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A phospholipase A₂-stimulating protein regulated by protein kinase C in *Aplysia* neurons

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We describe some properties of an M_r 30,000 thermolabile and trypsin-sensitive protein that activates phospholipase A₂ (PLA₂) and which was isolated from nervous tissue of the marine mollusk, *Aplysia californica*. A similar protein is present in rat cerebral cortex. This protein was partially purified from crude homogenates of nervous tissue by ion exchange chromatography on DEAE-Sephadex followed by size-exclusion high performance liquid chromatography (HPLC). It is loosely associated with membrane fractions, and is extracted by 0.05% Tween 20. Although similar in size to several previously described PLA₂-stimulating proteins from non-neural mammalian cells and tissues, it differs from them in some aspects of biological activity. The protein promotes the release of eicosanoids from the membranes of intact *Aplysia* neurons prelabeled with [³H]arachidonic acid and appears to be an *in vitro* substrate for protein kinase C (PKC). PLA₂-stimulating activity is greatly enhanced after exposing isolated ganglia to phorbol dibutyrate (PDBu) and is reduced by treatment with immobilized *E. coli* alkaline phosphatase. These observations suggest that phosphorylation of this stimulatory protein by PKC regulates PLA₂ in neurons.

Recent studies indicate that arachidonic acid and its metabolites might act as second messengers in nerve cells. Several neurotransmitters^{11,13,20,26,28} stimulate the release of arachidonic acid in nervous tissue. Application of arachidonic acid, as well as several of its metabolites formed through the 12-lipoxygenase pathway, modulate ion channels in identified *Aplysia* neurons^{2,7,21,23}. Arachidonic acid may also act as a retrograde messenger in long-term potentiation in the vertebrate hippocampus, an example of synaptic plasticity implicated in the formation of memory⁴. Since receptor-mediated activity of phospholipase A₂ (PLA₂) is thought to be the chief mechanism for releasing arachidonic acid from membrane phospholipids, a protein that regulates this enzyme in nervous tissue could alter synaptic efficacy and play a role in both short-term and long-term plasticity.

Although the mechanisms are not understood, several proteins affect the activity of PLA₂. Lipocortins are inhibitory, and it has been suggested that this inhibition is diminished when they are phosphorylated^{1,6,16,17}. Stimulatory proteins have also been isolated from non-neural mammalian cells, an M_r 28,000 PLA₂-stimulating protein, PLAP^{5,9,10} and a group of proteins called lipokinins¹⁵. We describe the partial purification of a similar stimulatory protein from *Aplysia* central ganglia

with somewhat different characteristics.

When crude homogenates from 100–200 *Aplysia* ganglia (25–50 animals) were subjected to ion-exchange chromatography, an activity that stimulates porcine pancreatic PLA₂ was eluted from the column in 0.3 M NaCl. We desalted and concentrated the proteins in this fraction for analysis by size-exclusion HPLC. Activity was eluted as one component with M_r 30,000 (Fig. 1A).

The increase in specific activity was 600-fold with recovery of all of the activity present in the initial homogenate. This high recovery may be misleading because inhibitory lipocortins, which are present in crude homogenates (ref. 8, and data not shown), are removed during the purification. The stimulation of the vertebrate PLA₂ observed was quite modest (Table I), and we would expect greater activity toward substrates other than *E. coli* membranes. We have not yet developed a convenient assay with *Aplysia* PLA₂ as enzyme or molluscan membranes as substrate. No intrinsic PLA₂ activity was present in the purified fraction. A distinct component with intrinsic phospholipase or lysophospholipase activity was eluted with a mobility of M_r 15,000 (data not shown). Fractionation of a homogenate from rat brain by ion-exchange chromatography followed by size-exclusion HPLC yielded similar results (Fig. 1B). The PLA₂-

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Fig 1 Size-exclusion HPLC of PLA₂-stimulating activities from *Aplysia* neural tissue and rat brain. With both *Aplysia* and rat, most of the activity, which was assayed as described in the legend to Table I, appeared in HPLC fraction 19, corresponding to a molecular weight of 30,000. A: *Aplysia* Central ganglia from specimens weighing 100–200 g were dissected out and placed in an artificial seawater supplemented with amino acids and vitamins containing high Mg²⁺ (in mM: NaCl 230, KCl 10, CaCl₂ 1, MgCl 220, HEPES 10, pH 7.6). The high concentration of Mg²⁺ blocks synaptic transmission. Each ganglion was rinsed briefly in homogenization buffer (Tris-HCl, 0.1 M, pH 7.5, phenylmethylsulfonyl fluoride (PMSF), 10 μ M; Tween 20, 0.05%; and soybean trypsin inhibitor, 5 μ g/ml, Sigma, St. Louis, MO) and immediately homogenized in a glass-glass tissue grinder. The homogenate was centrifuged for 4 min at 16,000 g to remove connective tissue and debris and the supernatant used for ion exchange chromatography on a DEAE Sephadex A-50 (Pharmacia, Piscataway, NJ) column (8 $\times$ 0.5 cm, bed vol 4 ml) equilibrated at room temperature with the homogenization buffer. The column was washed with buffer (10 vol) and proteins eluted stepwise with 0.1 M, 0.3 M and 1 M NaCl (each 40 ml) in the buffer. This procedure takes about 3 h. The 40 ml eluate from each step was dialyzed at 0–4 $^{\circ}$ C against either ammonium carbonate (0.1% w/v, pH 7.4) or Tris-HCl (pH 7.5, 25 μ M), and then freeze-dried. The dried protein was taken up in 0.5 ml of water for injection into a BioSil TSK-250 column (300 $\times$ 7.5 mm, Biorad, Richmond, CA). Protein was eluted at a flow rate of 1 ml/min with Tris-HCl, 0.1 M (pH 6.8) and PMSF, 10 μ M. Size was determined by reference to the elution of protein standards (bovine serum albumin, ovalbumin, carbonic anhydrase and α -lactalbumin, Sigma). B: Rat The activity in 0.4 g cortical slices from forebrain or hippocampus of male Wistar rats (200–300 g) with a razor blade and homogenized as above, was partially purified by the same procedure.

stimulating activity is not confined to the nervous system: *Aplysia* buccal muscle contains the protein with approximately the same specific activity (data not shown).

The protein has no effect on phospholipase C. In 6 experiments, the activity of the PLC from *Bacillus cereus* (Sigma), assayed⁹ with [³H]phosphatidyl inositol (Amersham) as substrate and incubated with 0.2 μ l of the purified protein (equivalent to the protein purified from about 30 ganglia) was 927 $\pm$ 325 cpm per 5 min; without addition of the protein, 905 $\pm$ 237 (S.E.M.).

To show that the stimulatory activity eluting with an M_r of 30,000 actually is a protein, samples that had been purified by HPLC were boiled for 5 min or incubated with trypsin. Both treatments abolished the protein's ability to stimulate PLA₂ (Table I). The PLA₂-stimulating activity from rat brain was also destroyed by boiling and by treatment with trypsin (not shown).

SDS-PAGE also showed that the partially purified *Aplysia* protein is present in the major component migrating with an M_r of 30,000 (Fig. 2). To verify that this band corresponds to the stimulatory protein, we electroeluted it from the polyacrylamide gel. The portion of the gel electrophoretogram containing material aligned with the M_r 30,000 protein standard was cut out, minced and electroeluted overnight (ISCO, Lincoln, NE) at low amperage in PAGE-running buffer (without SDS). The electroeluted material was assayed for its ability to stimulate PLA₂. Addition of the eluted protein increased PLA₂ activity to 234 $\pm$ 6% of control (n = 4). Eluates from adjacent portions of the gel were inactive (5 $\pm$ 0.7% of control). The material has not been subjected to two-dimensional gel electrophoresis, but by assuming that all of the M_r 30,000 component is the PLA₂-

TABLE I

Inactivation of PLA₂-stimulating activity by boiling and trypsin

Porcine pancreatic PLA₂ (Sigma) was assayed at 4 $^{\circ}$ C for 5 min as described by Touqui et al.²⁷ using as substrate a suspension of *E. coli* membranes that had been labeled by incubation with [³H]oleic acid (specific activity 5 Ci/mmol, Amersham, Arlington Heights, IL) but with half the concentrations of PLA₂ and of substrate specified. The PLA₂-stimulating protein was assayed by the increase in activity of the lipase. Values are reported as cpm per 5 min $\pm$ S.E.M. (n = 5) with no addition and after the addition of native HPLC fractions 19–20 (0.2 ml samples), the fractions after boiling, and the fractions after treatment with trypsin (10 mg/ml) for 30 min at 37 $^{\circ}$ C. The M_r 30,000 protein was partially purified as described in the legend to Fig 1.

Condition	PLA ₂ Activity (cpm/5 min)	Change (%)
No addition	2211 $\pm$ 175	-
Fractions 19–20	4540 $\pm$ 476	105
Boiled fractions	1820 $\pm$ 202	-18
Trypsin-treated fractions	2101 $\pm$ 147	-5

stimulatory protein, we estimate that each ganglion contains 0.50 ng (about 0.05% of total protein).

After developing the large-scale purification of the PLA₂-stimulating protein with 100–200 ganglia as starting material, we adapted the protocol for 8 pleural-pedal ganglia which were homogenized in 0.3 ml of the homogenization buffer but without Tween 20. The 16,000 g supernatant was then centrifuged for 90 min at 100,000 g and the pellet resuspended in 125 μ l of homogenization buffer containing 0.05% Tween 20. The extract was then centrifuged for 2 min at 16,000 g, and the supernatant injected into the HPLC column (omitting the column chromatography step on DEAE Sephadex used in the large scale protocol). When the detergent was absent in the homogenization buffer, essentially all of the PLA₂-activating activity sedimented with the 100,000 g pellet, but is readily extracted by brief exposure to 0.05% Tween 20.

We observed that ganglia from *Aplysia* which had been anesthetized by injection of isotonic MgCl₂ usually

contained much less PLA₂-stimulating activity than did ganglia from stressed animals. Since the synaptic discharge that occurs during the dissection to remove the central nervous system can cause the translocation of protein kinase C (PKC) to membrane and its activation, we suspected that this activity might be regulated by protein phosphorylation. Sacktor et al.²⁴ showed that, after treatment with phorbol ester, fractionated neural membranes contain a major phosphorylated component with an M_r of 30,000. We now find that the activity in extracts is increased when intact *Aplysia* ganglia are incubated with PDBu (Fig. 3). An inactive phorbol is ineffective.

As would be expected if protein phosphorylation activates the PLA₂-stimulating protein, we found that the activity from unanesthetized ganglia is greatly decreased after incubation for 30 min at 37 °C in 0.1 M Tris-HCl (pH 10) with *E. coli* alkaline phosphatase conjugated to agarose (Sigma). In two experiments, we found a 72% decrease in activity as compared to a 25% loss after incubations with agarose alone. In vitro phosphorylation of the partially purified protein with bovine PKC also leads to the labeling of an M_r 30,000 component (Fig. 4).

We next determined whether the *Aplysia* protein could cause release of arachidonic acid from membrane phospholipids in *Aplysia* nerve cells. For this purpose, we used isolated intact ganglia that had been labeled by incubation with [³H]arachidonic acid. Addition of the partially purified protein to the normal seawater (in mM: NaCl 460, KCl 10, CaCl₂ 11, MgCl₂ 55, HEPES 10, supplemented with amino acids and vitamins, pH adjusted to 7.6) greatly enhanced release of [³H]12-hydroxyeicosatetraenoic acid (12-HETE) (Fig. 5), a major arachidonate metabolite in *Aplysia* neural tissue

Fig. 2. SDS-PAGE of fractions resulting from the partial purification of the *Aplysia* PLA₂-stimulating protein. Protein on the electrophoretogram (12.5%, ref. 18) was stained with Coomassie blue, and represent the material purified from about 20 *Aplysia* central ganglia (see legend to Fig. 1). Lane 1: homogenate after removal of connective tissue and debris by low-speed centrifugation (the fraction shown is the resulting supernatant); Lane 2: chromatography on DEAE-Sephadex (the fraction shown is the material eluted by 0.3 M NaCl which has been desalted and freeze-dried); and Lane 3: fractions 19 and 20 from size-exclusion HPLC

Fig. 3. Enhancement of PLA₂-stimulating activity after exposing isolated *Aplysia* ganglia to phorbol ester. PLA₂-stimulating activity, isolated from 8 treated pleural-pedal ganglia (open bars) using the small-scale preparation was compared to activity from the 8 untreated contralateral ganglia from the same animals (filled bars). After treatment with PDBu (200 nM for 45 min; $n = 9$) in artificial seawater, the activity was increased ($P < 0.05$, paired t -test). The inactive 4- α -phorbol²⁵ caused no increase (200 nM for 45 min; $n = 8$). In this group of animals, the baseline activity was relatively high.

derived from the 12-lipoxygenase pathway²⁰. In 7 experiments, application of the protein led to the release of 3487 ± 1131 cpm per ganglion of [³H]12-HETE, 80 times greater than controls (45 ± 11 cpm per ganglion). Radioactive material eluting with the solvent front, which in this HPLC system contains polar metabolites of arachidonic acid (mainly prostaglandins and trihydroxy-eicosatrienoic acids^{20,23}) also was increased. Furthermore, in 3 experiments the M_r 30,000 protein, electroeluted from SDS-PAGE and diluted 1:1 in the seawater, also enhanced the release of [³H]12-HETE from ganglia prelabeled with [³H]arachidonic acid (treated ganglia, 1373 ± 517 cpm, controls, 49 ± 18 cpm). Adjacent portions of the electrophoretogram were also electroeluted and used as controls in these experiments.

We do not understand why the PLA₂-stimulating protein appears to be effective when applied extracellularly. One explanation is that the ganglia, which have had their connective tissue sheaths removed for labeling with

[³H]arachidonic acid, contain some damaged cells that permit entry of the protein. It is difficult to argue that the amounts of eicosanoids released are too great to be explained by a small amount of cell damage, since intracellular stimulation of a single identified neuron results in the release of detectable amounts of 12-HETE and prostaglandins^{20,23}. Nevertheless, it is intriguing to entertain the possibility that the protein might actually be effective on the outer leaflet of the neuron's plasma membrane. Other proteins that regulate PLA₂ may act in a similar manner. Lipocortins are secreted from lung and leukocytes in response to steroids^{3,12}. They may operate not only intracellularly, but also when applied outside cells¹². The M_r 28,000 PLAP was purified from fibroblasts by immunoaffinity chromatography using an antibody raised against the bee-venom toxin, mellitin⁹. Mellitin activates PLA₂ from the outside of cells because the peptide forms a pore through the membrane. Bomalaski et al.⁵ also reported that PLAP is effective when applied extracellularly.

We do not know how similar the *Aplysia* PLA₂-stimulating protein is to PLAP and the lipokins. The *Aplysia* protein (which, when further characterized, we would like to call lipotonin) enhances the activity of porcine pancreatic PLA₂ whereas PLAP is ineffective in this respect⁹. Furthermore, it is not yet known whether

Fig. 4 Partially purified *Aplysia* PLA₂-stimulating protein contains a substrate for PKC. The protein, purified by DEAE chromatography and size-exclusion HPLC, corresponding to the amount derived from 50 ganglia was incubated at 25 °C with rat brain PKC (0.12 U/ml, Lipidex, Westfield, NJ) in Tris-HCl, pH 7.5, 50 mM, MgCl₂, 10 mM, with phorbol 12-myristate 13-acetate (TPA), 200 nM, CaCl₂, 0.1 mM, phosphatidylserine (PS), 100 µg/ml or with EGTA, 0.5 mM. The reaction was initiated by adding 50 µM γ-[³²P]ATP and stopped after 15 min with SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, silver stained, and exposed overnight to X-OMAT AR film for autoradiography. [³²P] fractions 19–20 from the size-exclusion HPLC were incubated with PKC and [³²P]ATP in the presence of TPA, Ca²⁺ and PS or with EGTA. Autoradiography indicates that an M_r 30,000 component was phosphorylated by PKC. Silver stain the gel corresponding to the autoradiograph indicates the purity of the preparation and shows that equal amounts of the M_r 30,000 component were present in the samples used for protein phosphorylation.

Fig. 5 Release of 12-HETE from *Aplysia* neurons after treatment with the partially purified PLA₂-stimulating protein. Isolated neural components were incubated at 15 °C for 3 h with [³H]arachidonic acid (0.5 µCi/ml, Amersham, spec. act. 230 Ci/mmol) in normal artificial seawater; this procedure results in the incorporation of the radioactive fatty acid into the major phospholipid classes²⁰. After removal of any unincorporated label by washing, (A) 0.1 ml of the partially purified protein (fractions 19–20 reconstituted in artificial seawater, corresponding to 20 ganglia, or one-fifth of a preparation from 100 ganglia) or (B) an equal volume of an inactive HPLC fraction (control) were applied to the labeled cells. After 5 min, 50 µl samples of the seawater were collected, diluted with an equal vol of cold methanol, and centrifuged for analysis of labeled metabolites by reversed-phase HPLC using methanol/water/acetic acid (73:27:0.01, v/v) at a flow rate of 1 ml/min (ref. 20). Radioactivity in fractions was counted by liquid scintillation. The figure shows typical results from one of 7 independent experiments, each using the protein purified from 100 *Aplysia* ganglia.

the vertebrate proteins are regulated by PKC. Control by protein phosphorylation could explain the observation that specific activation of the α isozyme of PKC leads to the release of arachidonic acid from canine kidney cells¹⁴. Since the release of arachidonic acid may, in turn, stimulate other isozymes of PKC (for example, the γ form¹⁹ and possibly some of the Ca^{2+} -independent minor forms), this activity may be a step in the autoactivation

of the kinase, a possible molecular mechanism underlying the persistent synaptic enhancement mediated by PKC²⁵.

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ABBREVIATIONS

12-HETE	12-hydroxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
PDBu	phorbol dibutyrate
PKC	protein kinase C
PLA ₂	phospholipase A ₂

PLAP	PLA ₂ -activating protein
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
SDS-PAGE	polyacrylamide gel electrophoresis in sodium dodecyl sulfate
TPA	phorbol 12-myristate 13-acetate

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