RESEARCH

Persistent Activation of Protein Kinase C during the Development of Long-Term Facilitation in *Aplysia*

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Abstract

We investigated activation of the two major neuronal protein kinase C (PKC) isoforms in *Aplysia*, Ca^{2+} -activated Apl I and Ca^{2+} -independent Apl II, during the induction and maintenance of behavioral sensitization of *Aplysia* defensive reflexes. Activation of PKC occurred during the training stimulus and persisted for at least 2 hr thereafter but was not maintained for 24 hr. The persistent activation required protein synthesis and was blocked by cyproheptidine, an agent that also blocked the initial activation of PKC. Persistent activation involved both an increase in membrane-associated Apl I and an increase in an autonomous kinase activity that may be related to a post-translational modification of Apl II. These results are consistent with the hypothesis that in addition to its role in producing the presynaptic facilitation of mechanosensory-motor neuron synapses that underlie short-term facilitation, PKC is needed for maintaining synaptic changes in an intermediate period that precedes the modifications accompanying consolidation of memory.

Introduction

Protein kinases are important modulators of synaptic transmission (Byrne et al. 1991; Catterall et al. 1991; Swope et al. 1992; Greengard et al. 1993). By phosphorylating neuronal proteins, kinases produce both short- and long-term changes in the strength of the connections between neurons and their targets. Persistent activation of protein kinases at the synapse may constitute a molecular record of previous synaptic events that serves as an intermediate form of memory between short-term actions that are dependent on transient changes in concentration of second messengers and long-term structural changes at the synapse (Lisman 1985; Schwartz and Greenberg 1987; Schwartz 1993). We are therefore interested in understanding how protein kinases are regulated in neurons, and how persistent protein kinase activity is initiated and maintained.

Protein kinase C (PKC) is a family of multifunctional protein kinases (Nishizuka 1992; Dekker and Parker 1994) with an important role in regulating synaptic function. In neurons, PKCs are physiologically activated during several learning paradigms in vertebrates (Akers et al. 1986; Bank et al. 1989; Burchuladze et al. 1990; Klann et al. 1991; Sacktor et al. 1993; Sunayashiki et al. 1993) and invertebrates (Sacktor and Schwartz 1990; McPhie et al. 1993). Furthermore, activation of PKCs by pharmacological agents, such as phorbol esters, leads to an increase in synaptic strength seen in many different nervous systems, from invertebrates to mammals (Farley and Auerbach 1986; Malenka et al. 1986, 1987; Braha et al. 1990; Finch and Jackson 1990; Sugita et al. 1992).

There is also evidence that persistent activation of PKC is important for maintaining synaptic modifications. Increases in PKC activity can last

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long after the initial training stimulus has ended (Akers et al. 1986; Bank et al. 1989; Klann et al. 1991; Olds and Alkon 1991; Sacktor et al. 1993). In addition, inhibitors of PKC not only can block the immediate synaptic changes (Lovinger et al. 1987; Malenka et al. 1989; Braha et al. 1990; Farley and Schuman 1991; Sugita et al. 1992; Ghirardi et al. 1992) but also the maintenance of these changes (Malinow et al. 1988; Farley and Schuman 1991; Wang and Feng 1992). Molecular mechanisms causing persistent PKC activity may be changes in regulators of the kinase or post-translational modifications of the enzyme itself that make it less dependent on regulators. Recent evidence suggests that the enzyme molecule is modified during the maintenance phase of long-term potentiation of hippocampal CA1 neurons by phosphorylation of Ca^{2+} -activated PKCs (Klann et al. 1993) and formation of a constitutively active or autonomous kinase by specific proteolytic removal of the regulatory region of the Ca^{2+} -independent ζ isoform (Sacktor et al. 1993).

One impediment to examining the role of PKC in synaptic plasticity is that the multiplicity of isoforms present in the mammalian brain makes it difficult to assign specific functions to any one isoform (e.g., see Dekker and Parker 1994). In the marine mollusk *Aplysia*, we have shown that all of the PKC activity in neurons can be accounted for by two isoforms, the Ca^{2+} -activated Apl I and the Ca^{2+} -independent Apl II (Kruger et al. 1991; Sossin et al. 1993), and we have developed a kinase assay that allows the two isoforms to be measured independently (Sossin and Schwartz 1992). This makes *Aplysia* an attractive organism for studying the physiological roles of specific PKC isoforms.

In *Aplysia*, behavioral sensitization of the animal leads to an increase in the strength of mechanosensory-motor connections that can last from minutes to days (Frost et al. 1985; Scholtz and Byrne 1987). Originally, cAMP-dependent protein kinase (PKA) was shown to be involved in both short- and long-term increases in synaptic strength (Kandel and Schwartz 1982; Schacher et al. 1988; Sweatt and Kandel 1989). Recently, a role for PKC in short-term sensitization of sensory neurons has also been demonstrated. 5-HT, a facilitating neurotransmitter that mediates sensitization, activates PKC (Sacktor and Schwartz 1990; Sossin and Schwartz 1992). Some of the short-term changes mediated by 5-HT are mimicked by phorbol esters and are blocked by inhibitors of PKC (Braha et al. 1990, 1993; Ghirardi et al. 1992; Sugita et al.

1992). Little is known, however, about the activation of PKC in long-term sensitization. To address this issue, we examined PKC activity after treatments that stimulate long-term changes in synaptic efficacy in *Aplysia*.

Materials and Methods

Aplysia californica (70–100 grams; *Aplysia* research facility at the University of Miami, FL) were housed individually for 1–2 weeks before training. Animals that inked when gently handled or that did not eat regularly were excluded from these studies. Unless stated otherwise, all chemicals are from Sigma (St. Louis, MO).

BEHAVIORAL SENSITIZATION

Each training episode consisted of 10 shocks along one side of the animal. The long-term sensitization protocol consisted of four training episodes with an interval of 30 min between each episode (Scholtz and Byrne 1987). These unilateral stimulations result in sensitization of one side of the animal, allowing the unshocked side to be used as a paired control (Scholtz and Byrne 1987). Equal numbers of animals were shocked on the left and right sides to avoid the effects of any possible differences between the two sides. To examine the efficacy of behavioral sensitization, we measured the duration of siphon withdrawal after stimulation of the body wall with a jet of water. Each side was tested twice prior to training, and twice 24 hr after the training.

ISOLATION OF PLEURAL PEDAL GANGLIA

After training experiments, animals were first placed in a bath of isotonic MgCl_2 /artificial seawater (1:1, vol/vol) at the end of the experimental period and then anesthetized with an injection of isotonic MgCl_2 . Pleural-pedal ganglia were isolated rapidly from the animal and pinned to silicone plastic (Sylgard, Dow Corning, Midland, MI) in ice-cold dissecting seawater containing high Mg^{2+} and low Ca^{2+} [230 mM NaCl, 220 mM MgCl_2 , 1 mM CaCl_2 , 10 mM KCl, 10 mM HEPES, 0.2× minimal essential medium (MEM) amino acids (GIBCO BRL, Gaithersburg, MD), 0.2× MEM

nonessential amino acids, $0.5\times$ MEM vitamin solution, and 10 mM glutamine added just before use (final pH 7.8; Eisenstadt et al. 1973)].

Each pleural ganglion contains a cluster of 200 pleural sensory cell bodies with terminals in the pedal ganglion. These ganglia are connected by the short pleural–pedal connective. The pair of ganglia are symmetrical, and in all experiments the pair on one side of the animal is used as a control for the pair on the other. Kinase activity is measured in the entire pleural–pedal ganglia. Although we have shown previously that 5-HT can activate PKC specifically in the sensory cell cluster, with approximately twice the increase seen in the whole ganglia (Sacktor and Schwartz 1990), the number of clusters required for each PKC determination, particularly for the subtractive assay (see below), makes isolation of sensory cells impractical for the large number of determinations required for time-course and pharmacological experiments. In addition, other effects of long-term sensitization can be seen in measurements of the whole pleural ganglia (Kuhl et al. 1992).

For experiments with isolated ganglia, nervous tissue was placed in normal seawater [same as dissecting seawater but with 460 mM NaCl, 11 mM CaCl_2 , 55 mM MgCl_2 (Eisenstadt et al. 1973)] after the ganglia had been dissected from naive animals, and the ganglia were incubated at 15°C for 30 min. The ganglia were exposed to the media by slitting the connective tissue sheath, washed in normal seawater and incubated for at least another 30 min at 15°C before application of pharmacological agents or 5-HT.

PREPARATION OF EXTRACTS

Ganglia were washed twice with ice-cold dissecting seawater and twice with ice-cold 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 20 $\mu\text{g}/\text{ml}$ of aprotinin, 5 mM benzamide, and 0.1 mM leupeptin (homogenization buffer; Sacktor and Schwartz 1990). The neuronal components were then removed (Schwartz and Swanson 1987) and homogenized in 400 μl of the buffer in glass–glass homogenizers (Micro-metric, Tampa, FL). The homogenates were first centrifuged at 1,000g for 1 min to remove unbroken cells and debris, and the resulting supernatant was centrifuged for 30 min at 100,000g to isolate supernatant and particulate fractions.

SUBTRACTIVE ASSAY

The assay to measure Apl I and Apl II activity independently is essentially the same as described (Sossin and Schwartz 1992). The assay is based on the binding of the Ca^{2+} -activated PKCs, Apl I, but not the Ca^{2+} -independent PKC, Apl II, to lipid vesicles in the presence of Ca^{2+} ions, allowing the selective removal of Apl I by sedimentation of the vesicles. Recently, the selectivity of this assay was confirmed by showing the removal of Apl I, but not Apl II, after separation of the kinases by hydroxyapatite chromatography (Sossin et al. 1993).

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), hereafter referred to as PS] 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 PS 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 was 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. For assays using histone phosphorylation, the particulate fraction was suspended fivefold in homogenization buffer with 0.05% Triton X-100 and immediately used for the PKC assay (Sacktor and Schwartz 1990).

PKC ASSAY

Kinase activity was assayed either as described (Sacktor and Schwartz 1990) for histone phospho-

rylation or with the following modifications for the subtractive assay. The reaction mixture (30 μ l) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/ml of cAMP-dependent protein kinase inhibitor (Walsh), 10 mM EGTA, and 600 nM 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; Sossin et al. 1993) in the presence or absence of 50 μ g/ml of PS, and 20 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA). After addition of 10 μ l of extract (Ca^{2+} -independent PKC or total PKC), the reaction was started with 10 μ l of [γ - ^{32}P]ATP (New England Nuclear, Boston, MA) (1 μ Ci, 50 μ M final concentration). After 30 min at 20°C, 40 μ l of the 50- μ l reaction mixture was spotted onto a Whatman phosphocellulose paper disk, which was washed in 100 ml of 5% (wt/vol) ATP. The disks were then 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.

PKC activity is defined as the difference in phosphorylation between that obtained in the presence or absence of PKC activators (PS and TPA). Autonomous kinase activity is the amount of phosphorylation obtained without PKC activators but in the presence of EGTA and the specific inhibitor of the cAMP-dependent kinase. Averages for control particulate activities are Ca^{2+} -activated PKC activity, 172 ± 85 pmoles/min per mg; Ca^{2+} -independent PKC activity, 157 ± 66 pmoles/min per mg; autonomous kinase activity, 107 ± 45 pmoles/min per mg; S.D.; $n = 189$.

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 (15–20 μ g) was electrophoresed by SDS-PAGE and then transferred to nitrocellulose paper as described (Kruger et al. 1991). We detected Apl I and Apl II immunoreactivity using chemoluminescence (Amersham, Arlington

Heights, IL) with 10 μ g/ml of affinity purified anti-Apl II (Kruger et al. 1991), 10 μ g/ml of polyclonal anti-Apl I (Sossin et al. 1993), and 1:1000 goat anti-rabbit coupled to horseradish peroxidase (Pierce) The samples were quantitated using a Hoeffler (San Francisco, CA) GS 300 scanning densitometer.

IMMUNOPRECIPITATIONS

The data were obtained from the immunoprecipitation experiments as described (Sossin et al. 1993).

RESULTS

ACTIVATION OF PKCs

To begin elucidating the role of PKCs in long-term sensitization in *Aplysia*, we measured changes in PKC activity during the induction of long-term facilitation of sensory-to-motor synapses. We measured PKC activation as an increase in kinase activity in the particulate fraction, as activated PKC is presumed to translocate to the membrane (Thomas et al. 1987; Nishizuka 1992). These measurements reveal just one possible way that PKCs are activated in vivo, however, because specific isoforms of PKC may also translocate to cytoskeleton or nucleus, or not translocate at all after activation (Heidenrich et al. 1990; Kiley and Jaken 1990; Leach and Raben 1993).

We used two protocols to activate the kinase: behavioral sensitization (Scholtz and Byrne 1987) and prolonged application of 5-HT to isolated ganglia (Barzilai et al. 1989). PKC isozymes were activated in three distinct phases during the development of long-term facilitation as determined by our subtractive assay (see Materials and Methods). In the first phase, corresponding to short-term facilitation, Apl I was activated but Apl II was not (Fig. 1; Sossin and Schwartz 1992). This activation does not persist, even in the presence of continued stimulation (Fig. 1). In the second phase, which may be described as transitional, both Apl I and Apl II were activated ~ 2 – 2.5 hr after the beginning of the training protocol (Fig. 1). This result suggests that both isoforms of PKC become active as long-term facilitation develops. In the third phase, corresponding to intermediate facilitation, Apl I remained persistently activated for as

PERSISTENT ACTIVATION OF PKC

Figure 1: PKCs are activated in three phases during long-term facilitation. Experiments involved either treatment of intact isolated pleural–pedal ganglia with 5-HT (A,C) or training for behavioral sensitization of animals followed at various time intervals later by dissection of pleural–pedal ganglia (B,D). Both training protocols allowed the unstimulated pleural–pedal ganglion to be used as a control from the same animal. Activation of PKC was measured as the percent change in particulate kinase activity between stimulated and control sides using a subtractive enzyme assay that measures independently the two major PKCs in *Aplysia* nervous tissue, Apl I (A,B) and Apl II (C,D) (Sossin and Schwartz 1992; see Materials and Methods). Horizontal bars (A,C) represent the time during which the isolated ganglia were exposed to 5-HT (20 μ M). Vertical arrows (B,D) show the time of training episodes. Asterisks (*) indicate measurements where a comparison between the experimental and control ganglia (paired *t*-test) reached a significance value of $P < 0.05$. The phases indicated by the brackets at the top are described in the text. In experiments with behavioral sensitization, short-term sensitization from the last training episode is occurring with the development of long-term sensitization in the early part of phase 2. Values are mean $\pm$ S.E.M.; $n = 8$ –16 independent determinations.

long as 3.5 hr after the initial stimulus, whereas Apl II returned to its initial level (Fig. 1). This time course of Apl I activation during training was also seen in an independent series of experiments using histone phosphorylation as an assay for PKC (data not shown). Histone phosphorylation measures mainly Apl I, because Apl II phosphorylates histone poorly (Sossin and Schwartz 1992).

We then asked whether the different phases of Apl I activation could be dissociated pharmacologically. Anisomycin, an inhibitor of protein synthesis, blocked the later activation of Apl I (third phase) but did not affect its short-term activation

(first phase) (Fig. 2). This effect is similar to the actions of anisomycin on the biophysical changes observed at sensory-to-motor synapses, where the drug dissociates physiological responses by blocking long-term facilitation measured after 24 hr without affecting short-term facilitation (Montarolo et al. 1986). Cyproheptidine, a 5-HT receptor antagonist, also dissociates short- and long-term processes but in a reciprocal fashion to anisomycin, blocking short- but not long-term facilitation (Emptage and Carew 1993). We find that cyproheptidine blocked both the short-term and the later phases of Apl I activation (Fig. 2),

Figure 2: Short- and long-term activation of Apl I can be dissociated. Isolated pleural–pedal ganglia were used in all experiments. Activation of PKC was measured as the percent change in particulate kinase activity between stimulated and control ganglia using a subtractive assay that measures Apl I and Apl II independently (Sossin and Schwartz 1992; see Materials and Methods). Ganglia were assayed for PKC activation after a 30-min exposure to 5-HT (A) or 120 min after a 90-min application of 5-HT (B) with no drug (5-HT), or in the presence of 20 μ M anisomycin (5-HT + Aniso) or 200 μ M cyproheptidine (5-HT + Cyp). Drugs were applied at the times shown in the schematic (C). In each of these experiments, the only difference between control and stimulated ganglia was the presence of 5-HT (i.e., anisomycin vs. anisomycin + 5-HT). The drugs alone had no effect on the amount of Apl I activity [(A), 200 μ M cyproheptidine (Cyp); (B), 20 μ M anisomycin (Aniso)]. The values for 5-HT alone (5-HT) are the same as those shown in Fig. 1. Asterisks (*) indicate measurements in which a comparison between the experimental and control ganglia (paired *t*-test) reached a significance value of $P < 0.05$. We also did comparisons between exposure to 5-HT and exposure to 5-HT + drug. The difference between 5-HT and 5-HT + cyproheptidine after 30 min (A) and the difference between 5-HT and 5-HT + cyproheptidine or between 5-HT and 5-HT + anisomycin after 90 + 120 min (B) reached a significance value of $P < 0.05$ (unpaired *t*-test). Activation of Apl II was also monitored in these experiments (see Materials and Methods), but comparison between experimental and control ganglia did not reach significance values of $P < 0.05$ except for the treatment with anisomycin alone, where the increase in particulate Apl II activity in the experimental ganglia is significant ($23 \pm 12\%$ increase, $P < 0.05$ paired *t*-test). All values are mean $\pm$ S.E.M.; $n = 8$ –14.

suggesting that the initial phase of Apl I activation, or another process blocked by cyproheptidine is required for the later phase to occur.

The observation that cyproheptidine blocks Apl I activation but not the facilitation measured 24 hr later suggests that PKC activation is not needed to establish long-term sensitization measured after 24 hr. Consistent with this idea, we observed that PKCs are not activated 24 hr after behavioral sensitization (Table 1). Furthermore, there was no correlation between the activation of Apl I and the behavioral change seen after 24 hr.

These results argue that persistent activation of Apl I is not required at this stage of the long-term behavioral change. There is evidence that other 5-HT-mediated biochemical changes such as persistent activation of PKA (Ghirardi et al. 1994) and the activation of the ApC/EBP transcription factor (Alberini et al. 1994) also are no longer required after consolidation of the synaptic memory. We propose that persistent activation of Apl I is needed for the intermediate stages of facilitation, possibly participating in maintaining facilitation during the transition to more permanent modifi-

Table 1: Lack of correlation between *Apl I* activation and the duration of siphon withdrawal measured 24 h after training

Measurement	Change after 24 hr (%)
Particulate <i>Apl I</i> activity	1.8 ± 18
Particulate <i>Apl II</i> activity	-4.6 ± 9
Duration of siphon withdrawal	99 ± 21

Animals were trained for long-term behavioral sensitization as described in Fig. 1. A day later, the pleural-pedal ganglia were removed for dissection after the duration of siphon withdrawal was measured as described in Materials and Methods. There was no significant correlation between the two measures ($r^2 = 0.3$). The actual values for siphon withdrawal in seconds are trained side (before training) 3.9 ± 0.3 , (after training) 7.4 ± 0.7 ; untrained side (before training) 3.6 ± 0.3 , (after training) 2.5 ± 0.1 . Both the increase on the trained side and the decrease on the untrained side are significant at $P < 0.01$ (paired *t*-test).

cations of synaptic strength (Bailey and Kandel 1993; Alberini et al. 1994).

MECHANISMS OF PRODUCING PERSISTENT PKC ACTIVATION

Prolonged activation of PKC can be explained in two ways. First, changes in gene expression can lead to alterations in the balance between kinase activators and inhibitors, causing a persistent increase in the proportion of PKC associated with the membrane. Second, changes in gene expression can modify the enzyme molecule making it autonomous, no longer requiring exogenous activators after the original stimulus is removed.

We found evidence that both of these processes participate in the activation of PKC during the development of long-term facilitation in *Aplysia*. Consistent with the first explanation in which there is a change in the balance between PKC activators and inhibitors, we observed that increases in PKC activity are associated with increases in the proportion of particulate PKC as measured by immunoblotting (Figs. 3 and 4). We did not observe significant increases in the total amount of PKC, as there were no changes observed in the amount of supernatant PKC. This argues against the possibility that the persistent increase of PKC particulate activity results from an increase in synthesis of

PKC. We also found evidence for the second mechanism. There was an increase in constitutive kinase activity (the amount of phosphorylation of the PKC pseudosubstrate-based peptide measured without PKC activators). This increase required protocols that produce long-term sensitization and was blocked by both anisomycin and cyproheptidine (Fig 5).

For several reasons it is likely that the constitutive or autonomous phosphorylation observed results from activating a PKC rather than another kinase. The substrate peptide is used at concentra-

Figure 3: *Apl I* associated with the membrane increases after training for long-term sensitization. Isolated pleural-pedal ganglia were exposed to $20 \mu\text{M}$ 5-HT (+) or normal seawater (–) for 30 min (1), 90 min with a 30-min wash (2), or 90 min with a 2-hr wash (3), the times corresponding to phases 1, 2, and 3 as described in Fig. 1, and then extracted. The supernatant and particulate fractions were separated by centrifugation and assayed by immunoblotting (see Materials and Methods) using antibodies to *Apl I* (A) or *Apl II* (B). There is an increase in *Apl I* immunoreactivity in the particulate fraction, with no increase in the supernatant fraction, suggesting that the percentage of particulate *Apl I* is persistently increased by stimuli that result in long-term facilitation. In immunoblots using antibodies to *Apl II*, we observe increases in the particulate fraction only during phase 2.

Figure 4: Quantitation of immunoblot experiments. Data are expressed as the percent change in particulate (●) or supernatant (○) immunoreactivity for Apl I (A) or Apl II (B) measured at the times indicated. The horizontal bar represents the time during which the isolated ganglia were exposed to 5-HT (20 μ M). Asterisks (*) indicate measurements in which a comparison between the experimental and control ganglia (paired *t*-test) reached a significance value of $P < 0.05$. Values are mean $\pm$ S.E.M.; $n = 7-13$.

tions that we found to be specific for PKC (Sossin and Schwartz 1992), and the assay is performed in the presence of high concentrations of EGTA and the specific polypeptide inhibitor of cAMP-dependent protein kinase. Furthermore, in control experiments, approximately half of the constitutive activity was immunoprecipitated from either supernatant or pellet fractions by an affinity-purified antipeptide carboxy-terminal antibody to Apl II (Table 2). These results suggest that both isoforms of the kinase can be persistently activated by sensitizing stimuli, possibly through different mecha-

nisms: Apl I through a persistent change in the balance between activators and inhibitors and Apl II through a post-translational modification of the enzyme that creates an autonomous enzyme.

Discussion

BIOCHEMICAL EVIDENCE FOR AN INTERMEDIATE FORM OF MEMORY

We suggest that PKC is involved in the maintenance of synaptic strength in a stage of memory intermediate between short-term facilitation and the modifications that underlie the consolidation of synaptic memory (Greenberg et al. 1987; Schwartz and Greenberg 1987; Bailey and Kandel 1993; Alberini et al. 1994). Furthermore, we propose that this form of memory appears to be biochemically separable from the other forms. Recent evidence in *Aplysia* supports the idea that long-term facilitation is independent of short-term facilitation: Producing changes in gene expression is sufficient to mediate long-term facilitation even if the modifications underlying short-term facilitation are blocked pharmacologically (Emptage and Carew 1993) or do not occur because the stimulus is spatially remote from synaptic terminals (Clark and Kandel 1993). We provide evidence that changes in gene expression are required for biochemical changes (e.g., activation of PKC) during an intermediate phase of memory (Fig. 2) and suggest that this may also be independent of short-term facilitation. There is also physiological evidence for a separable intermediate form of synaptic facilitation in *Aplysia* that is sensitive to anisomycin (Ghirardi et al. 1994). Still further, we suggest that the changes in gene expression required for intermediate memory may be distinct from those required for long-term facilitation, as they are selectively blocked by cyproheptidine (Fig. 2; Emptage and Carew 1993).

There may be a correspondence at the molecular level between the separation of intermediate- and long-term changes in *Aplysia* and similar phases identified in hippocampal neurons. Activation of PKC has been shown to be required for the maintenance of synaptic changes during long-term potentiation (LTP) in the hippocampus (Malinow et al. 1988; Wang and Feng 1992), whereas activation of PKA is required in CA1 neurons for a late phase of synaptic potentiation but not at earlier times (Frey et al. 1993; Huang and Kandel 1994). This suggests that in hippocampal neurons, an in-

Figure 5: Autonomous kinase activity is increased during long-term facilitation. Experiments involved either exposure of isolated pleural-pedal ganglia to 5-HT (A,C) or behavioral sensitization of intact animals followed later by dissection of pleural-pedal ganglia (B). The horizontal bar (A) represents the time during which the ganglia were exposed to 5-HT (20 μ M). Vertical arrows (B) represent the time of each training episode. In C, ganglia were assayed for PKC activation 120 min after a 90-min application of 5-HT with no drug (5-HT) or in the presence of 20 μ M anisomycin added 1 hr before 5-HT (5-HT + Aniso) or 200 μ M cyproheptidine added 30 min before 5-HT (5-HT + Cyp). Constitutive kinase activation is the percent change in particulate kinase activity that was measured in the absence of PKC activators but in the presence of inhibitors of other kinases (EGTA and the polypeptide cAMP-dependent protein kinase inhibitor). All values are from the same ganglia used for measurements of Apl I and Apl II activity in Figs. 1, and 2. Asterisks (*) indicate measurements where a comparison between the experimental and control ganglia (paired t-test) reached a significance value of $P < 0.05$. (@) Indication of where a comparison between treatment with 5-HT and treatment with 5-HT + drug (Student's t-test) reached a significance value of $P < 0.05$.

intermediate memory dependent on activation of PKC can be separated from a later phase that is dependent on PKA. In *Aplysia*, activation of PKA has been shown to be sufficient for the induction of long-term structural changes in neurons (Nazif et al. 1991; Schacher et al. 1993). These observations suggest the possibility that a long-term program for structural changes is triggered by activating gene expression, predominantly through PKA, whereas PKC would play a more important role in the maintenance of synaptic changes during intermediate stages of memory.

Other biochemical changes occur during the development of long-term facilitation that may contribute to an intermediate phase of memory. There are changes in protein synthesis at 1 and 3 hr after application of 5-HT to isolated sensory neurons (Barzilai et al. 1989) that are analogous to the transitional (phase 2) and intermediate (phase 3) phases of PKC activation. Activation of ApC/EBP, a transcription factor that is required for long-term facilitation, is maximal 2 hr after application of 5-HT (Alberini et al. 1994) corresponding to the transitional phase.

The persistent activation of Apl I can be dissociated from its short-term activation. Most suggestions for the formation of a persistently active kinase have assumed that initial activation of the kinase coupled to an associated event (e.g., activation of a protease) or prolongation of the initial activation (e.g., resulting in more autophosphorylation) leads to a modification of the kinase that causes persistent activation (Lisman 1985; Schwartz and Greenberg 1987). Thus, the persistent activation might be expected to follow directly from the initial stimulation. In contrast, the persistent activation of Apl I appears not to depend directly on its short-term activation (Fig. 1). The persistent activation of PKA during long-term facilitation in *Aplysia* is also dependent on protein synthesis (Bergold et al. 1990) and thus may be separated from its short-term activation.

PHYSIOLOGICAL RELEVANCE OF PERSISTENT PKC ACTIVATION

Short-term activation of PKC by 5-HT leads to several physiological changes that are associated

Table 2. Antibodies to Apl II, but not Apl I, remove autonomous PKC from extracts of control ganglia

Antibody	Autonomous kinase activity remaining in supernatant	
	particulate fraction	supernatant fraction
Apl I	82 ± 6	110 ± 5
Apl II	52 ± 6	34 ± 3
Apl I + Apl II	56 ± 4	23 ± 3

Saturating amounts of a polyclonal rabbit antibody to Apl I (Sossin et al. 1993), an equal amount of control polyclonal normal rabbit serum, an affinity-purified rabbit antibody to Apl II (Kruger et al. 1991), an equal amount of control rabbit IgG, a combination of the two antibodies, or a combination of the two controls were used to immunoprecipitate PKC activity from a supernatant fraction of nervous system or from a 1% octyl- β -glucoside extract of a particulate fraction of nervous system. The antibodies were less efficient at immunoprecipitation in the presence of detergent (data not shown), and the percent immunoprecipitated from the particulate fraction should be taken as lower estimates. After precipitation, the resulting supernatants were assayed for the amount of remaining PKC activity. For each antibody, the amount of activity remaining in the appropriate control precipitation was set at 100%.

with increases in synaptic strength. These include spike broadening (Sugita et al. 1992), increases in Ca^{2+} current (Braha et al. 1993), and a presumed increase in vesicle mobilization associated with the reversal of synaptic depression (Braha et al. 1990; Ghirardi et al. 1992). Cyproheptidine blocks several aspects of short-term facilitation, presumably by blocking the activation of PKC (Fig. 2) and partially blocking the activation of PKA (Mercer et al. 1991; Goldsmith and Abrams 1992). Persistent activation of PKC during an intermediate phase of memory presumably would lead to the continued presence of the same physiological changes mediated by PKC during short-term facilitation. This is an idea that can be tested by examining the effects of inhibitors of PKC on the facilitation of the EPSP that can be measured during this time period (Ghirardi et al. 1994). At the present time, the psychological and behavioral correlates of intermediate memory are difficult to examine empirically, but are the subject of interesting theoretical models (see, e.g., Baddeley and Hitch 1993; McClelland et al. 1994).

MECHANISMS FOR PERSISTENT PKC ACTIVATION

There were two measures of PKC activation that persist after long-term stimulation: (1) an increase in particulate Apl I activity and (2) an increase in constitutive or autonomous kinase activity. Changes in PKC activators or inhibitors resulting from changes in gene expression might explain the increase in particulate Apl I activity but not the increase in autonomous PKC activity. Conversely, proteolysis of the regulatory region of Apl I or Apl II could explain the increase in autonomous PKC activity but not the increase in particulate Apl I activity that still requires PKC activators. A mechanism involving proteolysis would be similar both to models for persistent PKC formation during LTP (Sacktor et al. 1993) and protein synthesis-dependent degradation of regulatory subunits of the cAMP-dependent protein kinase in *Aplysia* sensory neurons (Bergold et al. 1990; Hegde et al. 1993). A modification of Apl I (e.g., phosphorylation) that increases both its activity at basal levels of diacylglycerol and its activity without activators could explain both results. Phosphorylation of PKCs has been proposed to explain autonomous activation in the hippocampus (Klann et al. 1993).

ACTIVATION OF APL II

Apl II is transiently activated by long-term but not short-term stimulation protocols (Sossin and Schwartz 1992; Fig. 1). This is puzzling because the Ca^{2+} -independent isoform might be expected to be activated by default under all conditions that stimulate the Ca^{2+} -activated kinase: Ca^{2+} -activated PKCs require both a lipid activator and Ca^{2+} ion, whereas Ca^{2+} -independent PKCs require only a lipid activator. We have suggested that the presence of endogenous PKC inhibitors prevents Apl II from being activated during short-term sensitization (Sossin and Schwartz 1994). A transient decrease in concentration of an inhibitor induced by long-term stimuli could explain the concomitant activation of both Apl I and Apl II shortly after the end of the long-term stimulus. Alternatively, an activator of Apl II, as yet unidentified, may be formed transiently by the prolonged stimulation that produces long-term sensitization.

PERSISTENT PHOSPHORYLATION: A COMMON MECHANISM FOR SYNAPTIC MEMORY

There are several similarities between activation of persistent phosphorylation during long-

term facilitation in *Aplysia* and in LTP in vertebrates. During LTP of CA1 neurons, PKC activity is persistently enhanced (Klann et al. 1991, 1992; Gianotti et al. 1992; Sacktor et al. 1993), and this is associated with the formation of autonomous PKC (Klann et al. 1993). Particulate PKCs do not appear to increase after LTP in the CA1 region of the hippocampus (Sacktor et al. 1993; Klann et al. 1993), but persistent increases in particulate PKCs have been reported in other vertebrate learning paradigms and in other brain regions (Akers et al. 1986; Bank et al. 1989; Olds et al. 1989; Burchuladze et al. 1990; Olds and Alkon 1991).

Persistent protein phosphorylation is an important way to generate long-term changes in synaptic strength (Schwartz and Greenberg 1987). All three of the multifunctional protein kinases (PKA, calcium-calmodulin-dependent protein kinase II, and PKC) can be activated in a persistent manner and by different molecular mechanisms (Greenberg et al. 1987; Kennedy 1989; Klann et al. 1992, 1993; Hanson and Schulman 1992; Fukunaga et al. 1993; Sacktor et al. 1993). Interestingly, it appears that different isoforms of PKC can be activated by different mechanisms (Klann et al. 1993; Sacktor et al. 1993; Dekker and Parker 1994). In the future it will be important to understand how these diverse ways of producing persistent kinases can be integrated into a sequential molecular mechanism for memory.

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