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Ubiquitin-Mediated Proteolysis in Learning and Memory

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Abstract

Sensitization of defensive reflexes in *Aplysia* is a simple behavioral paradigm for studying both short- and long-term memory. In the marine mollusk, as in other animals, memory has at least two phases: a short-term phase lasting minutes and a long-term phase lasting several days or longer. Short-term memory is produced by covalent modification of pre-existing proteins. In contrast, long-term memory needs gene induction, synthesis of new protein, and the growth of new synapses. The switch from short-term (STF) to long-term facilitation (LTF) in *Aplysia* sensory neurons requires not only positive regulation through gene induction, but also the specific removal of several inhibitory proteins. One important inhibitory protein is the regulatory (R) subunit of the cAMP-dependent protein kinase (PKA). Degradation of R subunits, which is essential for initiating long-term stable memory, occurs through the ubiquitin-proteasome pathway.

Introduction

Underlying the physiological changes that take place during sensitization is enhanced output of neurotransmitter (facilitation) at sensory-to-motor neuron synapses. Serotonin (5-HT), a major neurotransmitter released in response to sensitizing stimulation, produces the facilitation (Walters et al., 1983; Mercer et al., 1991; Emptage and Carew, 1993; Mauelshagen et al., 1996; Zhang et al., 1997). In short-term facilitation (STF), activation of adenylyl cyclase by 5-HT causes the increased output of neurotransmitter at the sensory-to-motor neuron synapses (probably glutamate; Dale and Kandel, 1993) through reversible phosphorylation of pre-existing proteins, including a K^+ channel (Kandel and Schwartz, 1982). In contrast, repeated stimulation induces the transcription of genes and synthesis of the proteins needed for an enduring increase in synaptic effectiveness (Goelet et al., 1996; Barzilai et al., 1989; Byrne et al., 1993; Byrne and Kandel, 1996).

The increased output of transmitter in long-term facilitation (LTF) is brought about by a

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molecular cascade involving protein phosphorylation, gene activation, and the growth and stabilization of new synapses. Induction begins when the catalytic (C) subunit of protein kinase A (PKA) moves into the nucleus (Bacskai et al., 1993) to phosphorylate the cAMP-responsive element-binding protein (1) CREB (Dash et al., 1990; Kaang et al., 1993; Bartsch et al., 1998, 1995). Proteins are synthesized in at least two distinct phases: an early period, during which early response genes are expressed (Alberini et al., 1994; Hegde et al., 1997) and a late period, during which effector proteins and proteins needed for the growth of new synapses are made (Bailey and Kandel, 1993; Barzilai et al., 1989; Ghirardi et al., 1995; Hegde et al., 1999). As in *Aplysia,* cAMP-dependent phosphorylation also appears to underlie learning in other animals (Bourtchuladze et al., 1994; Impey et al., 1996; Yin and Tully, 1996; reviewed by Schafe et al., 1999).

Modification of the cAMP-dependent PKA is a molecular correlate of LTF. Like all of the major multifunctional Ser/Thr protein kinases, PKA can become active in the absence of the second messenger. Typically, the activity of these kinases is blocked by an inhibitory domain of the enzyme or by independent inhibitory subunits. In the short term, the inhibition is released when the second messenger binds to the inhibitory component. In the long term, the kinase becomes autonomous when the inhibitory component is inactivated or removed. Thus, as a consequence of prolonged exposure to the second messenger, protein kinases undergo some structural modification that results in the persistence of activity even when the second messenger is absent. Autonomy serves to prolong the effect of the stimulus signal that had originally caused the rise in the intracellular concentration of second messenger. This mechanism also appears to operate in hippocampal long-term potentiation (LTP) (Sacktor et al., 1993; Osten et al., 1996), alcoholism (Dohrman et al., 1996; Pandey, 1998), and cocaine addiction (Boundy et al., 1998), suggesting that it is universal.

The capacity for autonomy makes Ser/Thr protein kinases central to the cellular mechanisms that underlie long-term memory and learning (Schulman and Hyman, 1998). We suggest that acquisition of persistence by a kinase is a molecular correlate of memory because the enzyme is altered and behaves as if it has been trained by experiencing the stimulus. When first described, Crick (1984) and Lisman (1985) proposed that stimulus-induced posttranslational modification of key regulatory enzymes is the molecular mechanism of memory. Several examples in which persistent protein kinases are produced support this view (Saitoh and Schwartz, 1985; Greenberg et al., 1987; Schwartz and Greenberg, 1987; Bergold et al., 1990; Martin et al., 1997). But the requirement for gene expression, especially cAMP-dependent CREB-activated protein synthesis, indicates that persistence by post-translational modification alone cannot be the sole molecular process.

In 1987, Schwartz and Greenberg reviewed three possible post-translational modifications that might produce autonomous kinases during the formation of long-term memory: autophosphorylation, changes in subcellular localization, and proteolytic processing. Among these modifications, auto-phosphorylation has now been shown to be particularly important for the persistence of Ca^{2+}/calmodulin-dependent protein kinase II during the induction of hippocampal LTP (Braun and Schulman, 1995). Changes in subcellular localization affect the action of most protein kinases studied, especially importation into the nucleus from the cytoplasm (Bacskai et al., 1993; Martin et al., 1997). Specific isoforms of PKA are tethered to the membrane by anchoring proteins in order to increase the activity of neuronal Ca^{2+} channels and to inhibit K^+ channels (*see* Fraser and Scott, 1999). But the activity of anchoring proteins has not yet been shown to be altered in neurons during the formation of memory. Proteolytic removal of inhibitory domains or inhibitory subunits of kinases also play a role in generating autonomous kinases. For example, PKC is per-

sistently activated by the loss of its regulatory domain (Inoue et al., 1977; Takai et al., 1977; Schapp et al., 1990; Sacktor et al., 1993). And an autonomous PKA results from the degradation of inhibitory regulatory (R) subunits.

Here we discuss how an autonomous PKA is generated by the ubiquitin-proteasome pathway during induction of LTF. Degradation of R must be regulated, since the subunit is typically stable. We compare the regulatory mechanisms by which R subunits are targeted and degraded with those involved in proteolytic removal of other physiologically important protein substrates such as the cell-cycle regulator cyclin and several transcription activators.

The Ubiquitin-Proteasome Pathway

R subunits of PKA are degraded through the ubiquitin-proteasome pathway (Hegde et al., 1993; Chain et al., 1995). Persistence occurs because the degradation of PKA regulatory subunits alters the ratio of R to C subunits, thereby partially relieving inhibitory control of the C subunits (Greenberg et al., 1987; Bergold et al., 1992). In the ubiquitin-proteasome pathway, substrate proteins must first be covalently linked to chains of multiple ubiquitins. This conjugation/ligation reaction marks the protein for degradation by the proteasome, a multi-catalytic proteolytic complex (reviewed by Coux et al., 1996; Hershko and Ciechanover, 1998; Peters et al., 1998a; Kornitzer and Ciechanover, 2000).

Conjugation/Ligation

Three kinds of enzymes catalyze ubiquitination, E1, E2, and E3. Ubiquitin, a highly conserved protein containing 76 amino-acid residues, is first activated in an ATP-dependent reaction by a single, common E1 (UBA, activating enzyme) (reaction 1 in Fig. 1). Next, the activated ubiquitinyl group is transferred to one of several E2s (UBC, conjugase, or carrier protein), where it is bound in thioester linkage (reaction 2, Fig. 1). E2s may then either alone or together with an E3 (UBR ligase, targeting factor) transfer the C-terminal glycine of the activated ubiquitin to the ε-amino group of a lysine residue in the protein substrate through a thioester cascade (Hershko et al., 1983; Scheffner et al., 1995) (reaction 3, Fig. 1). A second ubiquitin is then attached to the first and thus a multi-ubiquitin chain grows. An adaptor protein is required to complete some E2/E3 substrate protein complexes. An example is E6, a papilloma virus-induced protein needed to degrade the tumor-suppressor protein, p53 (Scheffner et al., 1994). There are many E2s that are highly conserved in eucaryotes from yeast to mammals (Jentsch, 1992; Scheffner et al., 1994; Haas and Siepmann, 1997; Haldeman et al., 1997). E2s often can work with more than one substrate, but E3s are believed to be substrate-specific. We found that a nervous-tissue fraction (Fraction II) that presumably contains all three types of enzymes (E1, E2, and E3) is capable of ubiquitinating R subunits (Hegde et al., 1993). The specific E2/E3 pair that recognizes the R subunit has yet to be identified.

Degradation

After they have been multi-ubiquitinated, substrate proteins are degraded by proteasomes (reaction 4, Fig. 1). Proteasomes are multi-catalytic complexes that can be sedimented by ultracentrifugation. Based on sedimentation value, the complex that degrades the multi-ubiquitinated substrates is designated as the 26 S proteasome. The 26 S proteasome is dumbbell shaped and has two components: a cylindrical 20 S core with 19 S caps at both ends. Substrates to be degraded are threaded into the core through a narrow opening (DeMartino and Slaughter, 1999). Several endopeptidase activities reside in the approx 14 subunits of the core component, while the subunits of the two 19 S caps recognize multi-ubiquitinated substrates (Hershko and Ciechanover, 1998). The substrate's polypeptide chain is then cleaved into peptides mostly 8 or 9 amino acids in length (De Mot et al., 1999; Rock and Goldberg, 1999).

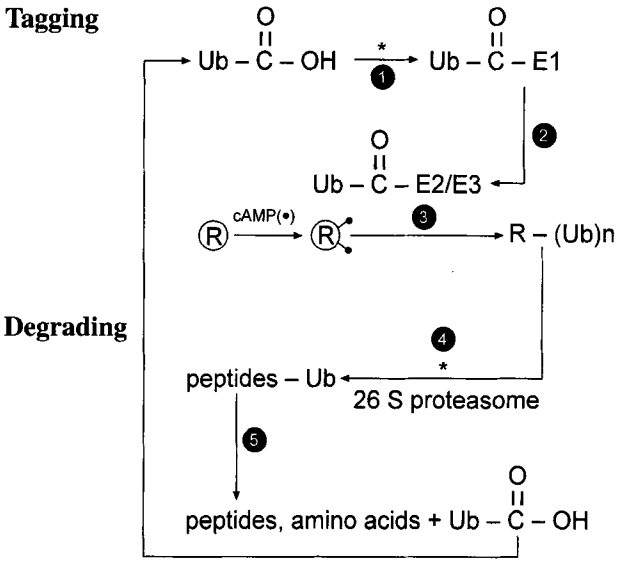

Fig. 1. The ubiquitin-proteasome pathway. R subunits of PKA become substrates for ubiquitination upon binding two molecules of cAMP. For tagging a substrate ubiquitin is first activated by an enzyme called E1. In the activation step a ubiquitinyl-AMP intermediate is formed; the ubiquitinyl group is then transferred to a Cys residue of E1 in a thioester bond with the release of AMP (reaction 1). Activated ubiquitin is next transferred to an active site Cys residue of the ubiquitin carrier protein E2 conjugating (UBC) enzyme. The E2 then alone or together with an E3 (UBR, ligase) attaches the C-terminus of ubiquitin in isopeptide linkage to an ε-amino group of a Lys residue in the substrate (reaction 2). For some substrates a specific adaptor protein is also required for the ligase reaction (not shown). Ligation of one ubiquitin to the substrate is then followed by the sequential addition of more ubiquitins, leading to the formation of multi-ubiquitin chains (reaction 3). The multi-ubiquitinated substrate is then degraded by the 26 S proteasome (reaction 4). Multi-ubiquitin chains are disassembled by the action of de-ubiquitinating enzymes, such as the ubiquitin C-terminal hydrolase, Ap-uch (reaction 5). Activation of ubiquitin as well as degradation of the substrate by the proteasome requires the hydrolysis of ATP (indicated by an asterisk*).

Multi-ubiquitin chains are disassembled by a family of ubiquitin-C-terminal hydrolases (reaction 5, Fig. 1). These reactions operate during the degradation of R subunits. We first found that the degradation of ubiquitinated R subunits depends on proteasomes (Hegde et al., 1993). Next, using in vitro reconstitution experiments, we showed that the proteasome can be a limiting step in the degradation of R subunits (Chain et al., 1995). Finally, the rate of R degradation is enhanced in the presence of a ubiquitin-C-terminal hydrolase (Hegde et al., 1997).

The Function of the Ubiquitin-Proteasome Pathway in the Nervous System

Ubiquitin-mediated proteolysis plays a role in many important cellular processes: transcriptional activation (Verma et al., 1995), the cell cycle (Pagano, 1997), antigen presentation (Rock et al., 1994), differentiation and growth (Huang et al., 1995; Zhu et al., 1996), apoptosis (Orlowski, 1999), metabolic hormonal responses (Mitch and Goldberg, 1996), tumorigenesis (Spataro et al., 1998; Schwartz and Ciechanover, 1999), and ion transport (Kopito, 1999). Although ubiquitinated proteins have been used as histological markers for pathological inclusions in the study of various neurodegenerative diseases (Arnold et al., 1998; Lowe et al., 1993), a physiological role for the pathway in the nervous system had not been established until recently. Our work on ubiquitin-mediated degradation of R subunits of PKA during induction of LTF suggested that this pathway plays a role in the physiological functioning of neurons. In addition, a ubiquitin-conjugating enzyme (E2) has been shown to be important in synaptogenesis (Muralidhar and Thomas, 1993; Oh et al., 1994). And proteasomes appear to be necessary for neurite outgrowth (Tsubuki et al., 1993). Still further, the E3 specific for degrading the tumor-suppressor protein, p53 (E6-AP, E6-associated protein) is mutated in Angelman's syndrome, a neurological disorder characterized by mental retardation (Albrecht et al., 1997; Kishino et al., 1997; Matsuura et al., 1997). Mice mutant in E6-AP learn poorly and are deficient in hippocampal LTP (Jiang et al., 1998).

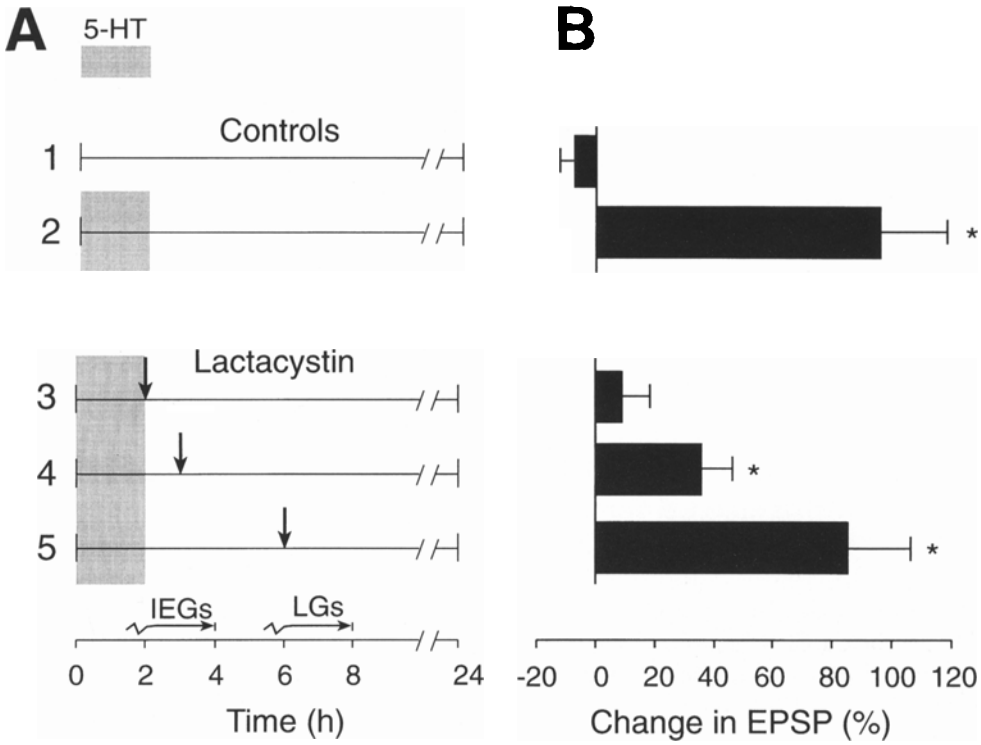

Fig. 2. Proteasomes function in a narrow time window early during LTF. The effect of lactacystin, a specific proteasome inhibitor, was tested using sensory-to-motor neuron synapses in culture. Excitatory postsynaptic potentials (EPSPs) were recorded from L7 motor neurons in response to extracellular stimulation of sensory neurons before and after exposure to 5-HT. Sensory neurons were either injected with lactacystin as indicated or left uninjected. **(A)** The duration of 5-HT treatment is indicated by a shaded box, and the times when the proteasome inhibitors was injected, by vertical arrows. Horizontal arrows indicate the two phases of protein synthesis, transcription of immediate-early genes (IEGs) and late genes (LGs) that are thought to be critical for LTF (Alberini et al., 1994; Barzilai et al., 1989; Hegde et al., 1997). **(B)** Bar graphs summarizing the changes in EPSP amplitude (mean ± SEM) at 24 h after the start of 5-HT treatment. (1) No 5-HT treatment (n = 13); (2) Five pulses of 5-HT (10 μ*M*) (n = 19); (3) Lactacystin (10 μ*M*) injected immediately after the last pulse of 5-HT (n = 14); (4) Lactacystin, 1 h after 5-HT (n = 9); (5) Lactacystin, 4 h after 5-HT (n = 8). Data are presented as mean percent change ± SEM in the EPSP amplitude measured 24 h after the treatment compared with the initial EPSP. An asterisk (*) indicates $p < 0.05$. (Adapted with permission from Chain et al., 1999.)

Degradation of R Subunits During LTF

Degradation of R subunits making PKA persistently active is a step that commits the sensory neuron to LTF. For LTF to be induced, PKA must phosphorylate CREB to activate the molecular cascade that eventually leads to the growth of new synapses. The degradation of R subunits is an example of how ubiquitin-mediated proteolysis provides both directionality and critical timing to a physiological process. Degradation occurs within 2 h of the start of sensitization treatment but never exceeds approx 20% of the R subunits initially present in the sensory neuron. Proof of the importance of proteasome action in vivo was provided by experiments with lactacystin, an irreversible and highly specific inhibitor of proteasomes (Chain et al., 1999). Lactacystin blocks LTF only if administered early and during a critical time window (Fig. 2). The molecular events

that underlie the acquisition of memory in neurons as well as other changes in cell state, for example, progression through the cell cycle, occur in a fixed order at specific times and cannot be reversed.

The key function of ubiquitin-proteasome-mediated proteolysis in LTF is to generate the persistently active protein kinase through the relief of negative regulation. Evidence for this conclusion came from experiments testing how the inhibition of LTF caused by inhibitors of the ubiquitin-proteasome pathway could be rescued (Chain et al., 1999). When lactacystin or antibodies against a critical enzyme in the ubiquitin pathway, *Aplysia* ubiquitin-C terminal hydrolases (Ap-uch) is injected into sensory neurons, LTF is blocked. If these reagents are injected along with free C subunits, however, LTF is not inhibited. Thus free C subunits or persistent activation of PKA is the important function of the ubiquitin-proteasome pathway during the induction of LTF (Fig. 3).

Degradation of R subunits by the ubiquitin pathway appears to be universally important. We showed that the vertebrate RI and RII type subunits can also be degraded by the ubiquitin-proteasome pathway (Hegde et al., 1993). Chronic administration of cocaine or morphine results in the phosphorylation of CREB in specific areas of the brain (Lane-Ladd et al., 1997; Barnhart et al., 1998). Boundy et al. (1998) showed that these treatments elevate cAMP and result in the downregulation of R subunits. The downregulation also was blocked by lactacystin, suggesting that regulated proteolysis by proteasomes is important in the formation of drug addiction.

The abrupt loss of R subunits during LTF is analogous to the programmed degradation of the cyclin-dependent kinase (CDK) inhibitor Sic1. Active CDK is necessary for DNA replication and cell division. To exit from the G1 phase of the cell cycle, Sic1 must be degraded to produce active CDK. Sic1 is degraded through the ubiquitin-proteasome pathway (Feldman et al., 1997; Verma et al., 1997; Nishizawa et al., 1998). The role of the PKA catalytic subunit during the formation of LTF is similar to that of CDK because its activation drives the gene expression required for LTF induction forward. R subunits block the activity of the kinase just as Sic 1 inhibits CDK.

The ubiquitin-proteasome pathway also mediates the programmed degradation of cyclin at specific stages of the cell cycle (Glotzer et al., 1991; Peters et al., 1998b). Cyclin is the activator of CDK. Activation of CDK drives the cell cycle: the inactivation of the kinase allows the cell to exit from mitosis and complete the cycle. Although the ubiquitin-proteasome pathway controls the action of both CDK and PKA through the degradation of their respective regulatory subunits, the biochemical result is different. The ubiquitin pathway *activates* PKA through degradation of its inhibitory R subunits. In contrast, ubiquitin-mediated degradation of cyclin, which is an activator of CDK, *inactivates* CDK.

How the Ubiquitin-Proteasome Pathway Can Be Regulated

The ubiquitin-proteasome pathway is said to be regulated because degradation frequently but not always occurs under specific conditions. Ubiquitin-mediated proteolysis can be regulated in four general ways: (1) by modifying the protein substrate; (2) by altering the activity of E2/E3s; (3) by modulating proteasome activity; and (4) by controlling the supply of the free ubiquitin available for conjugation/ligation.

Modifying the Substrate

Normally stable proteins that become damaged through wear and tear during stress or starvation were once thought to be the chief kind of substrate for ubiquitin-proteasome mediated degradation. Two other kinds of proteins are degradable. The first kind is needed only during specific phases of a sequential process, such as the cell cycle. At the appropri-

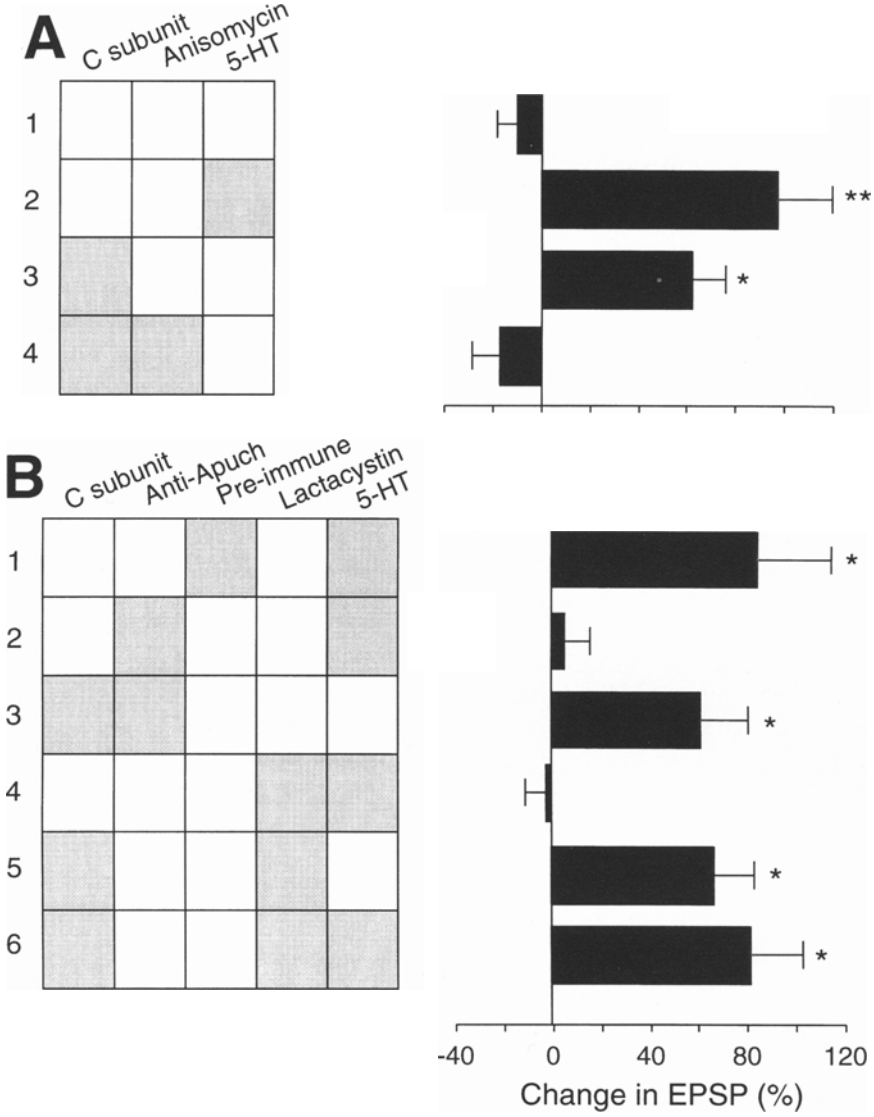

Fig. 3. The ubiquitin-proteasome pathway is critical only for producing a persistently active PKA. Intracellular injection of C subunits into sensory neurons results in LTF. If ubiquitin-proteasome-mediated degradation serves critically to produce the autonomous protein kinase, the block of LTF produced by agents that inhibit proteasome action (lactacystin and antibody against Ap-uch) should be circumvented by injecting C subunits. Changes in EPSP amplitude (mean ± SEM) at 24 h produced by the treatments indicated on the grid to the left by gray squares are summarized as bar graphs. **(A)** (1) Unstimulated neurons (no 5-HT treatment, $n = 24$); (2) LTF produced by five pulses of 5-HT ($n = 8$). (3) Injection of C subunits alone ($n = 22$); (4) Injection of C subunits in the presence of anisomycin (10 μM; added 1 h before the start of the experiment; $n = 8$). (This experiment shows that the LTF-produced by injecting C subunits is dependent on new protein synthesis and is not the result of residual kinase remaining in the neuron 24 h after the injection.) Injection of C subunits circumvents the effects of the inhibitors of the ubiquitin-proteasome pathway. **(B)** (1) Injection of pre-immune serum (control) followed by 5 pulses of 5-HT ($n = 6$); (2) Injection of Ap-uch antibody followed by 5 pulses of 5-HT ($n = 8$); (3) Ap-uch antibody injected together with C subunits ($n = 13$); Lactacystin (5 μM) injected before the treatment with 5-HT ($n = 7$); (5) Injection of C subunits together with lactacystin (n = 8); (6) Injection of C subunit together with lactacystin followed by five pulses of 5-HT ($n = 10$). A single asterisk (*) indicates $p < 0.05$; (**), $p < 0.01$ (from Chain et al., 1999).

ate time, these proteins are degraded abruptly and rapidly. Examples are transcription factors, whose action is needed only briefly. Thus Ap-C/EBP is expressed in *Aplysia* sensory neurons during the induction of LTF within 2 h of the start of stimulation. This factor presumably activates the expression of a distinct set of effector proteins, and is then degraded through the ubiquitin-proteasome pathway 2–4 h later (Yamamoto et al., 1999). Several other transcription factors, such as c-myc, c-fos, and c-jun, are regulated by ubiquitin-mediated degradation (Hochstrasser and Kornitzer, 1998). A second kind of protein also in normally stable but must be downregulated to effect sudden and irreversible changes in cell function. An example is the R subunit of PKA during LTF. R subunits are generally very stable. In unstimulated *Aplysia* sensory neurons, they have a half-life of several weeks (Pei, 1998). It is only after the prolonged stimulation used to produce LTF that R subunits are degraded.

There are several ways of changing a stable protein into a substrate for ubiquitin-mediated degradation. Phosphorylation is the major post-translational modification that makes proteins susceptible to ubiquitination. A well-studied example is cytokine-induced phosphorylation of IκB by a dedicated IκB kinase (Alkalay et al., 1995; Chen and Maniatis, 1998; Heilker et al., 1999). Like the R subunit, IκB is a negative regulator of NFκB and related Rel family transcription factors, which control the expression of genes that function in inflammation, cell proliferation, and apoptosis (Verma et al., 1995; Baldwin 1996; May and Ghosh, 1998). Phosphorylation causes IκB to dissociate from NFκB making the inhibitor susceptible to ubiquitination by the Skp1-cullin-F-box protein (SCF) complex, a recently discovered class of E3s (Krek, 1998; Kroll et al., 1999; Peters et al., 1998b; Tyers and Jorgensen, 2000). The released NFκB moves into the nucleus to activate transcription (Zhong et al., 1997). These events are similar to the release of the C subunit from the PKA holoenzyme and its translocation to the nucleus during the induction of LTF. While PKA C subunits are imported into the nucleus where they are needed to initiate and maintain gene expression, inhibitory R subunits, like IκB, remain in the cytosol, where they are degraded (Chain et al., 1999). Altered subcellular localization of a substrate protein can also control ubiquitin-ligation. Thus, the cyclin-dependent kinase inhibitory protein p27Kip1 is translocated from the nucleus to the cytosol, where it is rapidly degraded (Tomoda et al., 1999).

Like IκB, R subunits become susceptible to degradation through the receptor-mediated changes in the conformation of the molecule. In response to the neurotransmitter 5-HT, cAMP is elevated in the sensory neurons. R subunits with bound cAMP are substrates for ubiquitination and subsequent degradation. Both tandem binding sites must be occupied: R subunits mutant in cAMP-binding sites are not efficiently degraded. This requirement for cAMP suggests that a specific allosteric change in the molecule unmasks some recognition element for an E2/E3 pair in R subunits when cAMP binds. The role of allosteric change in causing susceptibility is illustrated by the inducible degradation of the photo-period protein, phytochrome (Shanklin et al., 1987). In its dark form, the phytochrome has a half-life of about 100 h; in the light, the photo-converted protein is degraded with a half-life of 1 h. Without bound cAMP, R subunits are quite stable, whereas with cAMP elevation in the sensory neurons, they are degraded within 2 h (Chain et al., 1999).

How are R subunits made susceptible to ubiquitination? Because of the allosteric change, some previous covered domain becomes accessible to the E2/E3 complex specific to R subunits. As with many short-lived proteins, the N terminus of R subunits from all animals contains a PEST region enriched in proline (P), glutamate (E), serine (S), and threonine (T) (Bergold et al., 1992; Chain et al., 1995; Takio et al., 1984; Clegg and McKnight, 1988; Kalderon and Rubin, 1988). PEST sequences are thought to target proteins for rapid destruction either as constitutive or as

conditional signals (Rogers et al., 1986; Rechsteiner and Rogers, 1996). The Clip-peptide (R with about 100 amino-acid residues removed from the N-terminus) that is produced by conventional proteases from all R subunits nonphysiologically during extraction of the enzyme from tissue lacks the PEST region. With *Aplysia* Rs, we find the Clip-peptide is quite stable, suggesting that PEST may be involved in R subunit degradation. Phytochrome is thought to be made susceptible to ubiquitination because its PEST region unmasked when light is absorbed (Reschsteiner, 1990).

Substrate modification also appears to be important for making proteins resistant to degradation. Presumably, for these proteins the modified molecule assumes a conformation that is unsuitable for ubiquitination. Even though many transcription activators are substrates for the ubiquitin-proteasome pathway, these regulatory proteins must be protected to initiate and sustain gene expression. Thus, the product of the *jun* gene is stabilized by MAP kinase phosphorylation (Musti et al., 1997). During induction of LTF, MAP kinase phosphorylation both stimulates the DNA-binding activity of Ap-C/EBP and renders it resistant to degradation, thereby prolonging its activity (Yamamoto et al., 1999).

Regulating Ubiquitination

A major control point for ubiquitin-proteasome-mediated degradation is the modulation of E2/E3s that ubiquitinate the substrate protein. One mechanism is through activation of E3s by phosphorylation and by association with other protein factors (adaptor proteins). The anaphase-promoting complex (APC), which controls the transition from metaphase to anaphase in mitosis, appears to be regulated by both mechanisms. APC is the complex that serves as the E3 for Pds1, a protein that inhibits the metaphase-to-anaphase transition in baker's yeast (Cohen-Fix et al., 1996). APC also acts as E3 for cyclin B ubiquitination at the end of mitosis. Ubiquitination of Pds1 by APC is regulated by the binding of a protein factor called Cdc20. Ligation of ubiquitin to cyclin B on the other hand requires binding of Hct1/Cdh1 to APC (Schwab et al., 1997); Visintin et al., 1997). Hct1/Cdh1 binding in turn is regulated by CDK-mediated phosphorylation. Phosphorylated Hct1/Cdh1 cannot bind to the APC and activate it (Jasperson et al., 1999; Zachariae et al., 1998). An additional layer of regulation is the control of APC activity by phosphorylation of its subunits. Cdc2-cylcin B kinase was shown to activate APC through reversible phosphorylation (King et al., 1995; Lahav-Baratz et al., 1995; Sudakin et al., 1995; Peters et al., 1996). A possible downstream component of Cdc2-cyclin B has been identified as the mammalian homolog of *Drosophila* Ser/Thr kinase (Polo), which mediates the effect of Cdc2-cylcin B (Kotani et al., 1998). Negative regulation of APC also occurs. For example, PKA inactivates APC (Yamashita et al., 1996). It would be interesting to see whether PKA phosphorylation has any role in ligating ubiquitin to R subunits or other substrates in neurons.

The requirement for an adaptor protein for ubiquitin ligation is illustrated by the degradation of the tumor-suppressor protein, p53 (Huibregtse et al., 1998). p53 is degraded in cells infected with papilloma virus. E6, a virus-specific protein forms a complex with E6-AP, a constitutive E3, that is capable of ligating p53 and ubiquitin. E6-AP alone is inactive with p53, but can serve as an E3 with other protein substrates. We have not had any indication for an adaptor protein in the ubiquitination of R subunits.

Another mechanism for regulating conjugation/ligation is the induction of specific E3 ubiquitin ligases. Thus far, the only example described is that of E3α, a ligase necessary for ubiquitination of proteins with basic, acidic, or large hydrophobic N-termini (N-end rule pathway; Varshavsky, 1996). E3α is rate-limiting during the protein breakdown that accompanies muscle atrophy (Solomon et al., 1998a, 1998b). Increased ubiquitination during muscle wasting that occurs during insulin defi-

ciency was correlated with an increase in E3α mRNA (Lecker et al., 1999). Unless extensive modification of its N-terminus takes place, degradation of the R subunit is unlikely to occur by the N-end rule pathway because it has an initiator methionine.

Regulation at the Proteasome Step

Proteasome activity can be regulated by the particular subunits that are part of the 20 S core or 19 S caps or by extrinsic proteins that associate with them, such as ubiquitin C-terminal hydrolases. The activities of proteasomes vary in response to the physiological condition of the cell. The capacity to degrade ubiquitinated proteins can be regulated by changes in: (1) proteasome concentration, (2) subunit composition, and (3) subcellular distribution. Increases in the amount of proteasomes have been documented in many physiological and pathological conditions (DeMartino and Slaughter, 1999). For example, during metamorphosis of the moth *Manduca*, flight muscles develop and intersegmental muscles are destroyed. The destruction of intersegmental muscles is brought about by an increased ubiquitin-dependent proteolysis as a result of extensive hormone-dependent reprogramming of the 19 S cap (Dawson et al., 1995; Takayanagi et al., 1996) that accompanies changes in ubiquitin-conjugating enzymes (Haas et al., 1995). It has been shown that the multi-ubiquitin binding subunit (S5a/MBP) and the ATPases MSS1 and S4 are induced during metamorphosis (Dawson et al., 1995; Takayanagi et al., 1996). Enhanced association of 19 S and 20 S particles results in enhanced proteasome activity (without an increase in the amount of proteasome subunits) during the transition from metaphase to anaphase in the ascidian meiotic cell cycle (Kawahara and Yokosawa, 1994). An overall increase in proteasome concentration was also observed during atrophy of skeletal muscle (Mitch and Goldberg, 1996).

Proteolysis by proteasomes can also be modulated by changes in the composition of its core subunits. For example, production of MHC Class I antibodies is regulated by various immunomodulatory cytokines that cause the induction of proteasome subunits (Driscoll et al., 1993; Gaczynska et al., 1993). Upon induction, these subunits assemble into newly synthesized proteasomes with altered catalytic characteristics. The activity of the proteasome can also be regulated by alterations in subcellular distribution. For example, during ascidian embryonic development, the distribution changes in a cell-cycle dependent manner. Proteasomes in the nucleus during interphase disappear during prophase; in telophase they reappear in the newly formed nucleus (Kawahara and Yokasawa, 1992).

Proteasome activity can also be controlled by enzymes that disassemble multi-ubiquitin chains. Accumulated polyubiquitin chains inhibit proteasome activity. Disassembly of multi-ubiquitin chains, which is rate-limiting, is regulated by de-ubiquitinating enzymes. These enzymes, which are cysteine proteases, consist of two categories: (1) ubiquitin C-terminal hydrolases (UCHs), and (2) ubiquitin-specific proteases (UBPs). UCHs are low molecular-weight (around 30,000 MW) proteins that cleave multi-ubiquitin chains from small peptides. UBPs, on the other hand, are heavier (around 100,000 MW) proteins that generally disassemble the multi-ubiquitin chain. When proteasomes degrade substrate proteins to small peptides, the peptide remnants attached to the multi-ubiquitin chain are cleaved by UCHs. The multi-ubiquitin chain is then is disassembled by UBPs. UCH function has been extensively studied in vitro (Wilkinson, 1997; Larsen et al., 1998). The only physiological example appears to be Ap-uch, which has been shown to act on the first ubiquitin attached to the substrate protein (Hegde et al., 1997). Mammalian isopeptidase-T and UBP Y are examples of UBPs that disassemble multi-ubiquitin chains (Wilkinson et al., 1995; Amerik et al., 1997). There is overlap in function between UCHs and some UBPs: for example, DOA4, a high molecular-weight enzyme, has been shown to hydrolyze the isopeptide

bond between the first ubiquitin in multi-ubiquitin chains attached to peptide remnants (Papa and Hochstrasser, 1993). Some isopeptidases may have an editing function at the proteasome step: Lam et al. (1997) suggested that a bovine isopeptidase associated with the proteasome removes ubiquitins from poorly ubiquitinated substrates, thus preventing their degradation.

UCHs can be expressed differentially during growth, development (for examples, *see* Huang et al., 1995; Naviglio et al., 1998), and oncogenesis (Papa and Hochstrasser, 1993; Gupta et al., 1994). Hadari et al. (1992) and Eytan et al. (1993) suggested that multi-ubiquitinated peptide remnants can clog proteasomes and slow the degradation process. Hydrolases associated with the proteasome are thus needed for efficient proteolysis (Papa et al., 1999). In accord with this idea, Hegde et al. (1997) showed that the proteolytic activity of *Aplysia* proteasomes is greatly enhanced by the induction of UCH, Ap-uch, a homolog of a vertebrate neuron-specific L1 hydrolase (Wilkinson et al., 1989). This enzyme is encoded by one of the two immediate early genes now known to be induced during LTF of *Aplysia* sensory neurons. The induction of the enzyme early in the formation of LTF is necessary for the degradation of R subunits. The expression of the hydrolase is coincident with the stimulus-induced elevation of cAMP. The resulting alteration in R subunit conformation when cAMP is bound coincident with the enhanced proteolytic activity of proteasomes enhanced by the hydrolase provides the necessary conditions for degrading R subunits.

The *Aplysia* hydrolase is specific to neurons and associates with proteasomes, remaining bound to the 26 S proteasome particle during differential centrifugation. As was found with other hydrolases, Ap-uch enhances protein degradation in vitro, presumably by catalyzing the removal of ubiquitin moities from ubiquitinated substrates (Eytan et al., 1993; Wilkinson, 1997; Hegde et al., 1997; Papa et al., 1999) (Fig. 4). Other proteins have been implicated in enhancing proteasome activity. Gonen et al. (1996) reported that elongation factor 1α (eEF1A) increases the degradation of N-acetylated protein, presumably by its isopeptidase activity. eEF1A, which is induced during LTF in *Aplysia* (Hegde et al., 1999), is a GTP-binding protein but with no similarity to the isopeptidases. If eEF1A has a role in protein degradation, it might be to enhance proteolysis by threading unfolded substrate proteins into the catalytic core of the proteasome.

Controlling the Supply of Free Ubiquitin Available for Conjugation/Ligation

Because free ubiquitin is needed for ubiquitinating substrate proteins, blockage of recycling would ultimately stop degradation. The hydrolases are important to the ubiquitin-proteasome pathway because the hydrolysis of multi-ubiquitin chains recycles ubiquitin. A diminished free-ubiquitin pool can also be replenished by the synthesis of the polyubiquitin precursor. Thus, during the programmed death of insect muscle, the rate of ubiquitination is regulated by the synthesis of polyubiquitin (Haas et al., 1995). Induction of polyubiquitin also occurs during denervation-induced atrophy of skeletal muscle (Medina et al., 1995). In *Aplysia* sensory neurons, however, the free-ubiquitin pool is not limiting (Chain et al., 1995) and polyubiquitin genes are not induced during LTF (Hegde et al., 2000). Therefore, an adequate supply of free ubiquitin is produced by recycling.

Conclusion

LTF of the sensory-motor neuron synapses occurs with repeated sensory stimuli. For facilitation to take place, genes must be induced and new protein synthesized. A molecular mechanism that commits the sensory neuron to the formation of LTF is the production of a persistently active PKA, which activates the first key transcription factor CREB. A persistently active PKA results from the ubiquitin proteasome-mediated degradation of R sub-

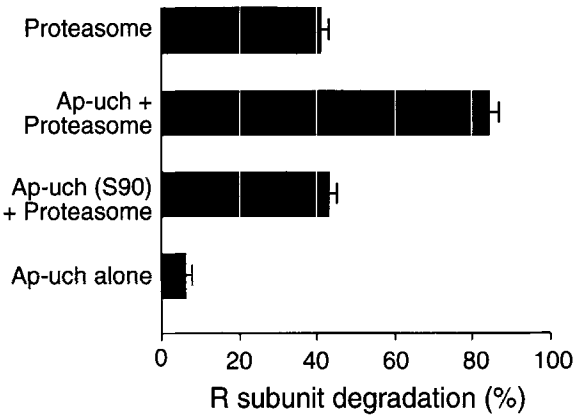

Fig. 4. *Aplysia* Ubiquitin C-terminal hydrolase (Ap-uch) enhances degradation by the proteasome. Ap-uch, a neuron-specific protein, is induced early during LTF. The enzyme binds to the proteasome and increases disassembly of multi-ubiquitin chains. To see if Ap-uch contributes to the increase in degradation of R subunit of PKA, proteasomes were prepared from *Aplysia* buccal muscle, which do not express Ap-uch. Degradation of 8-N_3-[^{32}P]cAMP-labeled *Aplysia* R subunit (N4) was assayed in the presence of affinity-purified, recombinant wild-type or mutant (S90) Ap-uch. After sodium dodecyl sulfates polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, the extent of degradation was determined by densitometry. Significant ($p < 0.01$ paired *t*-test) enhancement of degradation of N4 was observed when wild-type Ap-uch was added. The mutant hydrolase (S90) had no effect. No degradation was seen in incubations with the wild-type enzyme in the absence of the proteasome (Ap-uch alone). Values are mean ± SEM, $n = 4$.

units. At least two conditions must overlap for the degradation: elevation of cAMP and expression of Ap-uch. Stimulation of sensory neurons causes intracellular cAMP to increase. The resulting activation of PKA leads to phosphorylation of CREB and the induction of immediate early genes, one of which is Ap-uch. Induction of Ap-uch occurs within the narrow window in which cAMP is still elevated, resulting in limited R subunit degradation. The persistently active PKA is likely to produce continued CREB-mediated gene expression and keep important synaptic proteins phosphorylated.

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