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Mechanisms for Generating the Autonomous cAMP-Dependent Protein Kinase Required for Long-Term Facilitation in *Aplysia*

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Summary

The formation of a persistently active cAMP-dependent protein kinase (PKA) is critical for establishing long-term synaptic facilitation (LTF) in *Aplysia*. The injection of bovine catalytic (C) subunits into sensory neurons is sufficient to produce protein synthesis–dependent LTF. Early in the LTF induced by serotonin (5-HT), an autonomous PKA is generated through the ubiquitin–proteasome-mediated proteolysis of regulatory (R) subunits. The degradation of R occurs during an early time window and appears to be a key function of proteasomes in LTF. Lactacystin, a specific proteasome inhibitor, blocks the facilitation induced by 5-HT, and this block is rescued by injecting C subunits. R is degraded through an allosteric mechanism requiring an elevation of cAMP coincident with the induction of a ubiquitin carboxy-terminal hydrolase.

Introduction

The processes underlying short- and long-term sensitization in *Aplysia* are reflected in the short- and long-term facilitation (LTF) of sensory-to-motor neuron synapses; these can be examined not only in intact animals but also in cell culture (Frost et al., 1985; Montarolo et al., 1986). The application of one pulse of serotonin (5-HT), a transmitter released from interneurons activated during tail stimulation, causes brief facilitation by transiently increasing the release of transmitter from sensory neurons (Kandel and Schwartz, 1982). In contrast, five pulses initiate a molecular cascade leading to the synthesis of proteins needed for an enduring increase in synaptic effectiveness (Byrne et al., 1993; Byrne and Kandel, 1996). This cascade begins when the catalytic (C) subunit of the cAMP-dependent protein kinase (PKA) (Bacskai et al., 1993), together with MAP kinase (Michael et al., 1998), moves into the nucleus and phosphorylates cAMP response element–binding protein (CREB) activators and repressors (Dash et al., 1990; Kaang et al., 1993; Bartsch et al., 1995, 1998). The phosphorylation of CREB is also implicated in the formation of memory in *Drosophila* (Yin and Tully, 1996).

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What are the downstream events produced by CREB? In addition to the activation of genes that regulate the synthesis of new proteins, such as the transcription factor ApC/EBP (Alberini et al., 1994), Hegde et al. (1997) found that the induction of the immediate-early gene (IEG) ubiquitin carboxy-terminal hydrolase (*Ap-uch*) is essential for LTF. This deubiquitinase, which is neuron-specific, belongs to a diverse class of isopeptidases with several functions, among which is the enhancement of proteolysis by proteasomes (Eytan et al., 1993; Wilkinson, 1997).

The degradation of proteins by the ubiquitin–proteasome pathway has been implicated in regulating several important cellular processes (Mykles, 1998), including transcriptional activation (Verma et al., 1995), progression through the cell cycle (Pagano, 1997), apoptosis (Hale et al., 1996), antigen presentation (Rock et al., 1994), differentiation and growth (Zhu et al., 1996), muscle atrophy (Mitch and Goldberg, 1996), and synaptogenesis (Muralidhar and Thomas, 1993; Oh et al., 1994). In this multienzyme pathway (reviewed by Hershko and Ciechanover, 1998), protein substrates marked for degradation by ligation to ubiquitin molecules (Haas and Siepmann, 1997) are proteolyzed by the 26S proteasome (Baumeister et al., 1998).

What is the function of ubiquitin-mediated proteolysis in sensory neurons, and how does it operate? Previously, we proposed that one possible substrate of proteolysis is the regulatory (R) subunit of PKA (Hegde et al., 1993, 1997; Chain et al., 1995). Each R subunit contains two tandem cAMP-binding sites, one (B) a high-affinity site close to the carboxyl terminus, and the other (A) a low-affinity site near the domain masked by the C subunit in the holoenzyme (Taylor et al., 1990). Activation of the kinase occurs when the second messenger, cAMP, binds sequentially and cooperatively to these two sites, causing the C subunit to dissociate. Degradation of R subunits results in an excess of C and would generate a persistently active and autonomous kinase (Greenberg et al., 1987a; Schwartz and Greenberg, 1989). In support of this idea, Sweatt and Kandel (1989) found that several unidentified proteins remain phosphorylated for at least 24 hr in stimulated sensory neurons undergoing LTF. These observations raise two questions that we now propose to address: (1) is a persistently active PKA essential for LTF, and (2) is proteolysis of R required to establish a persistently active kinase and to produce LTF?

Here, we demonstrate that a persistently active PKA is sufficient to produce LTF using specific proteasome inhibitors. One of the principal actions of the ubiquitin–proteasome pathway is to generate the autonomous kinase. We find that ubiquitin-mediated proteolysis is essential for establishing LTF. This proteolytic action is restricted to the early consolidation phase of LTF. We characterize the conditions required for R subunits to be degraded and explain why only a fraction of the subunits is proteolyzed.

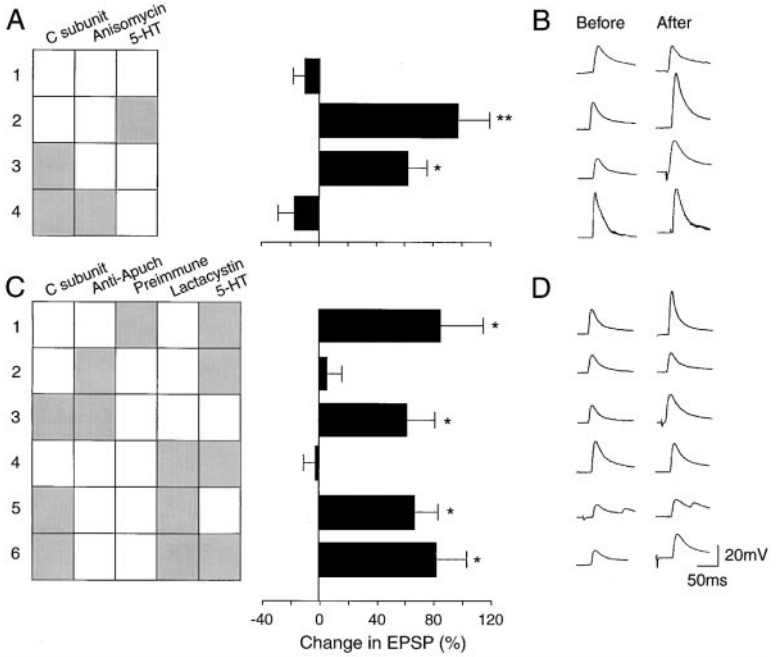

Figure 1. Intracellular Injection of PKA C Subunits Produces LTF

(A and C) Changes in EPSP amplitude (mean ± SEM) at 24 hr, produced by the treatments indicated in the grid on the left by gray squares, are summarized as bar graphs.

(B and D) Representative EPSP traces before and 24 hr after starting treatment with 5-HT.

(A and B) (1) shows unstimulated neurons (no 5-HT treatment, n = 24). (2) shows LTF produced by five pulses of 5-HT (n = 8). (3) shows the injection of C subunits alone (n = 22). (4) shows the injection of C subunits in the presence of anisomycin (10 μM; added 1 hr before the start of the experiment; n = 8). When administered, bovine C subunits were injected at the start of the experiment.

(C and D) Injection of C subunits overrides inhibitors of proteasomes. (1) shows injection of preimmune serum followed by five pulses of 5-HT (n = 6). (2) shows the injection of Ap-uch antibody followed by five pulses of 5-HT (n = 8). (3) shows Ap-uch antibody injected together with C subunits (n = 13). (4) shows lactacystin (5 μM) injected before treatment with 5-HT (n = 7). (5) shows the injection of C subunits together with lactacystin (n = 8). (6) shows the injection of C subunit, together with lactacystin, followed by five pulses of 5-HT (n = 10). As Castellucci et al. (1980) found for the production of short-term facilitation, some neurons injected with C subunits fail to show any response. About 10 ng of C subunit were injected into each cell indicated, but the exact amount delivered is difficult to determine because of protein stickiness in the glass electrode and differences in shape between sensory neurons. Because the amounts of enzyme injected are variable, failures probably result when insufficient amounts of C are injected. Nevertheless, the mean values shown include data from all of the cells injected. A one-way analysis of variance and Newman-Keuls' multiple range test were used to assess significance. A single asterisk indicates $p > 0.05$; double asterisks indicate $p > 0.01$. Castellucci et al. (1980) did not find LTF, because measurements were not made long enough in those early experiments.

Results

Injecting PKA C Subunits into Sensory Neurons Produces LTF

Greenberg et al. (1987a) found that an autonomous protein kinase could be formed in *Aplysia* sensory neurons that had been behaviorally sensitized. We now show that a persistently active PKA can produce LTF. In dissociated cell culture, five pulses of 5-HT produce facilitation that lasts longer than 24 hr. Typically, facilitation doubles the excitatory postsynaptic potential (EPSP) (Figures 1A and 1B, lines 1 and 2). A similar increase is produced by injecting free bovine C subunits into sensory neurons (Figures 1A and 1B, line 3). Castellucci et al. (1980) showed that injecting C can produce short-term facilitation. The increased synaptic potential observed 24 hr after injecting the subunit was not simply the persistence of a short-term facilitation mediated by excess kinase remaining in the injected neuron for a day, however, since no increase occurred after the injection of C if protein synthesis was inhibited by anisomycin (Figures 1A and 1B, line 4). Thus, protein synthesis is required for the C subunit to produce its long-term effect. These results suggest that the phosphorylation of substrate proteins by the injected C subunit is sufficient to induce and maintain the same cascade of gene activation for LTF as that produced by repeated pulses of 5-HT.

An Autonomous PKA Is Only Required Early during Formation of LTF

Protein kinase A inhibitor fragment 6–22 amide (PKI), a potent inhibitor of the C subunit, blocked LTF when injected before the 5-HT treatment and continued to

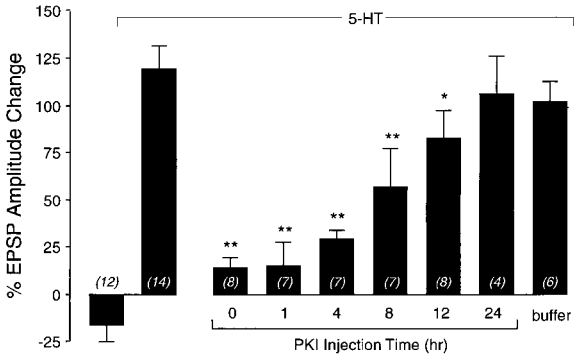

Figure 2. LTF Is Dependent on PKA Activity during the First 12 Hr after 5-HT Treatment

PKI, the peptide inhibitor of PKA, was injected at various intervals after the end of the 5-HT treatment. LTF was significantly diminished in neurons treated with five pulses of 5-HT and injected with PKI before 12 hr compared with either uninjected or buffer-injected (control) cells (Newman-Keuls' multiple comparison test). Control cells were not treated with 5-HT. Data are mean ± SEM. A single asterisk indicates $p > 0.05$; double asterisks indicate $p > 0.01$.

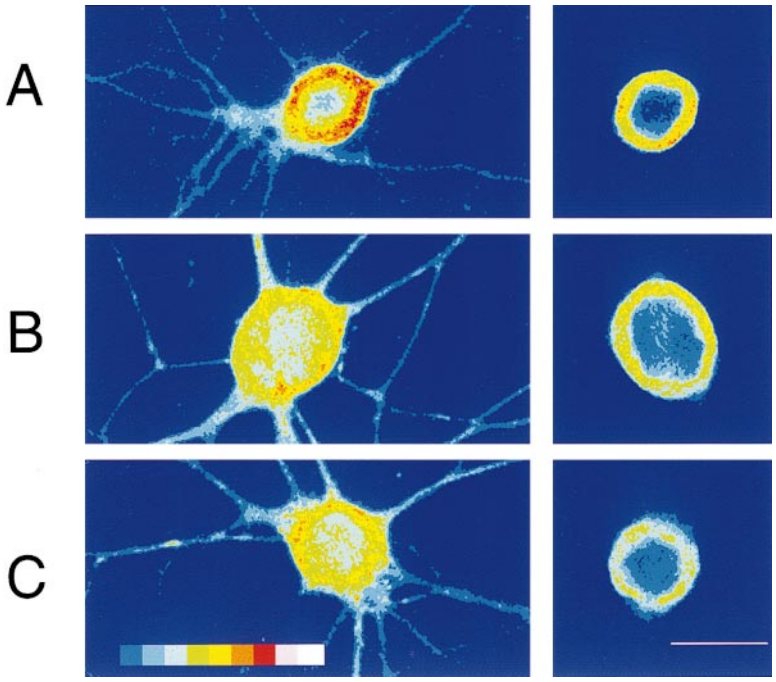

Figure 3. N4 R Subunits Are Rapidly Degraded in Sensory Neurons after Exposure to 5-HT

Cultured sensory neurons (6–8 per dish) were untreated (A) or subjected to 5 × 5 min pulses of 5-HT (20 μM) during a 2 hr period. After the treatment, the cells were incubated for 2 (B) or 24 hr (C) in normal culture medium, then fixed, immunostained with anti-N4-PEST antibody, and analyzed by confocal microscopy. Intensity of immunofluorescence is shown with artificial colors as indicated by the color wedge; blue (at the right) indicates lowest intensity, and white (at the left) indicates highest intensity. On the left is shown the fluorescence intensity in projections of individual sensory neurons examined for each experimental condition. The cells shown were selected at random from each dish. On the right are midline cross sections of the cells. No immunoreactivity was seen in cells treated only with preimmune sera. The experiment was performed four times. Scale bar, 50 μm.

inhibit when injected at various times for up to 12 hr. The inhibitor had no effect when injected after 12 hr, however (Figure 2). This result corroborates earlier experiments suggesting that kinase activity is needed only early during LTF (Alberini et al., 1994; O'Leary et al., 1995; Hegde et al., 1997).

R Subunits Are Rapidly Degraded after 5-HT Treatment

An autonomous PKA would be generated by a decrease in the R-to-C subunit ratio. We previously showed only that R subunits are degraded 24 hr after the induction of LTF (Greenberg et al., 1987a; Schwartz and Greenberg, 1989; Bergold et al., 1990). If a persistently active protein kinase is required early during the formation of LTF, then the degradation of R subunits should be an early event. To test this idea, we used confocal immunofluorescence microscopy with an antibody against N4, the major *Aplysia* R subunit, to determine the distribution and amount of the subunit in sensory neurons. N4 immunoreactivity was prominent in the cytoplasm of the cell body and its processes (Figures 3A, 3B, and 3C, left). It was absent from the nucleus both before and after the five repeated pulses of 5-HT, a treatment that elevates cAMP and produces LTF (Figures 3A, 3B, and 3C, right). The amount of N4 was diminished by 20% 2 hr after starting the 5-HT treatment (Figures 3A and 3B; Table 1) and was still decreased at 24 hr (Figure 3C; Table 1).

Mechanism of R Degradation: Ubiquitination Is cAMP-Dependent

The limited proteolysis of R subunits observed in the sensory cell raises two questions. First, what are the conditions that permit the degradation to occur, and second, what prevents it from going to completion as would be expected for proteins that are degraded through the ubiquitin pathway? The susceptibility of the normally stable R subunit to degradation increases with the degree to which R is saturated with ^{32}P-8N$_3$cAMP. It seems likely that cAMP causes this susceptibility by producing an R subunit with an appropriate conformation for ubiquitination. The conditions for saturating bovine R^I were ascertained with excess (micromolar) concentrations of the cAMP analog at pH values ranging from 6.2 to 9.5. Maximal incorporation occurred at pH 6.5 (Figure 4A, upper panel); R^I subunits labeled at this low pH were degraded more rapidly in reticulocyte lysates than were those labeled at pH 7.5 (Figure 4A, bar graph). These experiments were done in lysates depleted of C subunits; therefore, the difference in R subunit degradation observed implies that dissociation of the holoenzyme to release free R is not sufficient for degradation to occur. As described previously (Hegde et al., 1993,

Table 1. Decrease of R Subunits

Time (hr)	Intensity (arbitrary pixel units)	Decrease (%)
0	83.0 ± 3.5	0
2	66.5 ± 2.3	19
24	67.4 ± 2.3	18

Cultured sensory neurons were treated with five pulses of 5-HT as described in Experimental Procedures. The relative amount of R subunit immunofluorescence in untreated cells and in cells 2 and 22 hr after treatment was measured in four neurons selected at random from each dish. These were selected by using the mean fluorescence intensity over the largest ellipse that could fit within the projection profile of a cell (including the nucleus), which is shown here for the four neurons examined in a single dish. The pixel intensities were scaled on a linear range between 0 and 255. Intensity is expressed as mean ± SEM. The decreases in fluorescence measured after 2 and 22 hr were statistically significant (Student's t test, $p < 0.05$). The experiment was repeated four times, and a total of 16 neurons were examined for each condition.

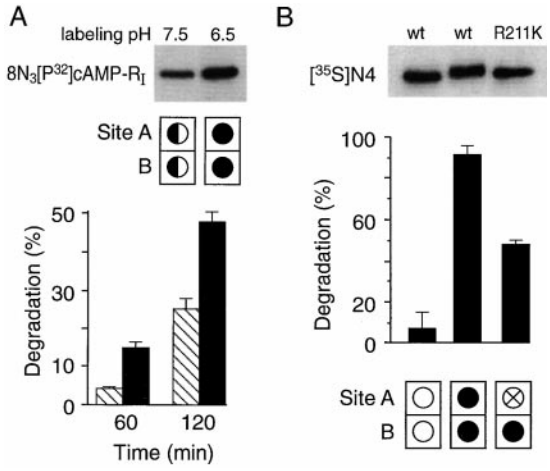

Figure 4. Degradation of R Subunits Depends on a cAMP-Bound Conformation

Degradation was assayed by the disappearance of bovine R^I with ^{32}P-8N_3cAMP (A) or with ^{35}SN-4 (B) by SDS-PAGE, autoradiography, and scanning densitometry. Diagrams to indicate the degree to which each of the two R cAMP–binding sites (A and B) are occupied (closed circles, fully occupied; half filled circles, partially occupied) are drawn on presumptions based on earlier studies of vertebrate R subunits (Bubis et al., 1988; Neitzel et al., 1991; Herberg et al., 1996).

(A) Bovine skeletal muscle R^I photoaffinity labeled with ^{32}P-8N_3cAMP at pH 7.5 (hatched bars) or pH 6.5 (closed bars) was incubated at 37°C for 1 or 2 hr in rabbit reticulocyte lysate (50 μl) containing 5 mM DTT, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, and 50 mM Tris/HCl (pH 8.0). The relative incorporation of ^{32}P-8N_3cAMP at pH 7.5 or pH 6.5 is shown in the autoradiograph (upper panel).

(B) Wild-type (wt) ^{35}S-N4 and ^{35}S-N4(R211K) were photoaffinity labeled with nonradioactive 8N_3cAMP at pH 6.5, producing a fully occupied wild-type subunit (closed circles) and R211K labeled only at site B (circle with an X). The different mobilities of the R subunits thus labeled is indicated in the autoradiograph (upper panel). The degradation of these subunit species is compared with the wild-type ^{35}S-N4 that had not been photoaffinity labeled (open circles). The subunits were incubated for 3 hr in extracts of pleural ganglia containing 0.2 mg protein, 5 mM DTT, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, and 50 mM Tris/HCl (pH 8.0). Mean values of the extent of degradation are presented ± SEM.

1997; Chain et al., 1995), degradation was measured using R subunits prelabeled with ^{32}P-8N_3cAMP.

We also measured the degradation of ^{35}S-methionine-labeled recombinant N4 (^{35}S-N4) free of bound cAMP in *Aplysia* nervous tissue extract that had been treated to remove endogenous PKA. Without cAMP, recombinant ^{35}S-N4 was stable (Figure 4B, bar graph). The recombinant protein was degraded only when it was prelabeled with 8N_3cAMP (Hegde, et al., 1993, 1997; Chain, et al., 1995) or incubated in the presence of cAMP or Sp-cAMP. Under these conditions, ubiquitin conjugates of R could be detected by immunoblotting with anti-ubiquitin antibody (data not shown). These results suggest that ubiquitin ligation requires a specific conformation of R, a conformation produced by binding cAMP (Su et al., 1995).

To obtain evidence that cAMP binding is necessary for R subunit degradation, we mutagenized cAMP binding site A in N4. The arginine residue at position 211 in N4 was replaced with lysine (R211K), since a mutation of the corresponding position in the vertebrate subunit R^I(R209K) abolishes the cooperative binding of cAMP to the lower affinity site A without affecting binding to site B (Bubis et al., 1988; Neitzel et al., 1991). About half as much ^{35}S-N4(R211K) photolabeled under saturating conditions was degraded in the 3 hr assay as the wild-type subunit (Figure 4B, bar graph), suggesting that both of the tandem cAMP-binding sites must be occupied if R is to be degraded efficiently. Thus, in the cell, degradation would occur when the concentration of cAMP is sufficiently elevated to saturate N4, producing the specific cAMP-bound conformation of R needed for ubiquitin ligation. In sensory neurons, this condition prevails during and after the administration of five pulses of 5-HT. Shortly after the 2 hr exposure, cAMP drops back to baseline (Bernier et al., 1982; Bacskai et al., 1993), and degradation of R subunits ceases.

Active Proteasomes Are Required for Persistent Activation of PKA

Lactacystin is an irreversible inhibitor that reacts selectively with catalytic threonine residues in the proteasome to block protein degradation in mammalian cells (Fenteany et al., 1995; Craiu et al., 1997). The injection of lactacystin (5 μM) into sensory cells before treatment with 5-HT blocked facilitation (Figures 1C and 1D, line 4). Proteasome-mediated protein degradation is thus essential for LTF. Lactacystin's inhibition of LTF can be overridden by injecting C subunits; injected together with C, lactacystin no longer was able to prevent LTF (Figures 1C and 1D, line 5). Moreover, injection of the C subunit also rescued lactacystin's block of LTF in neurons exposed to 5-HT (Figures 1C and 1D, line 6). Thus, generation of a persistently active PKA is the critical function of proteasomes in LTF.

Hegde et al. (1997) showed that the induction of *Ap-uch*, an IEG, is essential for LTF and that the hydrolase enhances proteasome-mediated degradation in vitro. An anti-peptide Ap-uch antibody blocks LTF (Hegde et al., 1997; and Figures 1C and 1D, line 2). When the C subunit was injected together with the hydrolase antibody, the antibody failed to block the LTF produced by exogenous C subunits (Figures 1C and 1D, line 3). These results indicate that phosphorylation by the C subunit is downstream to the step blocked by lactacystin and are consistent with the idea that proteasomes in the presence of the hydrolase generate an endogenous persistent kinase through the proteolysis of R subunits.

Proteasome Must Act Early during Formation of LTF

If the critical function of activated proteasomes is to generate an autonomous kinase, we would expect this function to take place early, overlapping the elevation of cAMP in time. To find the critical time window for proteasome action to establish LTF, we injected lactacystin at various intervals after pulsing the cells with 5-HT (Figures 5A, 5B, and 5C, lines 3, 4, and 5). Lactacystin injected immediately after the exposure to 5-HT blocked LTF. Lactacystin was less effective when injected 1 hr after the last pulse of 5-HT, and it had no

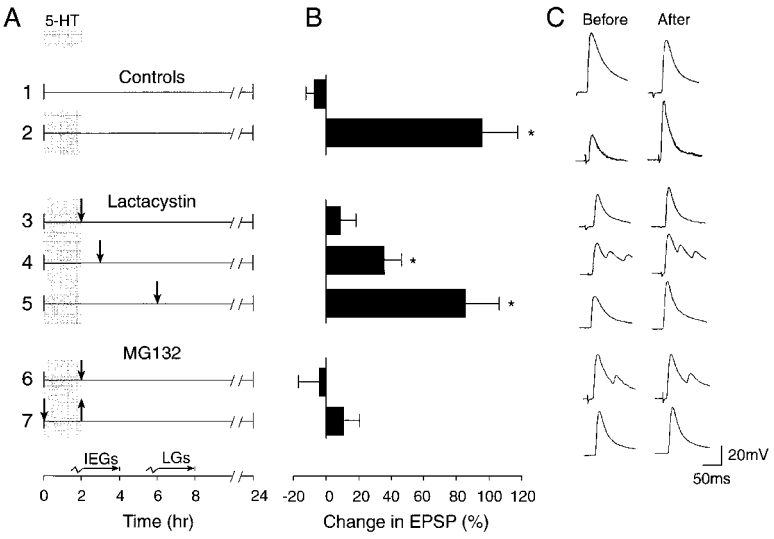

Figure 5. Proteasome Action Is Required Early during LTF

(A) The duration of 5-HT treatment is indicated by a shaded box, and the times when proteasome inhibitors were added, by vertical arrows. Horizontal arrows indicate the two phases of protein synthesis, the transcription of IEGs and late genes (LGs), that are implicated in producing LTF (Barzilai et al., 1989; Alberini et al., 1994; Hegde et al., 1997).

(B) Bar graphs summarizing the changes in EPSP amplitude (mean ± SEM) at 24 hr after the start of 5-HT treatment.

(C) Representative examples of EPSP traces before and 24 hr after the start of 5-HT treatment. (1) shows no 5-HT treatment (n = 13). (2) shows five pulses of 5-HT (10 μM) (n = 19). (3) shows lactacystin (10 μM) injected immediately after the last pulse of 5-HT (n = 14). (4) shows lactacystin, 1 hr after 5-HT (n = 9). (5) shows lactacystin, 4 hr after 5-HT (n = 8). (6) shows MG132 (5 μM) after 5-HT (n = 17). (7) shows MG132 added before (downward arrow) and removed after (upward arrow) the 2 hr exposure to 5-HT (n = 12). Data are presented as mean percent change ± SEM in the EPSP amplitude measured 24 hr after the treatment compared with the initial EPSP. The statistical analysis used is described in the legend to Figure 1.

effect when injected after 4 hr. Thus, proteasome action is required soon after the treatment with the facilitatory transmitter.

Since lactacystin binds proteasomes irreversibly, it could not be used to determine whether proteasome action is needed during the treatment with 5-HT. We therefore used a reversible, membrane-permeant proteasome inhibitor, carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132), to examine the role of protein degradation earlier in the development of LTF. MG132 binds reversibly to multiple active sites in proteasomes (Lee and Goldberg, 1996). Like lactacystin, MG132 blocked LTF when added after the last pulse of 5-HT (Figure 5, line 6). MG132, together with 5-HT, was applied and removed at the end of the 2 hr treatment. Cells exposed to MG132 during treatment with 5-HT showed no facilitation at 24 hr (Figure 5, line 7). In control experiments, MG132 was applied for 1 hr and then removed before the treatment with 5-HT (data not shown). Normal LTF (+84% ± 20%, n = 8) resulted, indicating that the effect of MG132 can be rapidly reversed in *Aplysia* neurons, as in other cells (Lee and Goldberg, 1996). Thus, proteasome action is required early in LTF, at a time that begins during the treatment with 5-HT and ends soon afterward, as shown with lactacystin (Figure 5, lines 4 and 5).

Lactacystin and MG132 inhibit the peptidase activities of *Aplysia* proteasomes in vitro (data not shown), as they do in the proteasomes of other species. *Aplysia* proteasomes are similar in subunit structure to those of other eukaryotes, although there are some quantitative differences in the spectrum of endopeptidase activities (Chain et al., 1995). Inhibiting proteasomes causes ubiquitinated protein conjugates to accumulate, with a corresponding decrease in free ubiquitin (Tawa et al., 1997). Accordingly, free ubiquitin decreased markedly in sensory neurons cultured for 22 hr in the presence of MG132 (data not shown).

Unlike lactacystin, MG132 is not specific for proteasomes. It is also an inhibitor of the calpains, which have been proposed to play a role in the formation of memory (see Vanderklish et al., 1996). The addition of 10 μM PD15060, an α-mercaptoacrylic derivative that inhibits calpains selectively in intact cells (Wang et al., 1996), did not block facilitation at 24 hr when applied immediately after sensitization treatment (+77% ± 18%, n = 11). Therefore, the effect of MG132, like that of lactacystin, is caused by inhibiting proteasomes, which are likely to be the major proteolytic system required for LTF. None of the protease inhibitors used affected the short-term process (Table 2), indicating that proteolysis is not needed in the synaptic mechanism of facilitation.

CREB Isoforms Are Phosphorylated by the Autonomous PKA

CREB-1a and CREB-1c are persistently phosphorylated after treatment with five pulses of 5-HT (Figure 6). Both

Table 2. Proteasomes Are Not Required for Short-Term Facilitation

Stimulation	Inhibitor	(n)	Change in EPSP at 10 min (%)
none	none	13	−18 ± 7
+	none	9	+115 ± 30
+	Lactacystin	8	+143 ± 17
+	MG132	8	+122 ± 35
+	PD150606	9	+131 ± 29

Facilitation was evoked in sensory motor neuron cocultures with one 5 min pulse of 5-HT (10 μM), and the EPSP was measured 10 min later. Lactacystin (10 μM) was pressure injected; MG132 (5 μM) and PD150606 (10 μM) were added 1 hr before 5-HT. We found no significant differences between the increases in EPSP produced in the presence of the inhibitors and the controls (t test). Cells exposed to the protease inhibitors for 24 hr are indistinguishable from untreated neurons in appearance. Treated cells retain normal biophysical properties. Also, none of the inhibitors interfered with basal synaptic transmission, suggesting that neither proteasomes nor Ca^{2+}-activated proteases participate importantly in the synaptic mechanisms of facilitation.

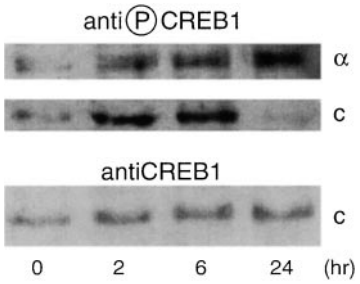

Figure 6. Persistent Phosphorylation of CREB Isoforms

The amounts of phosphorylated CREB-1a (top panel) and CREB-1c (middle panel) were determined at intervals after the treatment with 5-HT. For each time point, the immunoblots are from extracts of ganglia from one *Aplysia*; the data are representative of three similar experiments. Animals were sensitized with 5-HT as described by Alberini et al. (1994). After 2 hr, they were transferred to artificial sea water and the ganglia removed for extraction with Trizol at 2, 6, or 22 hr (Bartsch, et al., 1998). After SDS-PAGE, the proteins were analyzed by immunoblotting with polyclonal anti-phospho-CREB-1 (phospho-P-box) specific antibody that detects CREB-1a and CREB-1c. The two CREB isoforms were analyzed in parallel on different blots that were subjected to electrotransfer for 3 hr to optimize the signal of CREB-1a, and for 45 min for CREB-1c. The extracts were also analyzed for the amount of CREB-1c protein (bottom panel) with anti-CREB-1 (P-box) antibody.

isoforms are phosphorylated by PKA in vitro, but CREB-1a can also be phosphorylated by other protein kinases (Bartsch et al., 1998). The phosphorylation of CREB-1a persisted for 24 hr (Figure 6, top). Since CREB-1a expression is induced by the 5-HT treatment, the persistent phosphorylation observed may not be produced by PKA. In contrast, the phosphorylation of CREB-1c reliably reflects PKA activity. The phosphorylation of CREB-1c reached a maximum between 2 and 6 hr but was then dephosphorylated. CREB-1c was not induced (Figure 6, bottom). The duration of CREB-1c phosphorylation coincided with the period during which the autonomous PKA is required to produce LTF.

Discussion

cAMP Induces a Conformational Change in R Coincident with Proteasome Activation

We show that ubiquitin-mediated proteolysis is essential for developing LTF and that the proteasome inhibitors lactacystin and MG132 block the formation of LTF. A key substrate for proteolysis is the R subunit of PKA. The formation of a long-term memory for sensitization is initiated by prolonged elevation of cAMP in sensory neurons, leading to the phosphorylation of CREB activators and repressors (Dash et al., 1990; Bartsch et al., 1995, 1998; Yin and Tully, 1996) and the consequent induction of early response genes *ApC/EBP* and *Ap-uch* carboxy-terminal hydrolase (Alberini et al., 1994; Hegde et al., 1997). We find that one of the downstream molecular consequences of the induction of the hydrolase is the degradation of R subunits. In most tissues, R and C subunits are maintained in equal proportion (Hofmann et al., 1977). To preserve control of PKA by cAMP, mechanisms must exist to coordinate intracellular subunit stoichiometry (McKnight et al., 1988; Amieux et al., 1997). *Aplysia* and vertebrate R subunits can be proteolyzed in vitro through the ubiquitin pathway (Hegde et al., 1993), but increasing cAMP leads to the degradation of R only in neurons. Thus, R subunits are not degraded in *Aplysia* muscle even under conditions in which cAMP is greatly elevated for long periods of time (Bergold et al., 1990; Chain et al., 1995). Since the ubiquitin pathway operates in all tissues, why is the cAMP-induced degradation of R specific to neurons? Bergold et al. (1990) found that the degradation depends on the synthesis of new protein, and Hegde et al. (1997) provided evidence that a key component of the proteolytic machinery, a ubiquitin carboxy-terminal hydrolase, is specifically induced as an IEG only in neurons. It is not yet known whether this neuron-specific hydrolase is the only determinant of specificity or whether neurons might also express a special set of ligating and coupling enzymes (E2s and E3s) (Haas and Siepmann, 1997).

In *Aplysia* sensory neurons, only part of the total population of R subunits is lost; thus, the amount of R degraded never exceeds 30%, whether after behavioral training (Greenberg et al., 1987a) or exposure of isolated ganglia (Bergold et al., 1990) and cultured neurons to 5-HT (Figure 3). Dohrman et al. (1996) also observed R subunits to decrease in neuroblastoma X glioma hybrid cells treated with ethanol. Under these conditions, freed C subunits are imported into the nucleus. To maintain physiological regulation of PKA, it is necessary to limit the proportion of R subunits that is degraded (Schwartz and Greenberg, 1989), since there is no change in the amounts of R or C synthesized (Bergold et al., 1992), and since both R and C subunits are stable (half life $>$2 weeks). The degradation of protein substrates by the ubiquitin–proteasome pathway generally goes to near completion. How, then, is the degradation limited? Our data suggest that the loss of R subunits is limited in neurons, because a high concentration of cAMP is required to convert the normally stable R into a substrate for ubiquitin ligation, and because cAMP is sufficiently elevated for only a relatively short time in sensory neurons. The dissociation of holoenzyme is necessary but not sufficient for degradation; most of our in vitro studies were done with recombinant R alone and with nervous tissue extracts depleted of C. Without C, the protection of R subunits by the formation of holoenzyme cannot explain why R is not degraded.

The mechanism through which PKA becomes persistently activated is diagrammed in Figure 7; R subunit degradation requires at least two overlapping mechanisms, both caused by the elevation of cAMP in response to 5-HT. First, the binding of cAMP produces a conformation of R that is particularly susceptible to degradation. Second, by activating PKA and initiating its importation into the nucleus, cAMP promotes gene induction, leading to the expression of Ap-uch. The hydrolase, which is fully induced by 0.5 hr after the end of the 5-HT treatment (Hegde et al., 1997) in turn increases the degradative capacity of proteasomes. Only early in the development of LTF do these two mechanisms—cAMP binding to R and the induction of hydrolase—overlap.

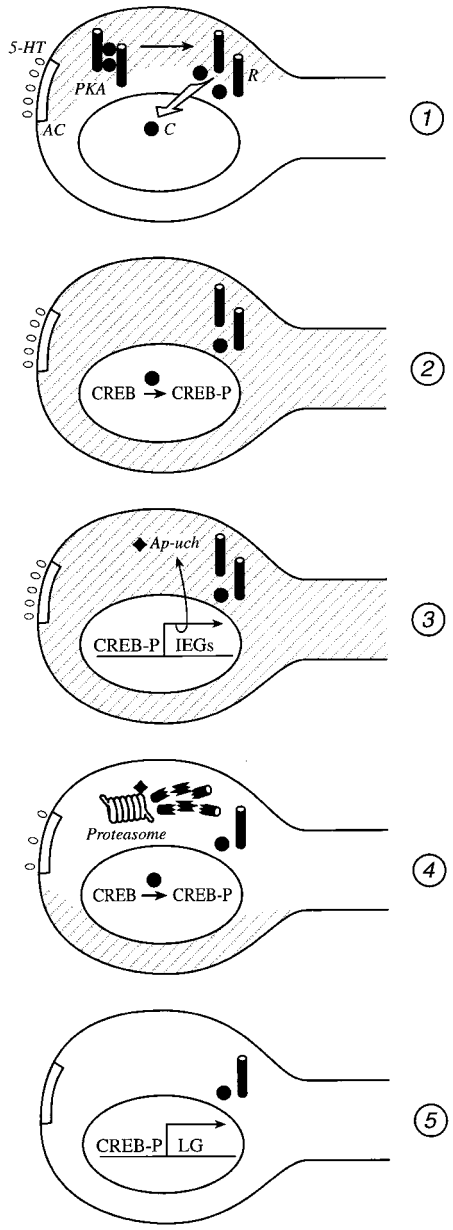

Figure 7. A Model Explaining the Role of the Ubiquitin–Proteasome Pathway in LTF

When 5-HT binds to its receptor in the sensory neuron (1), AC is activated to produce cAMP (shading). The elevated cAMP then causes the heterotetrameric PKA to dissociate. If the exposure to 5-HT is prolonged, some C subunit is imported into the nucleus (Bacskai et al., 1993), where it phosphorylates CREB (2). Phosphorylated CREB acts as a transcription activator to induce IEGs, one of which is a ubiquitin carboxy-terminal hydrolase (Ap-uch) (3). When the hydrolase is synthesized, it associates with proteasomes to enhance the proteasome's proteolytic activity to degrade the R subunit (4). Proteolysis of R can occur only when the subunit is dissociated from C and when its two cAMP-binding sites are occupied. Therefore, the degradation must occur in the brief interval after sufficient Ap-uch has been induced and before cAMP returns to baseline (5). As a result of the altered R-to-C subunit ratio, the kinase is active even in the absence of cAMP (decrease and absence of shading). Persistent PKA phosphorylation is essential for the formation of LTF. The injection of C subunits is sufficient to produce LTF and circumvents the need for Ap-uch or active proteasomes.

The Autonomous PKA Is Essential Early in Establishing LTF

Sweatt and Kandel (1989) found that several unidentified proteins remain phosphorylated for at least 24 hr in stimulated sensory neurons undergoing LTF. Phosphorylation by PKA probably accounts for these phosphoproteins, although other protein kinases are also activated (Saitoh and Schwartz, 1983, 1985; Sacktor and Schwartz, 1990; Sossin et al., 1994; Michael et al., 1998). One function of the persistent PKA would be synaptic, to maintain the potential for increased transmitter release from the sensory neuron through its action on the channels that are regulated by phosphorylation in both short- and long-term facilitation (Scholtz and Byrne, 1987; Byrne and Kandel, 1996). A second function would be nuclear: to phosphorylate CREB, initiating the molecular cascade of gene induction that results in LTF (Bartsch et al., 1998). During LTF, genes are activated sequentially in a cascade with at least two phases: an early period during which early response genes are induced (Alberini et al., 1994; Hegde et al., 1997) and a late period during which effector and structural proteins are synthesized (Barzilai et al., 1989; Ghirardi et al., 1995).

During the early phase, PKA can activate genes through CREB phosphorylation, because intracellular cAMP is elevated; later, when cAMP returns to basal levels, a persistently active kinase is formed that can continue to phosphorylate protein substrates in the absence of second messengers. We suggest that the autonomous PKA can serve as the simplest possible long-term memory mechanism. Through a reiterative function, a persistent kinase can maintain phosphorylation of transcription factors and cytoplasmic substrates, such as K^+ channels, long after the extracellular stimulation of adenylyl cyclase (AC) by 5-HT has stopped. This reiterative process is indicated in the diagram shown in Figure 7.

Even though PKA remains persistently active for at least 24 hr (Greenberg et al., 1987a; Sweatt and Kandel, 1989; Bergold et al., 1990; Müeller and Carew, 1998), our experiments with specific antagonists of PKA, Rp-cAMPS (Hegde et al., 1997) and PKI (Figure 2), indicate that persistent kinase activity is only needed for the first 12 hr.

Regulated Proteolysis Is Essential for Consolidating LTF

As in other instances of enduring alterations of cell state, for example, the cell cycle (Pagano, 1997), ubiquitin-mediated proteolysis during a critical time period commits the sensory neuron to the long-term change, converting a transient synaptic response into a stable memory. Transient second messenger modulation of metabolism and brief changes in enzyme and ion channel function are readily reversible, but proteolysis is irreversible, blocking old pathways and inducing new ones.

We propose that the persistently active PKA maintains transcription factors in their active state for the induction of LGs. In addition, the autonomous kinase would keep K^+ channels and other necessary cytoplasmic proteins phosphorylated, thereby maintaining the synapse in a facilitated state.

The consolidation of memory has often been shown to depend on the synthesis of new proteins (Squire, 1987). The degradation of critical inhibitory proteins is also important.

Experimental Procedures

Animals and Tissue Extracts

Aplysia (adult, 70–120 g; juvenile, 1–3 g) raised at the Mariculture Resource Facility of the University of Miami (FL) were rested in sea water for one week before an experiment. Pleural ganglia were homogenized in 8 mM KCl, 4 mM $MgCl_2$, 0.5 mM DTT, and 20 mM Tris-HCl (pH 7.6). The homogenate was centrifuged for 2 min at 3000 × g. Rabbit reticulocyte lysate was from Promega (Madison, WI). In some experiments, we extracted nervous tissue with a phenol/guanidine isothiocyanate solution (Trizol, Life Technologies, Grand Island, NY) as described by the manufacturer.

Reagents

Lactacystin was purchased from E. J. Corey (Harvard University, Cambridge, MA). MG132 was provided by Proscript (Cambridge, MA). Protein kinase A inhibitor fragment 6–22 amide was from Sigma (St. Louis, MO). Sp-cAMP was from Biolog, San Diego, CA. Ubiquitin aldehyde was from R. Cohen, University of Iowa (Iowa City). PD150606 was the gift of A. Saltiel, Parke-Davis (Ann Arbor, MI).

Bovine C subunits were from Promega. Bovine R^I subunits were purified from skeletal muscle (Chain et al., 1995). A polyclonal antibody, AbN4-PEST, was raised in rabbits against a peptide corresponding to 63–85, the PEST sequence in the N4 *Aplysia* R subunit (Chain et al., 1995). Polyclonal anti-CREB-1 (phospho-P-box) peptide and anti-CREB-1 (P-Box) peptide antibodies are described by Bartsch et al. (1998). A polyclonal ubiquitin antibody was from Sigma.

Cell Culture and Electrophysiology

Cultures were prepared and maintained for 4 days at 18°C (Montarolo et al., 1986; Dale et al., 1987). Briefly, *Aplysia* abdominal and pleural ganglia were treated with Type IX bacterial protease and desheathed. Individual sensory cells were gently teased apart with fine-tipped microelectrodes. Pleural sensory neurons from several adult animals were plated on 35 mm polylysine-coated glass coverslips with a single L7, the gill and siphon motor cell isolated from an abdominal ganglion of a juvenile *Aplysia*. The cells were cultured in *Aplysia* hemolymph: L15 medium (1:1), supplemented with salts consistent with *Aplysia*'s marine environment and containing penicillin (50 units/ml) and streptomycin (50 mg/ml) (Schacher and Proshansky, 1983).

EPSPs were recorded from L7, the postsynaptic motor cell, with intracellular glass microelectrodes filled with 2.5 M KCl (10–15 MΩ), and the membrane potential was held at −30 mV below its resting value. EPSPs were evoked by stimulating sensory neurons for 0.1–5 ms with an extracellular microelectrode. Results were stored on a four channel tape recorder. We induced LTF with five applications of 10 μM 5-HT, each lasting 5 min, followed by a 20 min interval. The amount of facilitation was calculated as the percent change in EPSP amplitude recorded 24 hr after the treatment with 5-HT compared with its value before treatment. Injection microelectrodes were beveled to a final resistance of 15–20 MΩ.

Immunocytochemistry and Confocal Microscopy

Cultured neurons were rinsed five times with phosphate-buffered saline (PBS)/30% sucrose and fixed for 1 hr with 4% paraformaldehyde in PBS/30% sucrose at 4°C. Subsequent steps were at room temperature unless otherwise indicated. Cells were rinsed three times with PBS/30% sucrose and permeabilized for 10 min with 0.1% Triton X-100 in PBS/30% sucrose. Free aldehyde groups were quenched with 50 mM NH_4Cl and rinsed three times in PBS. The cells were incubated with 10% goat serum in PBS for 1 hr to block nonspecific antibody binding and then at 4°C with anti-N4-PEST diluted (1:100) in 10% goat serum/PBS overnight. The cells were then washed five times with PBS for 30 min on a rotary shaker. The secondary antibody was Cy-3-conjugated goat anti-rabbit F(ab)2 (Jackson Immunoresearch, West Grove, PA) diluted 1:200 in 10% goat serum/PBS. No immunofluorescence was seen with preimmune serum. Controls were incubated with secondary antibody alone. In control experiments (data not shown), we saw a linear relationship between the amount of antigen and signal with the N4-PEST antibody. The antibody was also characterized by using immunoblots with both extracts of nervous tissue and purified R subunits (Chain et al., 1995).

Extended focus images were collected on a BioRad MRC1000 confocal system operating through a Zeiss Axiovert 100 microscope with a 40× 0.75 water immersion objective. The fluorophore was excited at 568 nm with a krypton/argon laser; 10% of the peak intensity was used for excitation. The final image was constructed from the maximum of corresponding pixels of 12 images obtained by focusing through the cells in 5.4 μm steps. Each of the stepped images were the average of four scans with the confocal iris completely open.

Site-Directed Mutagenesis

Single amino acid substitutions in N4 R subunits were made using QuikChange (Stratagene, La Jolla, CA) as described by the manufacturer. Complementary sense and antisense oligonucleotide primers were synthesized for PCR amplification of recombinant N4 encoded in pT7N4 (the gift of H. Bayley, University of Texas, Austin). This plasmid was derived from pKS (Stratagene, La Jolla, CA) and contains a T7 promoter sequence in the correct orientation for expressing N4. The sequences of the sense and antisense primers flanking the altered codon correspond to sequences in N4 (Bergold et al., 1992); thus, the sense primer used to generate pT7N4(R211K) was 5′-CTTATCTACGGCACGCCCAAAGCCGCCACTGTC-3′.

Preparation of ^{35}S-Methionine R Subunits

pT7N4 and pT7N4(R211K) were labeled with ^{35}S-methionine (Amersham, Arlington Heights, IL) and expressed in a coupled transcription–translation reticulocyte lysate system containing T7 polymerase (TNT-T7 from Promega). The lysate containing ^{35}S-N4 was diluted 20-fold and mixed with Cibachron Blue agarose (Sigma) equilibrated with 20 mM K phosphate (pH 6.4) containing 25 mM NaCl, 5% glycerol, 0.1 mg/ml ovalbumin, and 5 mM benzamidine. After the suspension was mixed for 30 min at 4°C, the gel was washed with 50 mM NaCl in the same buffer. R subunits were eluted twice at 30 min intervals with five washes of 0.1 mM cGMP at 23°C. Bound cGMP was removed by dialysis.

Depletion of R and C Subunits from Tissue Extract

Extracts were mixed with cAMP-agarose beads (Sigma). After the beads were sedimented by centrifugation, the supernatants were mixed with PKI-Affigel (from H. Bayley) and centrifuged again. The efficiency of depleting both R and C subunits (>97%) was determined at each step by measuring the amount of PKA activity with a Kemptide phosphorylation assay (Gjertsen et al., 1995) in the presence and absence of added cAMP.

Photoaffinity Labeling

We photoaffinity labeled recombinant N4 with nonradioactive $8N_3$cAMP (Sigma) or ^{32}P-$8N_3$cAMP (ICN, Irvine, CA; 60–70 Ci/mmol; 1 Ci = 37 GBq) (Greenberg et al., 1987b), adjusting the pH between 6.2 and 9.5. Samples were incubated for 30 min in the dark at 0°C and then exposed for 10 min to ultraviolet light.

Assays for Ubiquitin Conjugation and Degradation

Reactions were carried out with recombinant N4 (10 μg) bound to cAMP-agarose beads treated with lysozyme to block nonspecific binding. The conjugation reaction mixtures contained 2 mM ATP-γ-S (Boehringer Mannheim, Indianapolis, IN), 5 mM $MgCl_2$, 2 mM DTT, 50 mM Tris-HCl (pH 7.6), ubiquitin (2.5 μM), and 3 μM ubiquitin aldehyde. Nervous tissue extract (0.2 mg) was prepared in the presence of ATP-γ-S from *Aplysia* ganglia treated with 5-HT as described by Chain et al. (1995) and modified according to Sudakin et al. (1995). After they were incubated for 1 hr, the mixtures were centrifuged to sediment the agarose beads, which were then washed five times with 50 mM Tris-HCl/150 mM NaCl. N4 subunit conjugates were eluted with cAMP, separated by SDS-PAGE, and immunoblotted

with anti-ubiquitin antibody. The blots were then stripped of the antibody and probed again with anti N4-PEST to monitor elution of the R subunits.

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References

Alberini, C.M., Ghirardi, M., Metz, R., and Kandel, E.R. (1994). C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in *Aplysia*. Cell *76*, 1099–1114.

Amieux, P.S., Cummings, D.E., Motamed, K., Brandon, E.P., Wailes, L.A., Le, K., Idzerda, R.L., and McKnight, G.S. (1997). Compensatory regulation of R^{I} alpha protein levels in protein kinase A mutant mice. J. Biol. Chem. *272*, 3993–3998.

Bacskai, B.J., Hochner, B., Mahaut-Smith, M., Adams, S.R., Kaang, B.K., Kandel, E.R., and Tsien, R.Y. (1993). Spatially resolved dynamics of cAMP and protein kinase A subunits in *Aplysia* sensory neurons. Science *260*, 222–226.

Bartsch, D., Ghirardi, M., Skehel, P.A., Karl, K.A., Herder, S.P., Chen, M., Bailey, C.H., and Kandel, E.R. (1995). *Aplysia* CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. Cell *83*, 979–992.

Bartsch, D., Casadio, A., Karl, K., Serodio, P., and Kandel, E.R. (1998). CREB-1 encodes a nuclear activator, a repressor, and a cytoplasmic modulator that constitute a regulatory unit critical for long-term facilitation in *Aplysia*. Cell *95*, 211–223.

Barzilai, A., Kennedy, T.E., Sweatt, J.D., and Kandel, E.R. (1989). 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in *Aplysia* sensory neurons. Neuron *2*, 1577–1586.

Baumeister, W., Walz, J., Zühl, F., and Seemüller, E. (1998). The proteasome: paradigm for a self-compartmentalizing protease. Cell *92*, 367–380.

Bergold, P.J., Sweatt, J.D., Winicov, I., Weiss, K.R., Kandel, E.R., and Schwartz, J.H. (1990). Protein synthesized during acquisition of long-term facilitation is needed for the persistent loss of regulatory subunits of the *Aplysia* cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *87*, 3788–3791.

Bergold, P.J., Beushausen, S.A., Sacktor, T.C., Cheley, S., Bayley, H., and Schwartz, J.H. (1992). Identification of a regulatory subunit of the cAMP-dependent protein kinase down-regulated in *Aplysia* sensory neurons during long-term sensitization. Neuron *8*, 387–397.

Bernier, L., Castellucci, V.F., Kandel, E.R., and Schwartz, J.H. (1982). Facilitatory transmitter causes a selective and prolonged increase in adenosine 3′:5′-monophosphate in sensory neurons mediating the gill and siphon withdrawal reflex in *Aplysia*. J. Neurosci. *2*, 1682–1691.

Bubis, J., Neitzel, J.J., Saraswat, L.D., and Taylor, S.S. (1988). A point mutation abolishes binding of cAMP to site A in the regulatory subunit of cAMP-dependent protein kinase. J. Biol. Chem. *263*, 9668–9673.

Byrne, J.H., and Kandel, E.R. (1996). Presynaptic facilitation revisited: state and time dependence. J. Neurosci. *16*, 425–435.

Byrne, J.H., Zwartjes, R., Homayouni, R., Critz, S.S., and Eskin, A. (1993). Roles of second messenger pathways in neuronal plasticity and in learning and memory. Insights gained from *Aplysia*. Adv. Second Messengers Phosphoprotein Res. *27*, 47–108.

Castellucci, V.F., Kandel, E.R., Schwartz, J.H., Wilson, F.D., Nairn, A.C., and Greengard, P. (1980). Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase simulates facilitation of transmitter release underlying behavioral sensitization in *Aplysia*. Proc. Natl. Acad. Sci. USA *77*, 7492–7496.

Chain, D.G., Hegde, A.N., Yamamoto, N., Liu-Marsh, B., and Schwartz, J.H. (1995). Persistent activation of cAMP-dependent protein kinase by regulated proteolysis suggests a neuron-specific function of the ubiquitin system in *Aplysia*. J. Neurosci. *15*, 7592–7603.

Craiu, A., Gaczynska, M., Akopian, T., Gramm, C.F., Fenteany, G., and Goldberg, A.L. (1997). Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. J. Biol. Chem. *272*, 13437–13445.

Dale, N., Kandel, E.R., and Schacher, S. (1987). Serotonin produces long-term changes in the excitability of *Aplysia* sensory neurons in culture that depend on new protein synthesis. J. Neurosci. *7*, 2232–2238.

Dash, P.K., Hochner, B., and Kandel, E.R. (1990). Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. Nature *345*, 718–721.

Dohrman, D.P., Diamond, I., and Gordon, A.S. (1996). Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. Proc. Natl. Acad. Sci. USA *93*, 10217–10221.

Eytan, E., Armon, T., Heller, H., Beck, S., and Hershko, A. (1993). Ubiquitin-carboxy-terminal hydrolase activity associated with 26S protease complex. J. Biol. Chem. *268*, 4668–4674.

Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J., and Schreiber, S.L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science *268*, 726–731.

Frost, W.N., Castellucci, V.F., Hawkins, R.D., and Kandel, E.R. (1985). Monosynaptic connections made by the sensory neurons of the gill- and siphon-withdrawal reflex in *Aplysia* participate in the storage of long-term memory for sensitization. Proc. Natl. Acad. Sci. USA *82*, 8266–8269.

Ghirardi, M., Monterolo, P.G., and Kandel, E.R. (1995). A novel intermediate stage in the transition between short- and long-term facilitation in the sensory to motor neuron synapse of *Aplysia*. Neuron *14*, 413–420.

Gjertsen, B.T., Mellgren, G., Otten, A., Maronde, E., Genieser, H.G., Jastorf, B., Vintermyr, O.K., McKnight, G.S., and Doskeland, S.O. (1995). Novel Rp-cAMP analogs as tools for inhibition of cAMP-kinase in cell culture. J. Biol. Chem. *270*, 20599–20607.

Greenberg, S.M., Castellucci, V.F., Bayley, H., and Schwartz, J.H. (1987a). A molecular mechanism for long-term sensitization in *Aplysia*. Nature *329*, 62–65.

Greenberg, S.M., Bernier, L., and Schwartz, J.H. (1987b). Distribution of cAMP and cAMP-dependent protein kinases in *Aplysia* sensory neurons. J. Neurosci. *7*, 291–301.

Haas, A.L., and Siepmann, T.J. (1997). Pathways of ubiquitin conjugation. FASEB J. *11*, 1257–1268.

Hale, A.J., Smith, C.A., Sutherland, L.C., Stoneman, V.E.A., Longthorne, V.L., Culhane, A.C., and Williams, G.T. (1996). Apoptosis: molecular regulation of cell death. Eur. J. Biochem. *236*, 1–26.

Hegde, A.N., Goldberg, A.L., and Schwartz, J.H. (1993). Regulatory subunits of the cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: a molecular mechanism underlying long-term synaptic plasticity. Proc. Natl. Acad. Sci. USA *90*, 7436–7440.

Hegde, A.N., Inokuchi, K., Pei, W., Casadio, A., Ghirardi, M., Chain, D.G., Martin, K.C., Kandel, E.R., and Schwartz, J.H. (1997). Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. Cell *89*, 115–126.

Herberg, F.W., Taylor, S.S., and Dostmann, W.R. (1996). Active site mutations define the pathway for the cooperative activation of cAMP-dependent protein kinase. Biochemistry *35*, 2934–2942.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Hofmann, F., Bechtel, P.J., and Krebs, E.G. (1977). Concentrations of cyclic AMP-dependent protein kinase subunits in various tissues. J. Biol. Chem. *252*, 1441–1447.

Kaang, B.-K., Kandel, E.R., and Grant, S.G. (1993). Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in *Aplysia* sensory neurons. Neuron *10*, 427–435.

Kandel, E.R., and Schwartz, J.H. (1982). Molecular biology of learning: modulation of transmitter release. Science *218*, 433–443.

Lee, D.H., and Goldberg, A.L. (1996). Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. J. Biol. Chem. *271*, 27280–27284.

McKnight, G.S., Clegg, C.H., Uhler, M.D., Chrivia, J.C., Cadd, G.G., Correll, L.A., and Otten, A.D. (1988). Analysis of the cAMP-dependent protein kinase system using molecular genetic approaches. Rec. Prog. Hormone Res. *44*, 307–335.

Michael, D., Martin, K.C., Seger, R., Ning, M.-M., Baston, R., and Kandel, E.R. (1998). Repeated pulses of serotonin required for long-term facilitation activate mitogen-activated protein kinase in sensory neurons of *Aplysia*. Proc. Natl. Acad. Sci. USA *95*, 1864–1869.

Mitch, W.E., and Goldberg, A.L. (1996). Mechanisms of muscle wasting. The role of the ubiquitin–proteasome pathway. N. Engl. J. Med. *335*, 1897–1905.

Montarolo, P.G., Castellucci, V.F., Morgan, J., Kandel, E.R., and Schacher, S. (1986). A critical period for macromolecular synthesis in long-term heterosynaptic facilitation. Science *244*, 1249–1254.

Müller, U., and Carew, T.J. (1998). Serotonin induces temporally and mechanistically distinct phases of persistent PKA activity in *Aplysia* sensory neurons. Neuron *21*, 1423–1434.

Muralidhar, M.G., and Thomas, J.B. (1993). The *Drosophila* bendless gene encodes a neural protein related to ubiquitin-conjugating enzymes. Neuron *11*, 253–266.

Mykles, D.L. (1998). Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. Int. Rev. Cytol. *184*, 157–289.

Neitzel, J.J., Dostmann, W.R., and Taylor, S.S. (1991). Role of MgATP in the activation and reassociation of cAMP-dependent protein kinase I: consequences of replacing the essential arginine in cAMP binding site A. Biochemistry *30*, 733–739.

Oh, C.E., McMahon, R., Benzer, S., and Tanouye, M. (1994). bendless, a *Drosophila* gene affecting neuronal connectivity, encodes a ubiquitin-conjugating enzyme homolog. J. Neurosci. *14*, 3166–3179.

O'Leary, F.A., Byrne, J.H., and Cleary, L.J. (1995). Long-term structural rendering in *Aplysia* sensory neurons requires *de novo* protein synthesis during a critical time period. J. Neurosci. *15*, 3519–3525.

Pagano, M. (1997). Cell cycle regulation by the ubiquitin pathway. FASEB J. *11*, 1067–1075.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell *78*, 761–771.

Sacktor, T.C., and Schwartz, J.H. (1990). Sensitizing stimuli cause translocation of protein kinase C in *Aplysia* sensory neurons. Proc. Natl. Acad. Sci. USA *87*, 2036–2039.

Saitoh, T., and Schwartz, J.H. (1983). Serotonin alters the subcellular distribution of a Ca^{2+}/calmodulin binding protein in neurons of *Aplysia*. Proc. Natl. Acad. Sci. USA *80*, 6708–6712.

Saitoh, T., and Schwartz, J.H. (1985). Phosphorylation-dependent subcellular translocation of Ca^{2+}/calmodulin-dependent protein kinase produces an autonomous enzyme in *Aplysia* neurons. J. Cell Biol. *100*, 835–842.

Schacher, S., and Proshansky, E. (1983). Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. J. Neurosci. *3*, 2403–2413.

Scholtz, K.P., and Byrne, J.H. (1987). Long-term sensitization in *Aplysia*: biophysical correlates in tail sensory neurons. Science *235*, 685–687.

Schwartz, J.H., and Greenberg, S.M. (1989). Turtles all the way down: some molecular mechanisms underlying long-term sensitization in *Aplysia*. In Neural Models of Plasticity, J.H. Byrne, ed. (New York: Academic Press), pp. 46–57.

Sossin, W.S., Sacktor, T.C., and Schwartz, J.H. (1994). Persistent activation of protein kinase C during the development of long-term facilitation in *Aplysia*. Learn. Mem. *1*, 189–202.

Squire, L. (1987). Memory and Brain (New York: Oxford University Press).

Su, Y., Dostmann, W.R., Herberg, F.W., Durick, K., Xuong, N.H., Ten Eyck, L., Taylor, S.S., and Varughese, K.I. (1995). Regulatory subunit of protein kinase A: structure of deletion mutant with cAMP binding domains. Science *269*, 807–813.

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F.C., Ruderman, J.V., and Hershko, A. (1995). The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. Mol. Biol. Cell *6*, 185–197.

Sweatt, J.D., and Kandel, E R. (1989). Persistent and transcriptionally-dependent increase in protein phosphorylation upon long-term facilitation in *Aplysia* sensory neurons. Nature *339*, 51–54.

Tawa, N.E., Odessey, R., and Goldberg, A.L. (1997). Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. J. Clin. Invest. *100*, 197–203.

Taylor, S.S., Buechler, J.A., and Yonemoto, W. (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. *59*, 971–1005.

Vanderklish, P., Bednarski, E., and Lynch, G. (1996). Translational suppression of calpain blocks long-term potentiation. Learn. Mem. *3*, 209–217.

Verma, I.M., Stevenson, J.K., Schwartz, E.M., Van Antwerp, D., and Miyamoto, S. (1995). Rel/NF-κB/I-κB family: intimate tales of association and dissociation. Genes Dev. *9*, 2723–2735.

Wang, K.K., Nath, R., Posner, A., Raser, K.D., Buroker-Kilgore, M., Hajimohammadresza, I., Probert, J.W.A., Marcoux, F.W., Ye, Q., Takano, E., et al. (1996). An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. Proc. Natl. Acad. Sci. USA *93*, 6687–6692.

Wilkinson, K.D. (1997). Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. FASEB J. *11*, 1245–1256.

Yin, J.C., and Tully, T. (1996). CREB and the formation of long-term memory. Curr. Opin. Neurobiol. *6*, 264–268.

Zhu, Y., Carroll, M., Papa, F., Hochstrasser, M., and D'Andrea, A.D. (1996). DUB-1, a deubiquitinating enzyme with growth-suppressing activity. Proc. Natl. Acad. Sci. USA *93*, 3275–3279.