An Autonomous Kinase Generated During Long-Term Facilitation in *Aplysia* Is Related to the Ca^{2+} -Independent Protein Kinase C Apl II

Wayne S. Sossin

Department of Neurology and Neurosurgery
McGill University
Montreal Neurological Institute
Montreal, Quebec, Canada H3A-2B4

Abstract

Prolonged treatment with serotonin leads to long-term facilitation of sensory-to-motor neuron synapses in *Aplysia*. We have shown previously that there is a protein synthesis-dependent increase in an autonomous kinase activity that phosphorylates a protein kinase C substrate during an intermediate phase of this facilitation. Here, I report that the increase in autonomous activity was independent of RNA synthesis, suggesting it may play a role in the maintenance phase of synaptic facilitation. Immunoprecipitation experiments using an antibody specific to the Ca^{2+} -independent protein kinase C, Apl II, demonstrated that the autonomous kinase activity increased by serotonin emanated from Apl II. Chelerythrine, an inhibitor targeted to the substrate binding site of protein kinase C, also blocked the autonomous kinase activity increased by serotonin. Using immunoblotting experiments and calphostin-C, an inhibitor targeted to the regulatory domain of protein kinase C, the autonomous activity is shown not to be a catalytic fragment of Apl II. Furthermore, a higher concentration of calphostin-C was required to inhibit autonomous kinase activity than regulated kinase activity, suggesting that calphostin-C's binding site in the regulatory domain of Apl II is modified in the autonomous kinase. These data suggest that an autonomous kinase derived from Apl II

may play a role in synaptic facilitation in *Aplysia*.

Introduction

Behavioral memory is believed to be represented at a cellular level by changes in the synaptic strength between neurons. Changes in synaptic strength can be divided biochemically into a number of temporal phases. Neurotransmitters cause short-term changes in synaptic strength by modulating the activity of protein kinases through changes in second messenger levels that are independent of changes in protein synthesis. Prolonged applications of neurotransmitters or simultaneous pre- and postsynaptic activity can lead to long-term changes in synaptic strength that depend on RNA synthesis (Montarolo et al. 1986; Frey et al. 1988; Nguyen et al. 1994). Because signals derived from RNA synthesis must emanate from the cell body but synaptic strength can only be regulated at the synapse, there is a requirement for an intermediate form of memory that is local to the synapse and independent of transcription and that persists long enough for molecules synthesized in the cell body to arrive at the synapse (Sossin 1996). During this intermediate, or maintenance, period between short-term and long-term synaptic changes, modifications in synaptic transmission can be maintained by persistent activation of protein kinases, because during this period changes are still sensitive to kinase inhibitors (Malinow et al. 1988; Colley et al. 1990; Farley and Schuman 1991; Wang and Feng 1992; Huber et al. 1995).

Persistent kinase activity could result from changes in the balance of kinase activators and in-

hibitors or from modifications that allow kinases to become autonomous (i.e., active in the absence of activators). There are several examples of the latter mechanism occurring during long-term changes in synaptic strength. In the marine mollusk *Aplysia*, the inhibitory regulatory subunit of the cyclic AMP (cAMP)-dependent protein kinase (PKA) is degraded in a protein synthesis-dependent manner during long-term facilitation of sensory-to-motor synapses leading to more active catalytic subunits at basal levels of cAMP (Greenberg et al. 1987; Bergold et al. 1990). During long-term potentiation in rat hippocampal slices, there is an increase in an autonomous kinase that emanates from a Ca^{2+} /calmodulin-dependent protein kinase and can phosphorylate substrates in the absence of Ca^{2+} /calmodulin (Hanson and Schulman 1992; Fuku-naga et al. 1993). Several mechanisms for the persistent activation of protein kinase C (PKC) have been suggested. During long-term potentiation in hippocampal slices, it has been proposed that either a protein synthesis-dependent proteolytic cleavage of PKC creates an autonomous kinase by separating the regulatory and catalytic parts of the protein (Sacktor et al. 1993; Powell et al. 1994; Osten et al. 1996), or a phosphorylation of the protein leads to autonomous activity (Klann et al. 1993). One difficulty in elucidating the molecular mechanism of persistent PKC activation in the hippocampus is the presence of nine different isoforms of PKC in the mammalian nervous system.

In the marine mollusk *Aplysia californica*, the nervous system has only two phorbol ester-activated PKC isoforms, the Ca^{2+} -activated Apl I and the Ca^{2+} -independent Apl II (Kruger et al. 1991; Sossin et al. 1993). The simplicity of isoform distribution, coupled to physiological information about PKC's role in regulating synaptic transmission (Braha et al. 1990, 1993; Ghirardi et al. 1992; Sugita et al. 1992, 1994), makes *Aplysia* an attractive system for studying the regulation of PKCs during changes in synaptic strength. Behavioral sensitization of the whole animal and repeated or prolonged applications of serotonin to isolated ganglia lead to long-term facilitation of synaptic connections (Frost et al. 1985; Montarolo et al. 1986; Scholz and Byrne 1987; Emptage and Carew 1993). During an intermediate period of long-term facilitation that is induced either by behavioral sensitization or by application of serotonin to isolated ganglia, the activities of both particulate Apl I kinase and a particulate autonomous kinase (measured by phosphorylation of a PKC substrate pep-

tide in the absence of PKC activators) increase (Sossin et al. 1994). Apl II represents a good candidate for the autonomous kinase increased by serotonin, because a fraction of Apl II activity is autonomous both in control extracts from the *Aplysia* nervous system and in purified preparations of Apl II generated by expressing Apl II in *Spodoptera frugiperda* (Sf9) cells using the baculovirus system. (Sossin et al. 1994, 1996). However, there was no evidence that the autonomous kinase increased by serotonin emanates from Apl II.

In this study the autonomous kinase activities in *Aplysia* nervous system extracts and in *Aplysia* PKC isoforms purified from Sf9 cells have been characterized. I provide evidence that after serotonin treatment, regulated Apl II activity was converted to autonomous Apl II activity and this autonomous Apl II activity comprised the source of the autonomous kinase increased by serotonin. The autonomous and regulated kinase could be distinguished by the concentration of the PKC inhibitor calphostin-C required to inhibit them, suggesting models for the formation of the autonomous kinase. The autonomous kinase derived from Apl II may play an important role in maintaining synaptic strength during long-term facilitation.

Materials and Methods

A. californica (70–100 grams; Marine Specimens, Pacific Palisades, CA) were housed individually for 1 week before experiments. Animals that inked when gently handled were excluded from these studies.

PREPARATION OF NERVOUS SYSTEM EXTRACTS

Pleural-pedal ganglia were isolated as described (Sossin et al. 1994) and treated with 20 μM serotonin for 90 min, and the paired pleural-pedal ganglia from the same animal were treated with saline as an intra-animal control. After washing out the serotonin, ganglia were incubated for 2 hr followed by removal of the nervous tissue by dissection, homogenization, and isolation of the soluble or the particulate fraction as described (Sossin et al. 1994). Particulate fractions were resuspended in homogenization buffer (50 mM Tris-HCl at pH 7.5, 10 mM MgCl_2 , 1 mM EGTA, 5 mM 2-mercaptoethanol, 20 $\mu\text{g}/\text{ml}$ of aprotinin, 5 mM benzamidine, and 0.1 mM leupeptin). For immunoprecipitations, the 2-mercaptoethanol was removed to avoid inactivation of the antibodies. The long incubations in

the absence of 2-mercaptoethanol may be responsible for the approximately twofold lower kinase activities seen in these experiments compared with results using 2-mercaptoethanol (data not shown), and this may account for some of the differences between the data obtained from immunoprecipitations and the data obtained using the subtractive assay.

SUBTRACTIVE PKC ASSAY

The assay to measure Apl I and Apl II activity independently is essentially the same as described (Sossin and Schwartz 1992; Sossin et al. 1994). The assay is based on the finding that Ca^{2+} -activated PKCs, such as Apl I, but not Ca^{2+} -independent PKC, such as Apl II, bind to lipid vesicles in the presence of Ca^{2+} ions, allowing the selective removal of Apl I by sedimentation of the vesicles (Sossin and Schwartz 1992).

The particulate fraction was suspended in 150 μl of homogenization buffer by 10 passes through a 25-gauge needle. To remove PKC from the membrane, 72 μl of the suspended particulate fraction was added to 8 μl of 10% octyl- β -glucoside (Pierce, Rockford, IL; final concentration 1% octyl- β -glucoside) and incubated for 20 min at 4°C. The rest of the particulate fraction was saved for protein determination. To measure Ca^{2+} -independent PKC activity, Ca^{2+} -activated PKCs were removed by sedimentation: Twenty microliters of the suspended particulate fraction was diluted fivefold in homogenization buffer with lipid vesicles (125 $\mu\text{g}/\text{ml}$ of a mixture of dioleoyl phosphatidylserine (Avanti Polar Lipids, Alabaster, AL) and dioleoyl phosphatidylcholine (Princeton Lipids, Princeton, NJ) 9:1 wt/wt) and Ca^{2+} (125 μM final concentration; 1.125 mM Ca^{2+} with 1 mM EGTA in the buffer). After 5 min at 20°C, the samples were centrifuged at 100,000g for 30 min to sediment the vesicles. To measure total PKC activity, 20 μl of the suspended particulate fraction was diluted fivefold with just homogenization buffer. Fifty microliters of the resulting supernatants were then diluted threefold in homogenization buffer before being assayed for kinase activity. Ca^{2+} -activated PKC activity is measured as the difference between total PKC activity and Ca^{2+} -independent activity.

IMMUNOPRECIPITATIONS AND OTHER PKC ASSAYS

The particulate fraction from each paired ganglion was suspended in 150 μl of homogenization

buffer by 10 passes through a 25-gauge needle. To remove PKC from the membrane, 100 μl of the suspended particulate fraction was added to 10 μl of 10% octyl- β -glucoside (final concentration 1% octyl- β -glucoside) and incubated for 20 min at 4°C. The rest of the particulate fraction was saved for protein determination. The suspended particulate fraction was diluted 10-fold in homogenization buffer and centrifuged at 14,000g. The supernatant from this centrifugation (solubilized pellet) was either used immediately for experiments to measure inhibition of autonomous and regulated activities by pharmacological agents or used in immunoprecipitation experiments. In some experiments, the pellet from this centrifugation (nonsolubilized pellet) was suspended in homogenization buffer (same volume as solubilized pellet) by 10 passes through a 25-gauge needle and used to measure kinase activities in the octyl- β -glucoside pellet.

For immunoprecipitation experiments, the solubilized pellet was divided into three 300- μl fractions before incubation with control IgG, anti-Apl I (IgG fraction), or affinity-purified anti-Apl II for 3 hr at 4°C. Then, 10 μl of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden), equilibrated previously in homogenization buffer, were added for 1 hr, followed by sedimentation of the protein A-Sepharose beads by centrifugation (15,000g for 3 min). The resultant supernatant was assayed for kinase activity. All antibodies were used at 20 $\mu\text{g}/\text{ml}$, a saturating concentration of antibody for this amount of tissue (data not shown). The antibodies have been characterized previously, and they are highly specific for the individual isoforms (Kruger et al. 1991; Sossin et al. 1993).

PREPARATION OF PURIFIED PKCS FROM BACULOVIRUS

Purification of PKCs from Sf9 cells after baculovirus infection was as described (Sossin et al. 1996). The specific activity of Apl I and Apl II ranged from 1 to 40 nmoles/min per mg in the different experiments, and enzymes were diluted to remain within the linear range of the assay (Sossin and Schwartz 1992).

KINASE ASSAYS

The reaction mixture (30 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 5 mM EGTA, and

1 μM Ae-pep [LNRRRGSMRRRVHQVNGH, a synthetic peptide based on the pseudosubstrate peptide of Apl II shown to be phosphorylated well by both Apl I and Apl II (Sossin and Schwartz 1992) in the presence or absence of the PKC activators phosphatidylserine (50 $\mu\text{g}/\text{ml}$) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA; 20 nM). Some experiments included 0.1 mg/ml of cAMP-dependent protein kinase inhibitor (PKI; Sigma, St. Louis, MO), from 1 to 50 μM chelerythrine (LC Services, Waltham, MA), or from 0.1 to 5 μM calphostin-C (LC Services) in the reaction mixture. After addition of 10 μl of PKC-containing solution (extract or purified kinase), the reaction was started with 10 μl of [γ - ^{32}P]ATP (NEN, Boston, MA; 1 μCi ; 50 μM final concentration). After 30 min at 20°C, 40 μl of the 50- μl reaction mixture was spotted onto a Whatman phosphocellulose paper disk, which was washed in 100 ml of 1% (wt/vol) ATP. Then, the disks were rinsed four times for 5 min with 0.425% (vol/vol) phosphoric acid, and radioactivity was determined by scintillation. Each value is the average of duplicate determinations subtracted from a blank that contained no PKC.

Regulated PKC activity is defined as the difference in phosphorylation between that obtained in the presence or absence of PKC activators (phosphatidylserine and TPA). Autonomous kinase activity is the amount of phosphorylation obtained without PKC activators.

IMMUNOBLOTS

To obtain a signal from particulate fractions, we combined two or three pleural-pedal ganglia. The separation of supernatant and particulate fractions is described above. After suspension of the particulate fraction in homogenization buffer, a sample was removed for protein determination and sample buffer was added to the remainder and heated at 90°C for 7 min. Membrane and supernatant protein (10 μg) were electrophoresed by SDS-PAGE and then transferred to nitrocellulose paper as described (Sossin et al. 1994). To mark the location of protein kinase M, 500 ng of an extract from Sf9 cells infected with baculovirus encoding Apl II was loaded. We detected Apl II immunoreactivity using chemiluminescence (Amersham, Arlington Heights, IL) with 10 $\mu\text{g}/\text{ml}$ of affinity-purified anti-Apl II (Kruger et al. 1991) and 1:1000 goat anti-rabbit coupled to horseradish peroxidase (Pierce).

Results

PERSISTENT ACTIVATION OF PKC IS INDEPENDENT OF RNA SYNTHESIS

In previous experiments, using a subtractive assay to separate Apl I and Apl II activities, both Apl I and autonomous kinase activities were increased in the particulate fraction 2 hr after a 90-min application of serotonin to isolated pleural-pedal ganglia (Sossin et al. 1994). This increase was dependent on protein synthesis because it was blocked by 20 μM anisomycin (Sossin et al. 1994) (Fig. 1). Recently, an increase in sensory-to-motor neuron synaptic strength has been characterized during an intermediate phase of facilitation that is dependent on protein but not RNA synthesis (Ghirardi et al. 1995). To determine whether the persistent activation of PKC requires RNA synthesis, the effects of a transcriptional inhibitor, actinomycin D, were examined. Blocking RNA synthesis with actinomycin D had no effect on either the persistent increase in particulate Apl I activity or the persistent increase in the levels of the particulate autonomous kinase, suggesting that persistent activation of PKC may contribute to the RNA synthesis-independent phase of synaptic facilitation (Fig. 1). No significant changes in levels of Apl II activity were seen under any condition. Increases in particulate Apl I activity are associated with an increase in Apl I immunoreactivity in the particulate but not the supernatant fraction (Sossin et al. 1994). Similarly, the increase in autonomous activity was seen in the particulate but not the supernatant fraction (Fig. 1).

THE INCREASE IN AUTONOMOUS PKC ACTIVITY CAN BE REMOVED BY IMMUNOPRECIPITATION WITH AN ANTIBODY TO APL II

To determine whether the increase in autonomous activity seen after serotonin treatment derived from Apl II, particulate extracts were made from paired pleural-pedal ganglia treated with either serotonin or saline for 90 min and washed for 2 hr. Particulate extracts from each pleural-pedal ganglia were solubilized in 1% octyl- β -glucoside and divided into three equal aliquots, and kinase activity was removed by immunoprecipitating with saturating amounts of control IgG, anti-Apl I, or anti-Apl II antibodies. Autonomous kinase activity (Fig. 2A) or regulated kinase activity (activity stimulated in the presence of the PKC activators phos-

Figure 1: Actinomycin D does not prevent the increase in autonomous and Apl I kinase activities during long-term sensitization. Experiments involved treatment of intact isolated pleural-pedal ganglia with 5-HT or saline, allowing the unstimulated pleural-pedal ganglion from the same animal to be used as a control. Experiments with 5-HT alone or 5-HT + anisomycin are from Sossin et al. (1994) and are displayed for comparison. For experiments with actinomycin D, ganglia were preincubated for 1 hr with 50 μ g/ml of actinomycin D (Ghirardi et al. 1995), then treated with either serotonin (20 μ M) or saline in the presence of actinomycin D for 90 min followed by a wash period of 2 hr in the absence of pharmacological agents. Activation of PKC was measured as the percent change in particulate or supernatant kinase activity between stimulated and control ganglia using a subtractive enzyme assay that measures independently the two major PKCs in *Aplysia* nervous tissue, Apl I and Apl II, as well as the autonomous activity (Auton) (Sossin and Schwartz 1992; Sossin et al. 1994). Shown are values for serotonin alone (open bars), serotonin in the presence of anisomycin (heavily stippled bars), serotonin in the presence of actinomycin D (hatched bars), actinomycin D alone (solid bars), and for the autonomous kinase, serotonin alone in the supernatant fraction (lightly stippled bars). The value for the autonomous kinase with serotonin alone is a composite of earlier experiments (Sossin et al. 1994) and from experiments done in this paper ($n = 43$). The rest of the values are the average $\pm$ S.E.M. for 8–16 determinations. Asterisks indicate that a two-tailed paired t -test between control and serotonin-treated ganglia had a $P < 0.05$ (*) or $P < 0.01$ (**).

phatidylserine and TPA; Fig. 2B) was measured in the resulting immunodepleted supernatants. In these experiments, when control IgG was used for the immunodepletions, we observed an increase in autonomous activity from the serotonin-treated ganglia that was similar to that seen in earlier experiments (Fig. 2A, Total). Treatment with serotonin also increased the amount of autonomous kinase that was removed by immunodepletion with

an antibody to Apl II (Fig. 2A, Apl II; Fig. 2C). The amount of autonomous activity removed by immunodepletion with anti-Apl II completely accounted for the activity increased by serotonin, because after subtraction of this activity from the total activity there was no longer any significant increase in autonomous activity following serotonin treatment (Fig. 2A, Remainder). The antibody to Apl I did not remove any of the autonomous activity before or after treatment with serotonin (Fig. 2A, Apl I). This was not attributable to problems with the antibody to Apl I, because the same antibody did immunoprecipitate regulated kinase activity from the extracts (Fig. 2B, Apl I).

In these experiments, there was no significant increase in the amount of regulated kinase in serotonin-treated ganglia either before or after immunodepletion with antibodies against PKC (Fig. 2B). Interestingly, the amount of regulated Apl II activity immunodepleted after serotonin treatment decreased, suggesting that the autonomous activity may be formed from the regulated Apl II kinase. In support of this hypothesis, the decrease in the amount of regulated activity immunoprecipitated by anti-Apl II (15.3 ± 11 pmoles/min per mg) closely matched the increase in the amount of autonomous activity immunoprecipitated by anti-Apl II (15.8 ± 6 pmoles/min per mg). Furthermore, the percentage of kinase activity immunoprecipitated by anti-Apl II that was autonomous increased twofold after treatment with serotonin (Fig. 2D).

From our earlier experiments (Sossin et al. 1994; Fig. 1), one might have expected to see an increase in the amount of Apl I immunoprecipitated from the particulate fraction of serotonin-treated ganglia. If one combines the regulated activity immunoprecipitated by Apl I (Fig. 2B, Apl I) with the regulated activity that was not immunoprecipitated by either antibody (Fig. 2B, Remainder), there was a $13 \pm 8\%$ increase in the amount of non-Apl II-regulated kinase activity in serotonin-treated ganglia ($P = 0.07$, one-tailed paired t -test). It should also be noted that a number of procedural differences exist between the subtractive assay (Fig. 1; Sossin et al. 1994) and the immunoprecipitation assay (see Discussion).

DETERMINATION OF THE COMPONENTS OF THE AUTONOMOUS KINASE FROM EXTRACTS

A large amount of autonomous kinase activity could not be removed by antibodies to Apl I and

Figure 2: The autonomous activity that was increased by serotonin treatment can be immunoprecipitated by anti-Apl II. Immunoprecipitation of autonomous kinase activity (A) or regulated kinase activity (B) from pleural-pedal ganglia particulate extracts from control (lightly stippled bars) or serotonin-treated (heavily stippled bars) ganglia. The following activities are displayed: **Total**, activity remaining after immunodepletion with control IgG; **Apl II**, the amount of activity removed by an antibody to Apl II (calculated by subtraction of the activity in the presence of antibodies to Apl II from the activity in the presence of IgG); **Apl I**, same as Apl II but using an antibody to Apl I; and **Remainder**, the amount of activity remaining after subtraction of the activity removed by anti-Apl I and anti-Apl II. Asterisks (*) indicate that a two-tailed paired *t*-test between control and serotonin-treated ganglia had a $P < 0.05$. Error bars are S.E.M., $n = 11$. Neither the autonomous PKC nor the regulated PKC that remained after immunodepletion appeared to be owing to a lack of sufficient antibody as (1) larger quantities of antibodies resulted in no significant increase

in the amount of kinase activity precipitated (data not shown) and (2) there was no correlation between the percentage of activity removed and the total starting activity, as would be expected if the antibodies removed only a limited amount of kinase from each experiment (data not shown). (C) The kinase activity removed by anti-Apl II from the 11 individual trials with lines connecting values from the pleural-pedal ganglia from the same animals. Ganglia were treated with either saline (Control) or 20 μM 5-HT (5-HT) for 90 min followed by a 120-min wash. (D) The amount of autonomous activity immunoprecipitated by the antibody to Apl II was divided by the total activity (autonomous + regulated) immunoprecipitated by the antibody to derive the percentage of Apl II activity that was autonomous. Error bars are S.E.M., $n = 11$. The asterisks (**) indicate that a two-tailed paired *t*-test between the two values had a $P < 0.01$.

Apl II. In all the experiments so far, PKI was included to inhibit any autonomous kinase derived from the PKA. Surprisingly, removing PKI did not increase the autonomous kinase activity (as would be expected if part of the activity emanated from autonomously active PKA) but instead reduced autonomous kinase activity by $52 \pm 18\%$, S.D. ($n = 20$). When assayed in the absence of PKI, $67 \pm 3\%$ S.D. ($n = 3$) of the autonomous kinase in serotonin-treated extracts was immunoprecipitated by an antibody to Apl II. Thus, autonomous kinase that emanates from Apl II represents a large percentage of the autonomous kinase activity derived from extracts.

To determine whether the autonomous activity that is not immunoprecipitated by antibodies to

Apl II is attributable to another isoform of PKC, the autonomous activity was tested for sensitivity to the specific PKC inhibitor chelerythrine. Chelerythrine inhibited both the autonomous and the regulated PKC activities from extracts (Fig. 3A). Although the concentration of chelerythrine required to inhibit PKC was higher than reported previously [chelerythrine's K_i to inhibit histone phosphorylation is 1 μM (Herbert et al. 1990)], the same concentration of chelerythrine was required to inhibit PKC expressed in Sf9 cells using the baculovirus system as in extracts (Fig. 3A). The high concentration required may be owing to the use of a high-affinity peptide substrate, because chelerythrine inhibits PKC competitively with substrate (Herbert et al. 1990). Although 50 μM chel-

Figure 3: Chelerythrine inhibits the autonomous activity increased by serotonin. (A) Chelerythrine inhibits all PKC activities with a similar affinity: autonomous activity in particulate extracts (□), regulated activity in particulate extracts (○), autonomous activity from purified Apl II expressed in Sf9 cells (◇), regulated activity from purified Apl II expressed in Sf9 cells (Δ), and regulated activity from purified Apl I expressed in Sf9 cells (⊞). Data are averages of three to six independent experiments, and S.E.M.s are within 20% of the values shown. (B) Autonomous activity from pleural-pedal particulate extracts (soluble in 1% octyl-β-glucoside) or OG pellets (insoluble in 1% octyl-β-glucoside) from control (lightly stippled bars) or serotonin-treated ganglia (heavily stippled bars) either in the absence (Control) or presence of 50 μM chelerythrine (+ Chel). Asterisks (*) indicate that a two-tailed paired *t*-test between control and serotonin-treated ganglia had a *P* < 0.05. Error bars are S.E.M., *n* = 11 for extracted autonomous activity and *n* = 4 for the OG pellet autonomous activity. (C) The kinase activity removed by chelerythrine from the 11 individual trials with lines connecting values from pleural-pedal ganglia from the same animals. Ganglia were treated with either treated with saline (Control) or 20 μM 5-HT (5-HT) for 90 min followed by a 120-min wash. The amount of activity removed by chelerythrine increased after treatment with serotonin (*P* < 0.05; two-tailed paired *t*-test).

erythrine completely inhibited the regulated PKC from extracts and both the regulated and autonomous activities expressed in baculovirus, 50 μM chelerythrine inhibited only $73 \pm 17\%$ S.D. (*n* = 14) of the autonomous activity from extracts. This is not significantly different from the percentage of autonomous activity removed by the antibody to Apl II. Furthermore, 50 μM chelerythrine did not significantly inhibit the autonomous activity that remained after immunoprecipitation with the antibody to Apl II [$4 \pm 13\%$ S.D. (*n* = 3)]. Thus, the remaining autonomous activity in extracts may derive from non-PKC sources that can phosphorylate the pseudosubstrate-derived substrate.

If the chelerythrine-sensitive autonomous activity was equivalent to the autonomous activity immunoprecipitated by Apl II, chelerythrine should also be able to inhibit the autonomous kinase increased by serotonin. The increase in au-

tonomous activity induced by serotonin treatment was removed by incubation with chelerythrine (Fig. 3B,C). Note that these experiments were done in the absence of PKI, and, thus, a much larger percentage of the autonomous activity was removed. In some of these experiments, the autonomous kinase activity that was not removed from the particulate fraction with 1% octyl-β-glucoside (OG pellet) was also assayed. Although all the regulated kinase activity is removed by 1% octyl-β-glucoside (Sossin and Schwartz 1992; data not shown), there is a large fraction of autonomous activity that remains in the pellet (Fig. 3B). This activity, though, is largely insensitive to chelerythrine and is not increased by serotonin treatment (Fig. 3B). These results provide further confirmation that the autonomous activity increased by serotonin is attributable to an increase in the autonomous activity derived from Apl II.

IS THE AUTONOMOUS KINASE A PROTEIN KINASE M?

One attractive mechanism for the formation of an autonomous kinase is a serotonin-stimulated proteolysis between the regulatory domain and the catalytic domain of Apl II, which would be similar to autonomous PKCs formed during long-term potentiation in the hippocampus (Sacktor et al. 1993). The autonomous catalytic domain remaining after proteolysis is commonly called protein kinase M (PKM). Because our antibody is an anti-peptide antibody directed to the carboxyl terminus of Apl II, it should immunoprecipitate PKM as well. However, PKM is usually cytosolic, and the increase in autonomous kinase is restricted to the membrane (Fig. 1). Because almost 50% of the activity immunoprecipitated by Apl II is autonomous in serotonin-treated ganglia (Fig. 2D), if the autonomous activity is owing to formation of PKM, it should be detected easily by immunoblotting. Yet, no PKM was ever detected in immunoblots of the particulate fractions of control or serotonin-treated ganglia, even though PKM was detected in extracts from Sf9 cells that overexpressed Apl II (Fig. 4). In one out of four experiments, a significant amount

Figure 4: PKM is not detectable in particulate fractions of *Aplysia* extracts. Isolated pleural-pedal ganglia were exposed to 20 μ M 5-HT (+) or normal sea water (–) for 90 min with a 2-hr wash and then extracted. The supernatant (S) and particulate (P) fractions were separated by centrifugation and assayed by immunoblotting (see Materials and Methods) using an antibody to Apl II. There was no change in Apl II immunoreactivity in either fraction (data not shown, see Sossin et al. 1994). No PKM was detected in particulate extracts, although immunoreactive PKM was observed from Sf9 cell extracts after infection with a baculovirus encoding Apl II (Sf9) and migrates at the expected size of ~52 kD.

of PKM was detected in the supernatant fractions from ganglia, but the amount of PKM was not different in control or serotonin-treated ganglia (data not shown).

Calphostin-C, a specific inhibitor of PKC, acts by binding to the PKC regulatory domain and then irreversibly inactivating the kinase by generating high-energy free radicals in a light-dependent reaction (Gopalakrishna et al. 1992). Therefore, PKM that lacks the regulatory domain is insensitive to inhibition by calphostin-C (Kobayashi et al. 1989). Five micromolar calphostin-C inhibited the autonomous kinase activity in *Aplysia* extracts, whereas it did not inhibit Apl II-derived PKM purified from Sf9 cells expressing Apl II (Fig. 5A). Furthermore, the amount of autonomous activity inhibited by 5 μ M calphostin-C is similar to the amount of activity inhibited by chelerythrine or removed by antibodies to Apl II, suggesting that all of the Apl II-derived autonomous activity is calphostin-C sensitive. Thus, evidence from both immunoblotting and calphostin-C inhibition argue strongly against the possibility that the autonomous activity in *Aplysia* extracts derives from formation of a PKM.

INHIBITION BY CALPHOSTIN-C DIFFERENTIATES AUTONOMOUS AND REGULATED PKC ACTIVITIES

Autonomous and regulated PKC activities could be distinguished using calphostin-C, because low concentrations of calphostin-C inhibited regulated but not autonomous activity in nervous system extracts (Fig. 5B). The autonomous and regulated activities from purified preparations of kinases expressed in Sf9 cells using baculovirus could also be differentiated by calphostin-C. In purified Apl II preparations from Sf9 cells, autonomous activity was inhibited by 5 μ M calphostin-C (Fig. 5A,C) but not by concentrations <1 μ M, whereas regulated activity from these preparations was strongly inhibited by concentrations of calphostin-C <1 μ M (Fig. 5C). The similarity of inhibition by calphostin-C between the autonomous kinase in *Aplysia* extracts and the autonomous kinase generated by overexpression of Apl II in Sf9 cells is additional evidence that the autonomous kinase in extracts derives from Apl II. The autonomous kinase activity in Sf9 cells that is not inhibited by 5 μ M calphostin-C may be owing to some contaminating PKM in this preparation (data not shown). In purified Apl I preparations from Sf9 cells there is much less autonomous kinase than in

Figure 5: Calphostin-C inhibition of autonomous and regulated PKC activities. (A) Inhibition of autonomous kinases in extracts and in preparations purified from Sf9 cells. The amount of autonomous kinase remaining after inhibition by 5 μ M calphostin-C for extracts, for purified preparations of Apl II expressed in Sf9 cells using the baculovirus system (Sossin et al. 1996), or for PKM isolated from high-salt fractions of a Mono-Q column used for purification of Apl II from Sf9 cells (Sossin et al. 1996). The identity of PKM as a catalytic fragment of Apl II was confirmed by immunoblotting (data not shown). The small amount of inhibition of PKM by calphostin-C was mimicked by the carrier solution for calphostin-C, 0.5% DMSO (data not shown). Note that the activity from extracts includes ~30% of autonomous kinase that is not emanating from PKC, whereas in all of the purified preparations, 100% of the autonomous kinase emanates from PKC, as judged by complete inhibition by chelerythrine and the absence of autonomous activity in uninfected Sf9 cells. Error bars are S.E.M. for $n = 3$ –6 independent determinations. (B) The inhibition of kinase activities by calphostin-C is shown for autonomous (□) and regulated (◇) activities in particulate extracts from *Aplysia*. Error bars are S.E.M. for $n = 3$ –6 independent determinations. (C) The autonomous kinase is

not inhibited by low concentrations of calphostin-C in the presence of PKC activators. The effect of calphostin-C on kinase activity of a preparation of Apl II purified from Sf9 cells is, as follows: autonomous activity in the absence of PKC activators (□), total activity in the presence of PKC activators (◇), and regulated kinase activity (total activity–autonomous activity) (○). Note that the total activity in the presence of PKC activators does not decline below the original level of autonomous activity. Similar results were seen in three additional experiments.

Apl II preparations (Sossin et al. 1996), and this kinase was less sensitive to calphostin-C (data not shown), consistent with the isoform specificity of autonomous kinase activation.

The difference in inhibition of autonomous and regulated activities by calphostin-C suggests that the two activities emanate from separate molecular entities. An alternative explanation for the failure of low concentrations of calphostin-C to inhibit the autonomous activity is that calphostin-C requires PKC activators to be effective (Gopalakrishna et al. 1992; Rotenberg et al. 1995). More calphostin-C was required to inhibit the regulated kinase activity of purified preparations of PKC at

low concentrations of phosphatidylserine (data not shown). However, this hypothesis cannot explain the differential inhibition of autonomous and regulated activities, because low concentrations of calphostin-C did not inhibit the autonomous kinase even in the presence of PKC activators; in fact, the opposite is true, as the autonomous kinase was prevented from inhibition by calphostin-C in the presence of PKC activators. This could be seen most dramatically in preparations of purified Apl II with a large percentage of calphostin-C-sensitive autonomous activity (Fig. 5C). The amount of kinase activity in the presence of PKC activators did not decrease below the amount of autonomous ac-

tivity when inhibited by calphostin-C, contrary to the decrease expected if autonomous activity was being inhibited only in the presence of PKC activators. Furthermore, at high concentrations of calphostin-C, when the autonomous kinase was inhibited, the presence of PKC activators increased kinase activity by protecting the autonomous kinase from inhibition by calphostin-C. A similar situation is seen in nervous system extracts where PKC activators increase kinase activity even at concentrations of calphostin-C where all of the regulated kinase should be inhibited (Fig. 5B). Thus, not only are the autonomous and regulated kinases inhibited by different concentrations of calphostin-C, but they also differ in the interaction between PKC activators and calphostin-C. The regulated kinase is inhibited more in the presence of PKC activators, whereas the autonomous kinase is inhibited less.

Discussion

PHYSIOLOGICAL IMPORTANCE OF THE AUTONOMOUS KINASE

In this study, up to 50% of the particulate kinase activity generated by the Ca^{2+} -independent PKC in nervous system extracts is autonomous of PKC activators. The high percentage of autonomous PKC activity suggests that this activity is likely to be important in mediating PKC effects on synaptic function. Furthermore, the proportion of the kinase that is autonomous can be modulated by physiological stimuli, because it increased twofold after treatment with serotonin (Fig. 2D). The autonomous kinase generated from Apl II could contribute to the maintenance of synaptic changes during an intermediate phase of synaptic facilitation. The increase in autonomous kinase is dependent on protein but not RNA synthesis (Fig. 1), similar to the facilitation observed at this time (Ghirardi et al. 1995). There should also be higher levels of autonomous kinase activity from PKA at this time, owing to the protein synthesis-dependent down-regulation of the PKA regulatory subunit (Greenberg et al. 1987). It will be interesting to determine whether chelerythrine or calphostin-C can inhibit synaptic facilitation at this intermediate time point. Inhibition by chelerythrine but not by low concentrations of calphostin-C may be a signature for physiological events mediated by the autonomous kinase. Interestingly, in the rat hippocampus, kinase activity is required in an interme-

diate period after induction of LTP but is sensitive only to inhibitors that bind the catalytic region of PKC and not to inhibitors of the regulatory domain (Malinow et al. 1988; Colley et al. 1990; Wang and Feng 1992; Lopez et al. 1993).

One discrepancy in the results presented here is that Apl I activity increased after serotonin treatment (Fig. 1), but the amount of Apl I removed from the particulate fraction by immunoprecipitation after serotonin treatment did not change. Furthermore, 30% of the regulated PKC activity from the particulate fraction could not be removed by immunoprecipitation. In contrast, all of the regulated PKC activity in supernatant fractions was removed by immunoprecipitation (Sossin et al. 1993). This discrepancy could be interpreted in a number of ways. The increased Apl I activity found on the membrane after serotonin treatment may be more difficult to immunoprecipitate, perhaps because of association with other proteins or lipids. This is consistent with the increase in Apl I immunoreactivity that is observed in the particulate fraction after serotonin treatment using immunoblots, a technique that should not be sensitive to the effects of associated molecules (Sossin et al. 1994). It is also possible that the prolonged incubations required for immunoprecipitations and the decrease in total activity seen under these conditions (see Materials and Methods) rendered the increase in regulated Apl I activity too small to be detected. Alternatively, there may be an additional kinase activity expressed in the *Aplysia* nervous system, which (1) is completely membrane associated, (2) depends on either phosphatidylserine and/or TPA for activity, (3) is sensitive to chelerythrine and calphostin-C, and (4) phosphorylates a PKC substrate. However, an additional kinase is unlikely, because only two peaks, which correspond to Apl I and Apl II immunoreactivity, are seen when particulate extracts from *Aplysia* are fractionated on hydroxyapatite columns and measured for PKC activity (Sossin et al. 1993).

MECHANISM OF FORMATION OF THE AUTONOMOUS KINASE FROM APL II

Calphostin-C competes with phorbol esters and diacylglycerol for binding to the PKC regulatory domain, and, once bound, it irreversibly inactivates PKC by generating high-energy free radicals in a light-dependent reaction (Gopalakrishna et al. 1992). Inhibition by high concentrations of cal-

phostin-C restricts possible mechanisms for the formation of the autonomous kinase from Apl II. Most importantly, it excludes the possibility that a constitutively active proteolytic fragment of Apl II constitutes the autonomous kinase, because this fragment was not inhibited by calphostin-C, even at high concentrations (Fig. 5A).

Low concentrations of calphostin-C inhibit regulated but not autonomous activity. This may be because of a modification in the regulatory domain of the autonomous Apl II that affects the binding site for calphostin-C. A modification in the regulatory domain could also underlie the autonomous activation of Apl II, in some way preventing the regulatory domain from inhibiting the catalytic domain. Thus, the effect of serotonin would be to convert the regulated form of Apl II to the autonomous form of Apl II. One could imagine several mechanisms for this conversion including oxidation (Gopalakrishna and Anderson 1991; Palumbo et al. 1992), phosphorylation (Klann et al. 1993), tight binding of protein or lipid (Nelsestuen and Bazzi 1991), or an induced conformational switch that is difficult to reverse. Conclusive proof that the autonomous kinase is a modified form of Apl II will require separation of the two kinases and the ability to convert one form of the kinase to the other under controlled conditions.

ISOFORM SPECIFICITY OF THE AUTONOMOUS KINASE

The modification that underlies autonomous activation of PKC in extracts is highly isoform specific. After serotonin treatment, 50% of the particulate activity that can be immunoprecipitated by an antibody to Apl II is autonomous, whereas no autonomous activity is immunoprecipitated by antibodies to Apl I. This argues against a nonspecific mechanism for the generation of autonomous activity, as does the observation that the activity is induced by treatment with serotonin. Autonomous activity is also seen in purified preparations of PKC from Sf9 cells, and more autonomous activity was present in purified Apl II preparations than in Apl I preparations. Moreover, a higher percentage of the Apl II preparation's autonomous kinase was calphostin-C sensitive, suggesting that expressed Apl II retains a higher propensity to form this activity than does Apl I.

In summary, the results presented here suggest that an autonomous kinase derived from Apl II

was generated after serotonin treatment of pleural-pedal ganglia. The increase in autonomous activity required protein but not RNA synthesis, suggesting it may underlie the changes in synaptic strength seen during a maintenance phase of synaptic plasticity. Finally, the formation of the kinase appears to be associated with a modification in the regulatory domain of Apl II. Elucidation of the mechanism underlying generation of the autonomous kinase will allow for a better understanding of how kinase activity is regulated during the maintenance of synaptic changes required during an intermediate period of synaptic plasticity.

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W S Sossin

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