to determine whether a specific isoform of PKC

Table 1. Amino acid sequence of pseudosubstrate regions and synthetic peptides

Ca^{2+}-independent PKCs																															
Cloned sequences																															
Apl II	F	K	E	K	E	G	M	L	N	R	R	R	G	A	M	R	R	R	V	H	Q	V	N	G	H	K	F	M	A	M	F	F
RatPKCε	F	R	E	R	-	M	R	P	R	K	R	Q	G	A	V	R	R	R	V	H	Q	V	N	G	H	K	F	M	A	T	Y	L
dPKC98	F	K	E	R	A	G	F	-	N	R	R	R	G	A	M	R	R	R	V	H	Q	V	N	G	H	K	F	M	A	T	F	L
Synthetic peptides																															
ε-pep			E	R	-	M	R	P	R	K	R	Q	G	S	V	R	R	R	V													
Aε-pep								L	N	R	R	R	G	S	M	R	R	R	V	H	Q	V	N	G	H	-	NH_2				
Ca^{2+}-activated PKCs																															
Cloned sequences																															
Apl I				M	E	K	R	V	A	R	R	G	A	L	R	Q	K	N	V	H	E	V	K	N	H	K	F			L		
Rat β				S	T	V	R	F	A	R	K	G	A	L	R	Q	K	N	V	H	E	V	K	N	H	K	F			T		
Synthetic peptides																															
APKC-pep									R	V	A	R	R	G	S	L	R	Q	K	N	V	H	E	V	K	N	-	NH_2			

This table shows alignment of pseudosubstrate regions from cloned PKC isoforms and synthetic peptide substrates (–, gap). Amino acids, shown in the single letter code, that are identical within a family (Ca^{2+} independent or Ca^{2+} activated) are in boldface. The amino acid at the phosphorylation site is underlined [alanine (A) in the pseudosubstrate regions and serine (S) in the synthetic substrate peptides]. Aε-pep and APKC-pep are the new peptides used as substrates in this study; ε-pep is a commonly used substrate for measuring Ca^{2+}-independent PKCs (Schaap and Parker, 1990; Heidenrich et al., 1990; Kiley et al., 1990).

participates in presynaptic facilitation, we developed an assay for measuring the enzymatic activities of both Ca^{2+}-activated and Ca^{2+}-independent types of the kinase in homogenates of *Aplysia* ganglia. This asssay enables us to examine the selective activation of the two types in crude extracts obtained from small amounts of tissue. We show that only an isoform activated by Ca^{2+} is stimulated under conditions producing presynaptic facilitation. We also examine the biochemical properties of the Ca^{2+}-independent isoforms.

Materials and Methods

Aplysia californica (75–150 gm; *Aplysia* Research Facility at the University of Miami, Miami, FL) were bathed in isotonic $MgCl_2$:artificial sea water (1:1, v/v) and then anesthetized with isotonic $MgCl_2$; pleural-pedal ganglia were isolated from the animal and the sheaths removed to obtain neuronal components (cell bodies and neuropil) (Schwartz and Swanson, 1987). Unless stated otherwise, all chemicals are from Sigma (St. Louis, MO).

Preparation of extracts. One pleural-pedal ganglion was homogenized in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 5 mM benzamidine, and 0.1 mM leupeptin (400 μl) using glass–glass homogenizers (Micro-metric, Tampa, FL). Sacktor and Schwartz (1990) found that this high concentration of $MgCl_2$, which is close to physiological for marine mollusks (Hodgkin, 1964), increases the proportion of PKC in supernatant fractions of unstimulated cells, possibly by inhibiting Ca^{2+}-induced translocation of the enzyme during homogenization. The homogenate was first centrifuged to remove debris (1000 × *g*) and then at 100,000 × *g* for 30 min in a TL-100 (Beckman, Palo Alto, CA). The resulting particulate fraction was suspended in 150 μl of the homogenization buffer with 10 passes through a 25-gauge needle and diluted 10- to 20-fold; the supernatant fraction is diluted 20-fold before the assay.

PKC assays. Kinase activity was assayed as described by Sacktor and Schwartz (1990) with slight modifications. Unless otherwise stated, the reaction mixture (30 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg of cAMP-dependent protein kinase inhibitor (Walsh), 10 mM EGTA, 500 nM of the synthetic peptide substrate Aε-pep (see Table 1) in the presence or absence of 150 μg/ml of a mixture of dioleol phosphatidylserine (Avanti, Alabaster, AL) and dioleol phosphatidylcholine (Princeton Lipids, Princeton, NJ; 9:1, w/w; hereafter called PS), and 200 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). The phospholipids were stored in small batches. Before each experiment they were dried down under N_2 gas, suspended in water, vortexed vigorously for 30 sec, and sonicated twice for 30 sec in a cup sonicator (Laboratory Supplies Co., Hicksville, NY).

In some experiments the EGTA and TPA in the reaction mixture were replaced with 0.4 mM Ca^{2+}; because of the EGTA carried over from the homogenization buffer, this results in an approximate final free Ca^{2+} ion concentration of about 0.2 mM. After addition of diluted nervous system extracts (10 μl; 40–100 ng of protein), an equal volume of γ-^{32}P-ATP (New England Nuclear, Boston MA; 1 μC, 50 μM) was added to start the reaction. After 30 min at 20°C, 40 μl of the 50 μl reaction mixture was spotted onto a Whatman phosphocellulose paper disk, which was dropped rapidly into 100 ml of 5% ATP. The disks were then rinsed four times for 5 min with 0.425% phosphoric acid, and radioactivity was determined by scintillation. PKC activity is defined as the difference in phosphorylation between that obtained in the presence of PKC activators and that obtained in their absence. Baseline values in the absence of activators ranged from 10% to 50% of the stimulated values. Over 90% of the stimulated activity was due to phosphorylation of the synthetic peptides as opposed to phosphorylation of endogenous substrates. In the figures, each data point is the result of two or three independent determinations that did not vary by more than 20% and usually by less than 10%. For experiments to examine activation by PS and Ca^{2+}, PKC activity was measured as the difference in protein phosphorylation between that obtained in the presence of PS and that obtained in its absence. This minimizes any contribution from the Ca^{2+}/calmodulin-dependent protein kinase, which is present in *Aplysia* neurons (DeRiemer et al., 1984; Saitoh and Schwartz, 1985).

Protein was measured in triplicate by a modification of the Bradford reaction (Sacktor and Schwartz, 1990).

Results

Characterization of Aplysia *pseudosubstrate peptides.* In order to develop an assay for differentiating between Ca^{2+}-activated and Ca^{2+}-independent types of PKC, we first synthesized peptide substrates inferred from the cDNA sequences of pseudosubstrate regions in the different isoforms (Ono et al., 1987; Nishizuka, 1988; Schaeffer et al., 1989; Kruger et al., 1991). Since the region C-terminal to the pseudosubstrate is the same in the *Aplysia* and vertebrate kinases, but distinct in the two types of PKC (Table 1), we expected that peptides including this region might have served to distinguish the isoforms on the basis of substrate specificity. We found that the difference in affinities of these substrates is insufficient for use in discriminating among their corresponding isoforms, however. A ver-

Table 2. Binding of pseudosubstrate peptides

Kinase	Synthetic peptide K_m (μM)	
	Aε-pep	APKC-pep
Vertebrate		
PKC βI	0.50 ± 0.17	0.24 ± 0.05
PKA	24 ± 7.5	3.3 ± 1.9
Aplysia		
Ca^{2+}-independent PKC		
Supernatant	0.28 ± 0.21	0.88 ± 0.11
Particulate	0.32 ± 0.06	ND

The PKC assay is described in Materials and Methods. All values are the mean ± SE of either two or three independent experiments done in duplicate or triplicate and are calculated from computer-generated linear fits to Lineweaver–Burke plots. It is difficult to assess the actual affinity of the Ca^{2+}-activated PKCs for the peptides because they display substrate inhibition kinetics for both Aε-pep and APKC-pep (Fig. 2*A*). The apparent Michaelis constants of the vertebrate DEAE-purified βI PKC (gift of S. Rotenberg and I. B. Weinstein, Columbia University; Rotenberg et al., 1990) were calculated from experiments using low concentrations of substrate (20–200 nM) to minimize substrate inhibition. The catalytic subunit of PKA was assayed as described for PKC, except for the use of 50 mM MOPS (pH 6.8) instead of Tris-HCl, the absence of PKA inhibitor, and the presence of 0.1% BSA. ND, not determined.

tebrate Ca^{2+}-activated βI PKC (Rotenberg et al., 1990) has only a twofold higher affinity for the major-form pseudosubstrate peptide, APKC-pep; the *Aplysia* Ca^{2+}-independent PKC, assayed selectively as described below, has a threefold higher affinity for the minor-form pseudosubstrate peptide, Aε-pep (Fig. 1; Tables 1, 2).

The two substrates, Aε-pep and APKC-pep, have high affinities (Michaelis constants of 0.2–0.9 μM) for all *Aplysia* and vertebrate PKCs tested (Table 2), while a pseudosubstrate peptide (ε-pep) extending to the nonconserved N-terminal (Table 1) was reported to have a lower affinity (68 μM; Schaap and Parker, 1990). This suggests that the C-terminal sequences of these peptides are important for binding. Assays with the new peptide substrates also revealed a difference in the properties of the two types of PKC, since we found substrate inhibition with Ca^{2+}-activated PKCs but not with Ca^{2+}-independent PKCs (Fig. 1). Substrate inhibition is also seen with *Aplysia* and vertebrate Ca^{2+}-activated PKCs purified by ion-exchange chromatography as described by Thomas et al. (1987) (data not shown).

The new peptide substrates, APKC-pep and Aε-pep, can also be phosphorylated by the catalytic subunit of bovine PKA, although with lower affinity (Table 2). This lack of specificity would not affect our kinase assays in crude homogenates, however, since they were done in the presence of the specific PKA inhibitor.

Assay for Ca^{2+}-independent PKCs. Ca^{2+}-activated PKCs bind to phosphatidylserine vesicles in the presence of Ca^{2+} (Uchida and Filburn, 1984), while the Ca^{2+}-independent PKCs, because they are not modulated by Ca^{2+}, should not. Since assays with the synthetic peptide substrates did not discriminate between the two types sufficiently, we made use of this difference for a subtractive assay to distinguish the two types of PKC.

Supernatant fraction. Ca^{2+}-activated PKCs were removed by diluting nervous system supernatants (see Materials and Methods) with an equal volume of homogenization buffer containing PS vesicles (125 μg/ml) and Ca^{2+} (125 μM final concentration; 1.125 mM Ca^{2+} with 1 mM EGTA in the buffer). The mixture was incubated for 4 min at 20°C and then centrifuged at 100,000 × *g* for 30 min. Pure phosphatidylserine vesicles are unstable

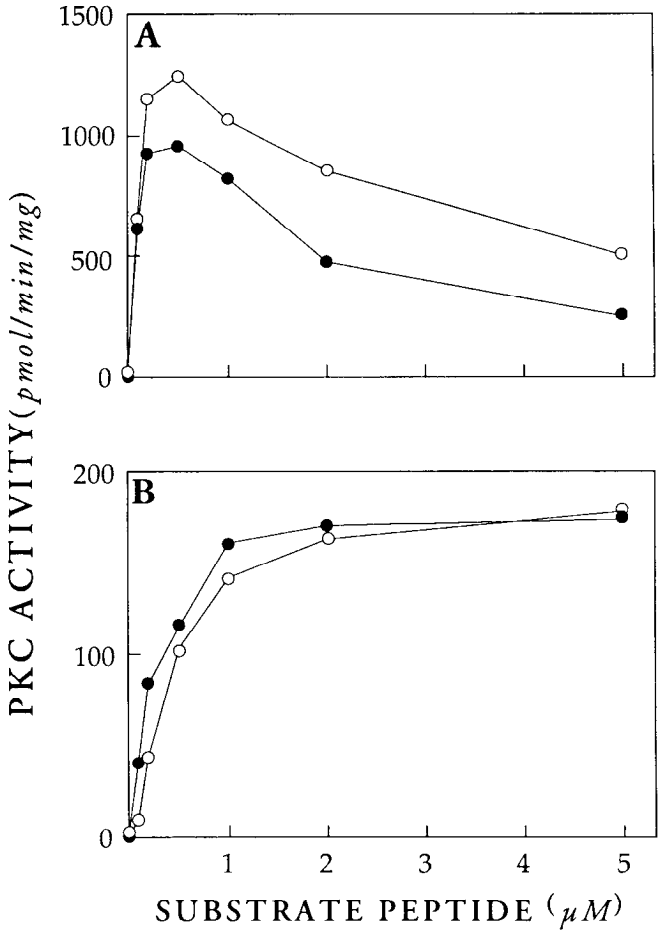

Figure 1. Substrate dependence of Ca^{2+}-activated and Ca^{2+}-independent PKCs in supernatant fractions of *Aplysia* ganglia. *A*, Activation of total PKC by PS and Ca^{2+} (○, APKC-pep; ●, Aε-pep). Phosphorylation of both peptide substrates is markedly inhibited at higher concentrations. *B*, Activation of Ca^{2+}-independent PKCs (supernatant after sedimentation with PS vesicles in the presence of Ca^{2+}) by PS and TPA (○, APKC-pep; ●, Aε-pep). This PKC is not inhibited at high substrate concentrations. Also note the higher affinity of this PKC for the synthetic peptide Aε-pep as compared to APKC-pep.

and fuse in the presence of divalent cations (Duzgunes et al., 1981; Boni and Rando, 1985). To obtain reproducible results in the subtractive assay, we used a 9:1 mixture of phosphatidylserine and phosphatidylcholine; although a higher proportion of phosphatidylcholine would result in more stable vesicles (Boni and Rando, 1985; Pelosin et al., 1987), vesicles with higher proportions of phosphatidylcholine do not sediment completely during the 100,000 × *g* centrifugation step. The mixture containing 10% phosphatidylcholine appears to be adequate, though, since it inhibits Ca^{2+}-stimulated vesicle fusion significantly (Duzgunes et al., 1981). Moreover, the 10 mM $MgCl_2$ present in the sedimentation step also inhibits the destabilizing effects of Ca^{2+} (Duzgunes et al., 1981).

The removal of Ca^{2+}-activated PKCs from the supernatant by sedimentation can be demonstrated either by immunoblotting with an antibody to the Ca^{2+}-activated PKC Apl I (Kruger et al., 1991; Fig. 2*B*) or by assaying the kinase activity remaining that is activated by PS and Ca^{2+} in the absence of phorbol ester (Fig. 2*A*), a means of stimulating Ca^{2+}-activated forms of PKC.

Under these conditions, the kinase reaction in the supernatant fraction occurs at a constant rate and is dependent on the amount

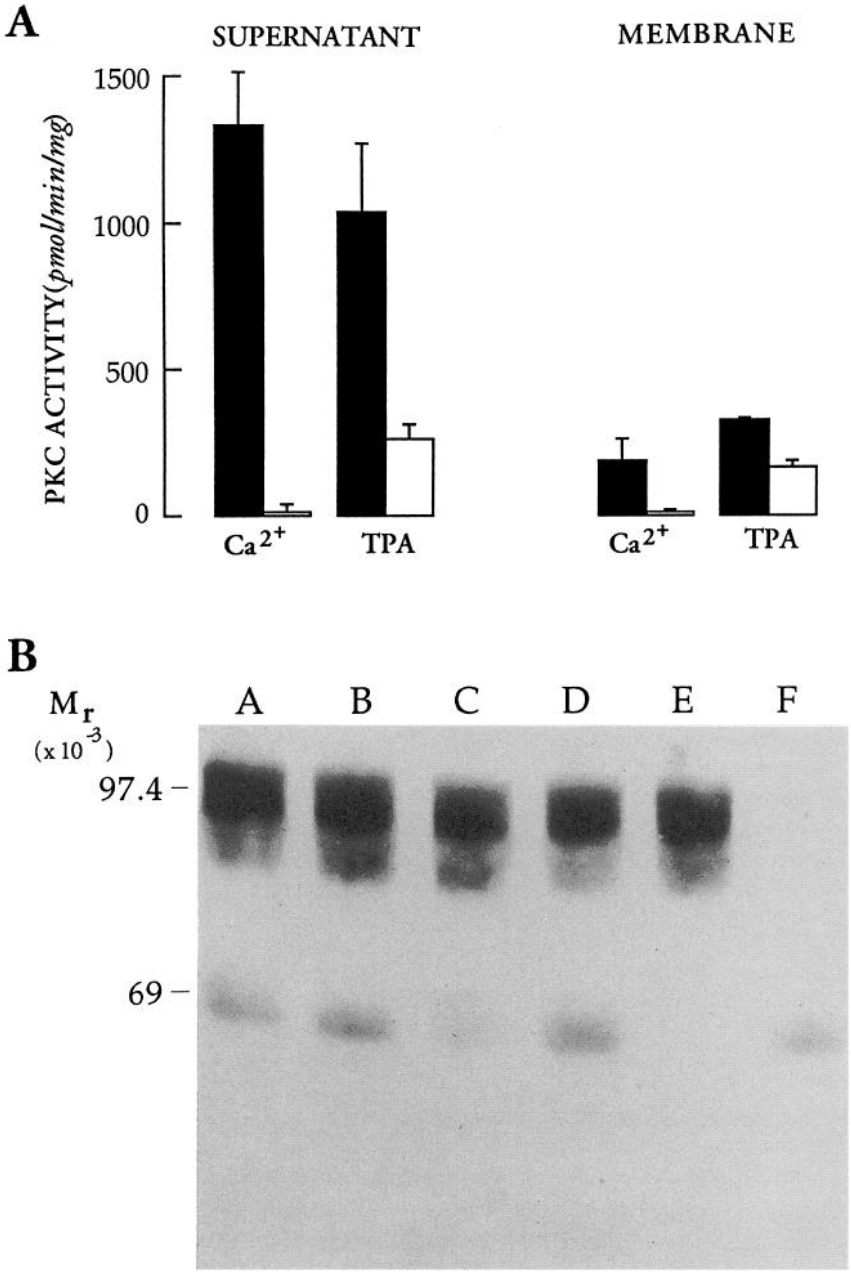

Figure 2. Selective assays for Ca^{2+}-activated and Ca^{2+}-independent PKCs using sedimentation with PS vesicles in the presence of Ca^{2+}. *A,* Evidence for separation by measurement of PKC activity. *Solid bars* represent total PKC activity, and *open bars* represent PKC activity measured after sedimentation with PS vesicles in the presence of Ca^{2+}. The total PKC activity (*solid bars*) was also subjected to centrifugation at 100,000 × *g* but in the absence of PS vesicles and Ca^{2+}. PKC activity was measured in the presence of 150 μg/ml PS and either Ca^{2+} (0.2 mM) or TPA (200 nM). In these experiments, the synthetic peptide Aε-pep (500 nM) was used as substrate, except for the supernatant activation by Ca^{2+}, in which 500 μg/ml histone III-S was used. In later experiments, Aε-pep gave similar results under these conditions (data not shown). Values are means ± SE from five experiments. *B,* Evidence for separation from immunoblotting. Supernatant fractions from *Aplysia* neuronal components (30 μg/100 μl) were incubated for 5 min at 20°C with the following additions: *lane A,* none; *B,* 125 μM Ca^{2+}; *C,* 125 μM Ca^{2+}, 5 μg/ml PS; *D,* 125 μg/ml PS; *E,* 125 μM Ca^{2+}, 125 μg/ml PS; *F,* 125 μg/ml PS, 500 nM TPA. The supernatant fractions were then centrifuged at 100,000 × *g* for 30 min at 4°C, electrophoresed in 9% SDS-polyacrylamide gels (Laemmli, 1970), immunoblotted with antisera to Apl I at a dilution of 1:500 together with antisera to Apl II at 1:1000 (Kruger et al., 1991), and developed with alkaline phosphatase (Promega, Madison, WI). The high molecular weight bands (at approximately *M,* 90,000) are Apl II and the lower bands (at approximately *M,* 70,000) are Apl I (Kruger et al., 1991). Neither Ca^{2+} (*lane B*) nor PS (*lane D*) alone caused any loss of Apl I or Apl II. Even small amounts of PS in the presence of Ca^{2+} (*lane C*) reduced the amount of Apl I in the supernatant. With larger amounts of PS (the normal assay conditions), Apl I was completely removed without any loss of Apl II from the supernatant (*lane E*). The Apl II antigen was removed by TPA (*lane F*). The Apl II antigen was more readily translocated to phosphatidylserine vesicles by the phorbol ester in the presence of EGTA than was the Apl I antigen (Kruger et al., 1991). These experiments were done three times with similar results. Molecular weight rainbow markers are from Amersham (Arlington Heights, IL).

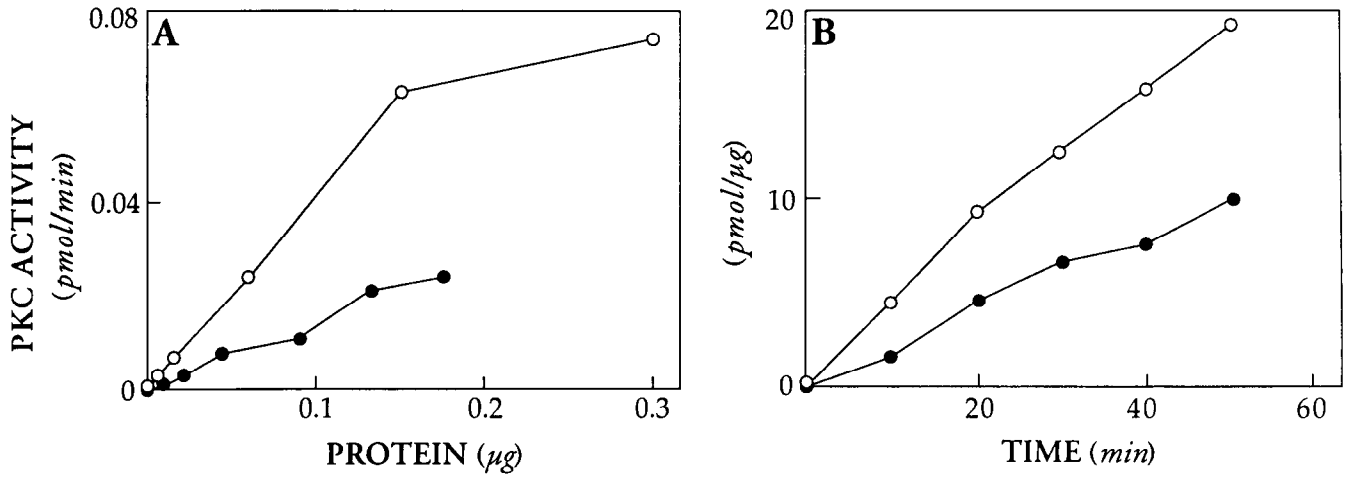

Figure 3. Dependence of Ca^{2+}-independent PKC activity on protein and time. *A*, Dependence on extract protein. Different amounts of supernatant (○) or detergent-solubilized membrane extract (●) were added to PS vesicles in the presence of Ca^{2+} for sedimentation. After sedimentation, the remaining kinase activity was measured by activation with PS (50 μg/ml) and TPA (20 nM). The value shown for protein is the amount of protein added to the kinase assay following dilution of the supernatant after sedimentation. With amounts of protein greater than 200 ng, the assay is no longer linear. *B*, Dependence on time. After sedimentation with PS vesicles in the presence of Ca^{2+}, the remaining kinase activity was measured by activation with PS (50 μg/ml) and TPA (20 nM) for various times; ○, supernatant fraction, 60 ng; ●, solubilized membrane fraction, 44 ng. Both of these experiments were done twice with similar results.

of extract protein added, suggesting that endogenous inhibitors and activators of PKC have been diluted effectively (Fig. 3). The enzyme remaining in the supernatant (presumed to be Ca^{2+}-independent PKCs) phosphorylates histone III-S poorly, with an approximately 10-fold lower maximum rate than with Aε-pep. This is in agreement with previous characterizations of Ca^{2+}-independent PKCs (Kiley et al., 1990; Schaap and Parker, 1990). Thus, after incubation with PS vesicles in the presence of Ca^{2+}, centrifugation appears to remove the Ca^{2+}-activated isoforms of PKC selectively, allowing the Ca^{2+}-independent forms to be assayed.

We do not know if removal of the Ca^{2+}-stimulated enzyme results entirely from binding to PS vesicles or from proteolysis that is stimulated by PS (Huang and Huang, 1990) because only 30% of this kinase activity can be recovered from the sedimented vesicles. The lack of complete recovery of Ca^{2+}-activated PKCs does not affect the subtractive assay, however, since the total Ca^{2+}-activated activity is measured as the difference between total activity and the activity remaining in the supernatant after sedimentation. There is no indication that the unregulated proteolyzed enzyme protein kinase M (Kishomoto et al., 1983; Lee and Bell, 1986) is formed during the treatment with PS vesicles and Ca^{2+}: the activity measured in the absence of lipid activators did not increase after the sedimentation and no immunoreactive protein corresponding to protein kinase M was detected on Western blots. Furthermore, Ca^{2+}-independent PKCs were not cleaved after the treatment with PS vesicles in the presence of Ca^{2+} as indicated by the lack of degradation assessed by immunoblotting (Fig. 2*B*). Still further, Ca^{2+} in the absence of PS did not cause the degradation of the Ca^{2+}-activated PKC Apl I (Fig. 2*B*).

Particulate fraction. In order to assay translocation as well as kinase activity, the Ca^{2+}-independent PKC on the membrane must also be measured. To do this, PKC activity was first removed from the resuspended particulate fraction by incubation with 1% octyl-β-glucoside for 20 min at 4°C [Triton X-100, a detergent generally used for solubilizing membrane-bound PKC (Thomas et al., 1987), interferes with the subsequent translocation to PS vesicles and is a more potent inhibitor of the kinase assay than octyl-β-glucoside]. After the incubation with 1% octyl-β-glucoside, no kinase activity remained in the pellet resulting from centrifugation at 100,000 × *g*. The extracted kinase was then diluted fivefold with homogenization buffer containing PS vesicles (125 μg/ml) and Ca^{2+} (125 μM final concentration; 1.125 mM Ca^{2+} with 1 mM EGTA in the buffer), incubated for 4 min at 20°C, and then centrifuged at 100,000 × *g* for 30 min to remove the Ca^{2+}-activated PKCs selectively. Since the critical micelle concentration of octyl-β-glucoside is 25 mM (0.73%), the fivefold dilution disrupted the detergent vesicles, allowing the extracted enzyme easier access to the PS vesicles.

The kinase remaining in the supernatant after this sedimentation was similar to the Ca^{2+}-independent activity in the original supernatant extract described above. Thus, it is not stimulated by PS in the presence of Ca^{2+} (Fig. 2*A*), its affinity for Aε-pep is similar to that of the Ca^{2+}-independent PKC in the original supernatant extract (Table 2), and it is not inhibited at high concentrations of the synthetic peptide substrates. Furthermore, similar to the supernatant PKC, phosphorylation with the Ca^{2+}-independent PKC extracted from the particular fraction occurred at a constant rate and depended on the amount of extract protein added (Fig. 3).

Characterization of the Ca^{2+}-independent PKC. The activity remaining in the supernatant fraction after sedimentation has the expected properties of a Ca^{2+}-independent PKC: (1) it is stimulated by phorbol esters at low concentrations (K_a, 1.8 ± 0.9 nM, n = 2, Fig. 4*A*); (2) it is stimulated by PS at low concentrations (K_a, 0.19 ± 0.03 μg/ml, n = 2, Fig. 4*B*); and (3) it is not influenced by free Ca^{2+} over a wide range of concentrations (data not shown). The Ca^{2+}-independent PKC is stimulated by PS alone only at very high concentrations, and unlike the total PKC activity in *Aplysia* nervous tissue, Ca^{2+} does not decrease the requirement of the Ca^{2+}-independent activity for PS (Fig. 4*B* and data not shown).

Physiological activation of PKC. 5-HT increases the specific

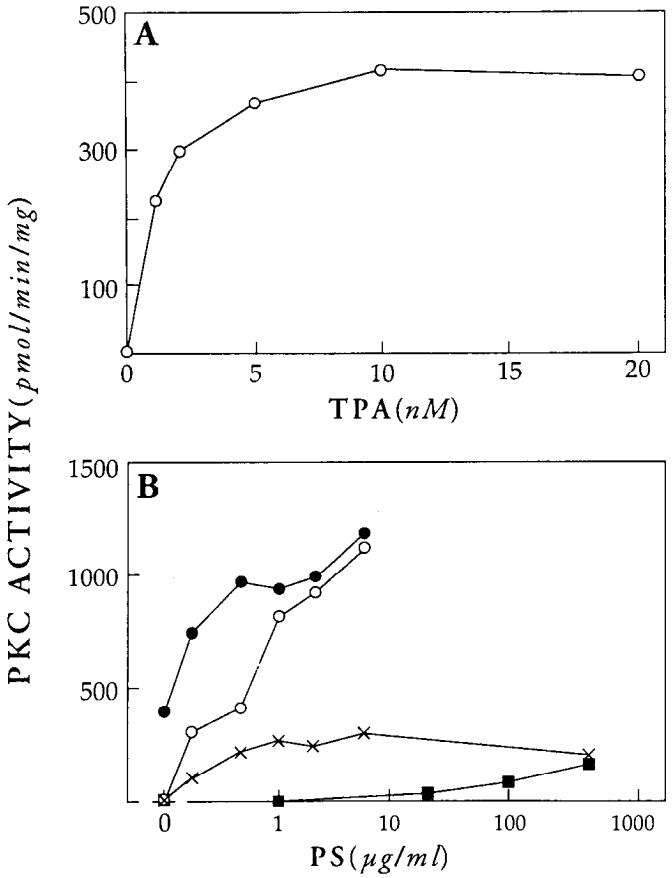

Figure 4. Characterization of the Ca^{2+}-independent PKC in supernatant fractions of *Aplysia* ganglia. *A*, Activation by TPA. Ca^{2+}-activated PKCs were removed by sedimentation with PS vesicles in the presence of Ca^{2+}. The resultant supernatant was assayed in the presence of 50 μg/ml PS. *B*, Activation of total and Ca^{2+}-independent PKCs by PS. Stimulation by PS is shown for total PKC (no sedimentation) in the presence of 20 nM TPA and 10 mM EGTA (○), or 0.2 mM Ca^{2+} (●). Activation of the Ca^{2+}-independent PKC (supernatant after sedimentation with PS vesicles in the presence of Ca^{2+}) was measured in the presence of 20 nM TPA (×), or in its absence (■). All data are from a single experiment that was typical of two other independent experiments, except for the stimulation by PS alone (■) and the last point (500 μg/ml) of the Ca^{2+}-independent PKC (×), both of which are the average of four independent experiments. The variation of these data was less than 20%. PKC activity measured in the presence of Ca^{2+} was subtracted from the phosphorylation stimulated by 0.2 mM Ca^{2+} alone. Even in the absence of added PS, TPA and Ca^{2+} stimulated a substantial amount of PKC activity, suggesting that the requirement for PS is low in the presence of Ca^{2+}.

activity of PKC associated with membranes of neurons in pleural-pedal ganglia (Sacktor et al., 1989–1990). Because histone III-S was used as substrate in those experiments, the contribution of Ca^{2+}-independent PKCs was underrepresented. The subtractive assay described above permits us to measure *both* types of PKC separately. We found that the amount of Ca^{2+}-activated PKC associated with membrane increases without a concomitant increase in Ca^{2+}-independent PKC (Table 3). This is in agreement with our previous indication that Ca^{2+}-independent PKCs are not translocated using immunoblotting with an antibody against Apl II (Kruger et al., 1991). In the experiments reported above, we did not detect a decrease in the amount of Ca^{2+}-activated PKC in the supernatant (Table 3), even though a small decrease was detected by Sacktor et al. (1988–1989).

Table 3. Selective translocation of the Ca^{2+}-activated PKC by 5-HT

Condition	PKC activity (pmol/min/mg)	
	Ca^{2+} independent	Ca^{2+} activated
Membrane fraction		
Control	162 ± 13	272 ± 32
5-HT	179 ± 14	352 ± 41*
Supernatant fraction		
Control	285 ± 39	666 ± 84
5-HT	301 ± 23	708 ± 71

Pairs of intact pleural-pedal ganglia were dissected, pinned to silicone plastic in high-$MgCl_2$ seawater, and then rinsed twice for 5 min in normal seawater (Sacktor et al., 1988–1989). The isolated ganglia were then exposed for 5 min with 20 μM 5-HT (experimental) or normal seawater (control) after the connective tissue sheath of the ganglia were slit to assure access to the neurons (Sacktor et al., 1988–1989; Sacktor and Schwartz, 1990). The ganglia were then washed in ice-cold homogenization buffer, and the neural components were dissected and separated into supernatant and membrane fractions as described in Materials and Methods. These fractions were then subjected to the sedimentation assay described in Results. In these experiments, the concentrations of activators were 50 μg/ml PS, 20 nM TPA, and 10 mM EGTA. Under these conditions, Ca^{2+}-activated and Ca^{2+}-independent PKCs are activated maximally. Ca^{2+}-activated kinase was measured as the difference between the total PKC activity (before treatment with PS vesicles in the presence of Ca^{2+} followed by sedimentation) and the Ca^{2+}-independent activity. Values are the mean ± SE from 15 experiments with membrane and from 9 experiments with supernatant.

* Significantly different, $p < 0.01$, paired t test.

This result is not surprising because the decrease expected is modest (less than 15% of the total supernatant activity) and is probably below the limit of detection for this assay. Alternatively, the increase observed in the membrane-associated activity after 5-HT treatment could be due to some mechanism other than translocation, for example, activation of dormant enzyme already associated with membrane.

Discussion

Substrate specificity of Ca^{2+}-activated and Ca^{2+}-independent PKCs. Even though distinct in the two types of PKC, pseudosubstrate regions do not provide a basis for the synthesis of highly specific substrates for these kinases. In assays for both types of PKC, we used a novel peptide whose sequence matches the pseudosubstrate domain of Apl II and the conserved C-terminal adjoining amino acids. We found that this peptide is not a sufficiently specific substrate for Ca^{2+}-independent PKCs since it is also phosphorylated nearly as well by Ca^{2+}-activated PKCs. Thus, substrate specificity may not account for for the complete conservation of this region throughout phylogeny. Perhaps this region also plays a role in the regulation of PKC activation.

The small differences in specificity displayed by the synthetic pseudosubstrate peptides need not imply that the enzymes phosphorylate proteins indiscriminately. On the contrary, use of these peptides provides two arguments for differences in the substrate binding sites in the two types of PKC. First, there is a two- to threefold preference by each type for the peptide derived from its pseudosubstrate region. Second, the Ca^{2+}-independent PKCs are not inhibited at high concentrations of these substrates, while Ca^{2+}-activated PKCs, of both *Aplysia* and vertebrates, are inhibited (Fig. 1). The inhibition can be explained if the substrate binds to the catalytic site in a nonproductive manner (Dixon and Webb, 1964). This would suggest that the site in the catalytic region important for binding substrates is different in the two types of PKC and is consistent with the conservation of distinct amino acid sequences in the catalytic

region of Ca^{2+}-independent PKCs throughout phylogeny (Kruger et al., 1991).

A substractive assay for PKC. Parallel signal transduction pathways provide many distinct ways of modifying neuronal properties. A goal of these experiments was to assay PKC in crude extracts of nervous system tissue and thus be able to discriminate among the actions of various stimuli and neurotransmitters on the different types of PKC present in *Aplysia.* This should provide insight into the distinctive roles that the different types might play in neuronal plasticity. There are several criteria that must be met for an assay to be useful for this purpose: (1) Ca^{2+}-activated PKCs must be removed by sedimentation with PS vesicles in the presence of Ca^{2+}, (2) the Ca^{2+}-independent PKC should not be sedimented by this treatment, and (3) the residual activity measured after sedimentation should be an authentic PKC.

Immunoblot experiments have shown that the Ca^{2+}-activated PKC Apl I is removed from the supernatant by sedimentation (Fig. 3*A*; Kruger et al., 1991). Furthermore, the activity stimulated by PS and Ca^{2+} is removed by this procedure. This is important since there is evidence for Ca^{2+}-activated isoforms other than Apl I in *Aplysia* neurons (Kruger et al., 1991).

The amount of Ca^{2+}-independent PKC that sediments with the PS vesicles is difficult to assess quantitatively without an independent way to determine the amount of Ca^{2+}-independent PKC present before sedimentation. Some experimental evidence suggests that the loss of Ca^{2+}-independent PKC during the sedimentation is small. After sedimentation with PS vesicles in the absence of Ca^{2+}, there is no loss of total PKC activity (99 ± 11%; $n = 4$) or of Apl II, a Ca^{2+}-independent PKC (Fig. 2*B*). These results indicate that if Ca^{2+}-independent PKCs are lost during the sedimentation, the loss would be produced paradoxically by a Ca^{2+}-dependent mechanism. Furthermore, no loss of Apl II immunoreactivity is seen after sedimentation with PS and Ca^{2+} (Fig. 2*B*). Therefore, most of the activity removed by sedimentation is Ca^{2+} activated, even though this activity is stimulated by PS-TPA-EGTA in the absence of Ca^{2+} ions. This is consistent with our observation that, at *saturating concentrations* of TPA and PS, there is little stimulation of PKC activity by Ca^{2+} ions (Fig. 4*B*). Even though these data suggest that Ca^{2+}-independent activity is stable during the sedimentation with PS and Ca^{2+}, they do not rule out the possibility that some Ca^{2+}-independent PKC is lost during the sedimentation. Further experiments using purified Ca^{2+}-independent PKCs will be necessary to show definitively that none of this kinase is lost.

The phosphorylating activity that remains after sedimentation satisfies the requirements of a Ca^{2+}-independent PKC. It is stimulated by PS and TPA at low concentrations of the activators, but not by low concentrations of either PS or TPA alone. The residual activity is not stimulated by Ca^{2+}, and its affinity for PS is also not affected by Ca^{2+}. Finally, immunoblots show that Apl II, a Ca^{2+}-independent PKC, is present in the supernatant after the sedimentation with PS vesicles in the presence of Ca^{2+} (Fig. 2*B*).

Only Ca^{2+}-activated PKC activity is increased by 5-HT. 5-HT applied to intact isolated ganglia increases the PKC activity associated with membranes in ganglia and in sensory cells (Sacktor et al., 1988–1989; Sacktor and Schwartz, 1990). The use of histone III-S as substrate in these studies underrepresented Ca^{2+}-independent PKCs and left open the question of whether this type of PKC is also translocated by 5-HT. Using an assay optimized for measuring the Ca^{2+}-independent PKCs, we found no increase in the amount of Ca^{2+}-independent activity in the particulate fraction, while in the same experiment a significant increase of Ca^{2+}-activated activity was observed. This is consistent with previous experiments using immunoblotting with an antibody to Apl II that also revealed no increase in the amount of this PKCε homolog translocated after exposure to 5-HT (Kruger et al., 1991). Thus, we found that a transmitter stimulates Ca^{2+}-activated PKCs without activating Ca^{2+}-independent PKCs concomitantly.

The lack of activation of the *Aplysia* Ca^{2+}-independent PKC by 5-HT is surprising given the translocation of PKCε by transmitters in other systems (Kiley et al., 1990; Strulovichi et al., 1991). One possibility is that neurons that contain the Ca^{2+}-independent PKC do not respond to 5-HT. This is unlikely because Apl II is present in most neurons of pleural-pedal ganglia including sensory neurons (Kruger et al., 1991; W. Sossin, A. Elste, and J. H. Schwartz, unpublished observations), and most neurons in the ganglia are innervated by 5-HT (see Goldstein et al., 1984). Another possibility is that 5-HT causes the Ca^{2+}-activated PKC to be translocated solely by increasing the concentration of free Ca^{2+}-ions, and thus would not be expected to translocate Ca^{2+}-independent PKCs. This explanation is unlikely since 5-HT does not raise Ca^{2+} concentrations in sensory cells significantly (Blumenfeld et al., 1990). Furthermore, Apl I, a Ca^{2+}-activated PKC, also is not translocated by 5-HT, even though this isoform is translocated in homogenates to neuronal membranes by Ca^{2+} (Kruger et al., 1991).

An attractive explanation for the lack of translocation of the Ca^{2+}-independent PKC is that, in addition to diacylglycerol, this isoform may need to be modified (e.g., by being phosphorylated) or may require an additional factor that is absent from 5-HT–stimulated cells. In GH4C1 cells, PKCε *is* translocated to membranes by thyrotropin-releasing hormone, an activator of phospholipase C (Kiley et al., 1990). One indication for the presence of a modification or an additional factor in GH4C1 cells is that the Ca^{2+}-independent PKC isolated from these cells is stimulated to phosphorylate substrates by PS alone (Kiley et al., 1990), while in extracts from insect cells, in which this same enzyme is overexpressed, activation is not caused by PS alone (Schaap and Parker, 1990). In *Aplysia,* we see stimulation by PS alone only at high concentrations of phospholipid (Fig. 4*B*). The difference in activation by PS alone between Ca^{2+}-independent PKCs in GH4C1 cells and *Aplysia* ganglia may be related to the difference in the ability of the enzymes to be translocated. For example, in the presence of Ca^{2+}, the Ca^{2+}-activated PKCs can be activated by PS alone and are translocated more readily to membranes. A possible site for specific regulation of the Ca^{2+}-independent PKCs is the conserved N-terminal region (Osada et al., 1990; Kruger et al., 1991).

The reason for the large number of kinase isoforms is an unanswered question in understanding signal transduction. One possibility is that the presence of several isoforms with subtle differences in substrate specificity and activation parameters enables the cell to respond with a greater dynamic range and a more robust response to external stimuli. Alternatively, each isoform may have a dedicated physiological function, and the differences in substrate specificity and activation are indications of the specialized modifications that are needed for fulfilling that role. Our results support the latter hypothesis. We found that 5-HT stimulates only a Ca^{2+}-activated isoform of PKC and, presumably through this activation, causes presynaptic facilitation of depressed sensory-to-motor neuron synapses. Other

transmitters, or combination of stimuli, may activate Ca^{2+}-independent PKCs and modulate some other process in sensory cells. Identifying these stimuli and the function of that signal transduction pathway is an important goal for further research.

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