Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7436–7440, August 1993
Neurobiology

Regulatory subunits of cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: A molecular mechanism underlying long-term synaptic plasticity

ASHOK N. HEGDE*, ALFRED L. GOLDBERG†, AND JAMES H. SCHWARTZ*

*Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, 722 West 168th Street, New York, NY 10032; and †Department of Cellular and Molecular Physiology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115

Communicated by Gerald D. Fischbach, May 20, 1993

ABSTRACT **In *Aplysia*, behavioral sensitization of defensive reflexes and the underlying presynaptic facilitation of sensory-to-motor neuron synapses lasts for several minutes (short term) or days to weeks (long term). Short-term sensitization has been explained by modulation of ion-channel function through cAMP-dependent protein phosphorylation. Long-term facilitation requires additional molecular changes including protein synthesis. A key event is the persistent activation of the cAMP-dependent protein kinase at baseline concentrations of cAMP. This activation is due to selective loss of regulatory (R) subunits of PKA without any change in catalytic (C) subunits. To understand the molecular mechanisms that produce the loss of R subunits in long-term facilitation, we investigated how R subunits are degraded in extracts of *Aplysia* nervous tissue and in rabbit reticulocyte lysates. Degradation of *Aplysia* R subunits requires ATP, ubiquitin, and a particulate component that appears to be the proteasome complex. Degradation is blocked by hemin, which causes the accumulation of high molecular weight derivatives of R subunits that are likely to be ubiquitin conjugates of R subunits and intermediates in the degradative pathway. We also show that vertebrate R_I and R_{II} subunits can be degraded through the ubiquitin pathway. We suggest that degradation is initiated by cAMP, which causes the holoenzyme to dissociate and, further, that the altered R-to-C ratio in *Aplysia* sensory neurons is maintained in long-term facilitation by newly synthesized proteins that help target R subunits for accelerated degradation.**

In sensory-to-motor neuron synapses in *Aplysia*, cAMP-dependent protein kinase (PKA) plays an important role in the presynaptic facilitation that underlies both short-term (1, 2) and long-term (3, 4) sensitization of defensive reflexes. In short-term facilitation, PKA modulates the function of ion channels directly (2, 5). Several additional changes are needed to produce long-term facilitation, however, including protein synthesis (6, 7). A key molecular event is the persistent activation of PKA in sensory neurons at basal concentrations of cAMP. Greenberg *et al.* (8) and Bergold *et al.* (9) showed that the amounts of regulatory (R) subunits of PKA are diminished in trained animals or after treatments that produce long-term facilitation in isolated ganglia. Since the amount of catalytic (C) subunits is not altered under these conditions, the loss of R subunits should lead to enhanced protein phosphorylation for prolonged periods (8, 9). Earlier studies suggested that loss of R subunits results from accelerated proteolysis rather than from reduced synthesis (8–10). We therefore examined how R protein is degraded in *Aplysia* neurons.

Aplysia nervous tissue contains five isoforms of R, designated N_1–N_5 (11). The structures of *Aplysia* R subunits have been examined biochemically (12, 13) and by molecular cloning (10). Antisera raised against recombinant N_4 cross-react with all the other isoforms except N_2, which might be similar to vertebrate R_{II} (10, 13). The other isoforms, N_1, N_3, N_4, and N_5, although distinctly different molecules, resemble each other and are similar to vertebrate R_I (10).

Both *Aplysia* N_4 and vertebrate R subunits contain two N-terminal domains that are likely to be important for determining susceptibility to proteolysis (14). One domain consists of ≈30 aa and is enriched in proline, glutamate, serine, and threonine (PEST) residues (10, 14). Rechsteiner and coworkers (15) have shown that PEST domains are frequently found in proteins that are subject to rapid degradation. The other domain is the hinge region, which is immediately adjacent to the PEST domain. It is particularly sensitive to cleavage *in vitro*, probably because it contains pairs of dibasic amino acids (10, 14). In extracts, proteolysis at this site is inhibited by agents that block trypsin-like serine proteases and calpains (13).

The loss of R subunits during facilitation of *Aplysia* sensory neurons could be caused by decreased synthesis or by increased degradation. Since Bergold *et al.* (10) did not detect a decrease in the synthesis or processing of the mRNA encoding N_4, the neuronal R isoform, accelerated proteolysis appears more likely. Even though free R-subunit proteins are susceptible to proteolytic attack at the hinge region (14), Schwartz and Greenberg (4) noted that degradation of R subunits in sensory neurons does not generate the smaller cAMP-binding fragments, which are produced by limited proteolysis at the hinge region. Also, preliminary experiments with permeabilized *Aplysia* synaptosomes did not support the idea that loss of R subunit protein from sensory neurons *in vivo* results from proteolytic cleavage at the hinge region (4). On the other hand, several regulatory proteins that are under physiological control appear to be degraded by the ATP-ubiquitin-dependent proteolytic pathway (16). In this pathway, proteins to be degraded are conjugated to many molecules of ubiquitin by a series of ATP-dependent coupling enzymes (16, 17). Multiubiquitination marks the polypeptide for rapid hydrolysis by a 26S (1500 kDa) complex that requires ATP and contains the proteasome [multicatalytic protease complex (18, 19)]. In the present study, we tested whether the differential breakdown of R subunits is catalyzed by this ATP-ubiquitin-requiring pathway.

MATERIALS AND METHODS

Aplysia californica weighing 70–100 g were obtained from the Mariculture Resource facility at the University of Miami (Miami, FL). Neural components were dissected from the connective tissue sheath of ganglia (9).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PKA, cAMP-dependent protein kinase; R subunit, regulatory subunit; C subunit, catalytic subunit; DTT, dithiothreitol; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate; PEST, proline, glutamate, serine, and threonine.

R Subunits. Bovine R_I subunits (20) (Sigma) were affinity-purified using cAMP-agarose (21). R_{II} subunits were from Promega. Recombinant N_4, expressed in Sf9 cells in a baculovirus system and purified to homogeneity, was provided by Hagan Bayley and Stephen Cheley (Worcester Foundation for Experimental Biology, Shrewsbury, MA).

Photoaffinity Labeling. Neural components were homogenized in glass–glass tissue grinders (Micrometric Instruments, Tampa, FL) in 150 mM NaCl/3 mM EGTA/0.5 mM 2-mercaptoethanol/5 mM benzamidine/50 mM Tris·HCl, pH 7.6. We labeled R subunits in homogenates of neural components by incubation with 8-N_3-[^{32}P]cAMP (ICN; 60–70 Ci/mmol; 1 Ci = 37 GBq) in the buffer for 90 min in the dark at room temperature and by subsequent photolysis for 5 min at a wavelength of 254 nm as described (8). Purified R subunits were labeled by incubation for 10 min on ice.

Degradation Assays. *Aplysia* extracts [10 μg of protein; estimated by the Bradford method (22)] containing the five photoaffinity-labeled R subunits were incubated in rabbit reticulocyte lysate (50 μl; Promega)/5 mM dithiothreitol (DTT)/10 mM $MgCl_2$/10 mM KCl/50 mM Tris·HCl, pH 8, in a total volume of 100 μl. Before use, reticulocyte lysates were dialyzed against 8 mM KCl/4 mM $MgCl_2$/0.5 mM DTT/20 mM Tris·HCl, pH 7.6 (23). In some experiments, we added 0.5 mM ATP, 10 mM creatine phosphate, and creatine phosphokinase (Sigma; 100 μg/ml) as an ATP regenerating system. Where indicated, action of ATP was inhibited by omitting $MgCl_2$ and adding 10 mM EDTA or by adding 4 mM adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA) (Boehringer Mannheim). In some instances, we blocked the action of ubiquitin by including 10 μg of methylated ubiquitin prepared by treating bovine ubiquitin (Sigma) with 12 mM formaldehyde and 20 mM sodium cyanoborohydride (24). After incubation for 3 h at 37°C, reactions were stopped by adding 50 μl of 3× SDS/PAGE (25) sample buffer [2% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol/10% (vol/vol) glycerol/0.001% bromophenol blue/6.25 mM Tris·HCl, pH 6.8] and heating for 3 min at 98°C. Degradation was assayed by the disappearance of the labeled R subunits on one-dimensional gels. [Although five labeled isoforms can be separated by two-dimensional gel electrophoresis, these appear as three distinct bands on SDS/PAGE because N_2 migrates with N_5 (M_r, 47,000), N_3 migrates with N_4 (M_r, 52,000), and N1 migrates separately (M_r, 105,000) (11–13).] We used scanning densitometry (Hoefer) of autoradiographs to measure the intensity of bands corresponding to the R subunits. In some experiments, the proteasome complex (18, 19) was removed from lysates by centrifugation at 100,000 × *g* for 6 h in the presence of 2 mM ATP/5 mM $MgCl_2$ (ref. 26; N. Tawa E. Folco, and A.L.G., unpublished data).

For degradation experiments in *Aplysia* extracts, ganglia were homogenized in 8 mM KCl/4 mM $MgCl_2$/0.5 mM DTT/20 mM Tris·HCl, pH 7.6. The homogenate was centrifuged at 3000 × *g* for 2 min. In some experiments, to remove the proteasome, the supernatant was further centrifuged at 100,000 × *g* for 6 h in the presence of 2 mM ATP/5 mM $MgCl_2$ (ref. 26; N. Tawa *et al.*, unpublished data). Photoaffinity-labeled *Aplysia* nervous tissue R subunits (5 μg of total protein) were incubated for 3 h at 15°C in 100 μl containing the supernatant fraction (200 μg protein), 5 mM DTT, 10 mM $MgCl_2$, and 50 mM Tris·HCl (pH 8); 3 h is the time of incubation usually used in previous studies (27, 28).

Ubiquitination. Conjugation assays were carried out with a fraction from reticulocyte lysate, which is depleted of ubiquitin, called fraction II [225 μg of protein; prepared according to Ciechanover *et al.* (29)] in 100 μl containing 5 mM DTT, 10 mM $MgCl_2$, 5 mM ATP, ATP-regenerating system, and 50 mM Tris·HCl (pH 8) for 1 h at 37°C. We added 2 μg of ubiquitin where indicated. To observe formation of R-subunit-ubiquitin conjugates, we added hemin (Sigma, 100 μM) because it inhibits proteolysis without affecting conjugation of ubiquitin to proteins (30–32). In some experiments, ATP was depleted from fraction II by prior treatment (20 min at 37°C) with apyrase (Sigma, 5 units/ml).

RESULTS

Degradation of R Subunits by the Ubiquitin-ATP-Proteasome Pathway. The soluble ATP-ubiquitin pathway has been characterized most thoroughly in rabbit reticulocyte lysates (16, 17, 31). In these lysates, radiolabeled *Aplysia* R subunits, both recombinant N_4 and from crude extracts, were degraded during incubation for 3 h at 37°C (Figs. 1*A* and *B* and 2*A*). In the ubiquitin pathway, both the conjugation of the protein substrate to ubiquitin and its subsequent hydrolysis require ATP (16, 17). We therefore tested whether degradation of *Aplysia* R subunits also depends upon ATP. R subunits were degraded only when Mg^{2+}-ATP was present (Figs. 1 *A* and *B*, lanes 3, and 2*A*), and degradation was inhibited by EDTA or by the addition of the nonhydrolyzable ATP-analog p[CH_2]ppA (Figs. 1 *A* and *B*, lanes 2 and 4, and 2*A*). Ubiquitin conjugates are degraded by the 26S proteasome complex (18, 19, 33). Because of its size, this structure can be removed by ultracentrifugation (ref. 26; N. Tawa *et al.*, unpublished data). After the lysates had been ultracentrifuged, the degradation of R subunits was greatly dimin-

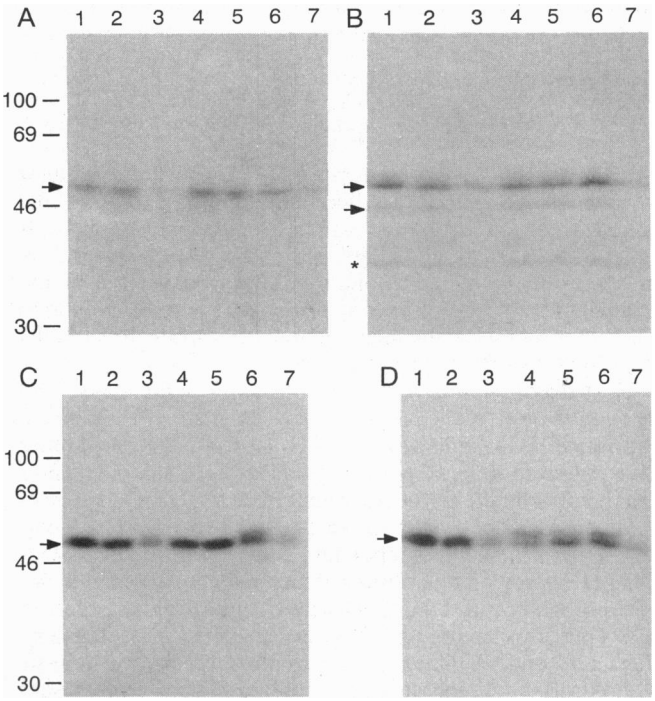

FIG. 1. Requirements for degrading *Aplysia* and vertebrate R subunits in rabbit reticulocyte lysates. (*A*) Recombinant *Aplysia* N_4 (20 ng). (*B*) *Aplysia* nervous tissue R subunits (10 μg of total protein). Arrows point to M_r 52,000 and M_r 47,000 R subunits. The M_r 105,000 protein binds very little 8-N_3-[^{32}P]cAMP and becomes visible only after long exposures. Asterisk, M_r 37,000 proteolytic product of R subunits that results from the cleavage at the hinge region. (*C*) Rabbit muscle R_I subunits (50 ng). (*D*) Bovine heart R_{II} subunits (50 ng). R subunits were ^{32}P-affinity-labeled and incubated in the lysates; after 3 h, samples were separated by SDS/PAGE, and the gels were autoradiographed. Lanes: 1, sample before 3-h incubation; 2, 10 mM EDTA; 3, 0.5 mM ATP; 4, 0.5 mM ATP/4 mM p[CH_2]ppA; 5, 0.5 mM ATP/10 μg of methylated ubiquitin; 6, 0.5 mM ATP in a lysate depleted of proteasome by ultracentrifugation (100,000 × *g* for 6 h); 7, 0.5 mM ATP, sedimented pellet containing proteasomes (0.5 mg of total protein) resuspended in reticulocyte supernatant. Molecular weight markers (×10^{-3}) are indicated at left of *A* and *C*.

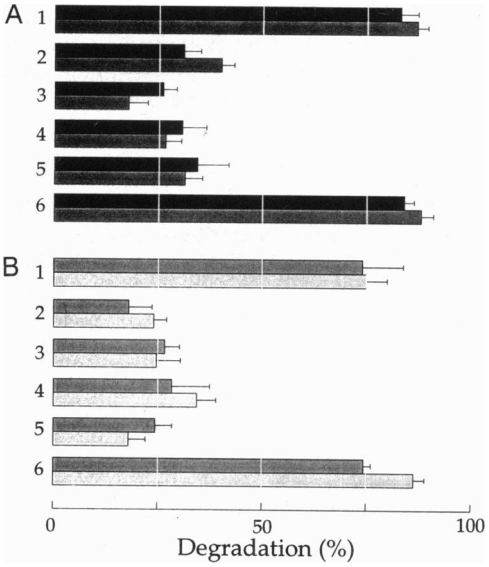

FIG. 2. R-subunit degradation in reticulocyte lysates at 3 h. Values for the percent degradation are the means ± SEM of three experiments similar to those presented in Fig. 1 *B–D* in which the amounts of R subunits before a 3-h incubation are set at 100%. (*A*) *Aplysia* nervous tissue R subunits (upper bar, M_r 52,000 subunit; lower bar, M_r 47,000 subunit). (*B*) Vertebrate subunits (upper bar, R_I; lower bar, R_{II}). Pairs of bars: 1, 0.5 mM ATP; 2, 10 mM EDTA; 3, 0.5 mM ATP plus 4 mM p[CH_2]ppA; 4, 0.5 mM ATP plus 10 μg of methylated ubiquitin; 5, 0.5 mM ATP plus reticulocyte lysate depleted of proteasomes by ultracentrifugation (100,000 × *g* for 6 h); 6, 0.5 mM ATP plus sedimented pellet containing proteasomes (0.5 mg of total protein) resuspended in reticulocyte supernatant.

ished, even though ATP was present (Figs. 1 *A* and *B*, lanes 6, and 2*A*). When the pellet containing the proteasome complex was added back to the lysate supernatants, R subunits were degraded (Figs. 1 *A* and *B*, lanes 7, and 2*A*). In addition, methylated ubiquitin, which lacks free amino groups and, therefore, does not support multiubiquitination (24), also inhibited the degradation of *Aplysia* R subunits (Figs. 1 *A* and *B*, lanes 5, and 2*A*).

The structure of the R subunits of PKA is highly conserved through evolution (14). Much of the sequence of the *Aplysia* R isoform N_4 is similar to mouse R_I (10). We tested whether the vertebrate R_I and R_{II} are degraded in reticulocyte lysates in a manner similar to the *Aplysia* R isoforms. Degradation of commercially obtained purified R_I and R_{II} was ATP-, ubiquitin-, and proteasome-dependent (Figs. 1 *C* and *D* and 2*B*). Furthermore, we often observed high molecular weight components when p[CH_2]ppA was used to block the action of ATP (Fig. 1*D*, lane 4) and in lysates depleted of proteasome (Fig. 1 *C* and *D*, lanes 6). p[CH_2]ppA blocks proteolysis without affecting conjugation of ubiquitin to a protein substrate (16, 17, 34). Since the high molecular weight components appear under conditions in which the proteolytic step is selectively blocked, these extra components are likely to be ubiquitin conjugates of R subunits.

Although we found that R subunits were degraded by the ubiquitin-ATP-proteasome pathway in reticulocyte lysates, if this pathway is actually used to produce long-term facilitation in sensory neurons, it is important to show that this pathway operates in *Aplysia* nervous tissue. We observed that the *Aplysia* R subunits in extracts of nervous tissue were degraded in the presence of ATP and that their hydrolysis was inhibited by p[CH_2]ppA, EDTA, and methylated ubiquitin. Furthermore, as in reticulocyte lysates, the degradation was inhibited when proteasomes were removed by ultracentrifugation and was restored by adding back the sedimented proteasome (Table 1).

Table 1. Degradation of ^{32}P-photoaffinity-labeled *Aplysia* R subunits in extracts of nervous tissue

Condition or addition	Degradation, %	
	M_r 47,000	M_r 52,000
Complete mixture	77.7 ± 5.5	73.7 ± 5.9
+ EDTA	31.3 ± 4.8	16.7 ± 1.9
+ p[CH_2]ppA	27.0 ± 7.1	23.3 ± 1.5
+ methylated Ub	38.0 ± 1.7	32.3 ± 1.8
Proteasome removed	36.0 ± 3.5	22.7 ± 1.9
Proteasome restored	84.3 ± 2.2	77.0 ± 2.1

Experiments were carried out for 3 h as described in the text. The complete incubation mixture contained ATP, $MgCl_2$, DTT, KCl, and Tris·HCl, pH 8. Action of ATP was inhibited by adding EDTA (and by omitting Mg^{2+}) or by adding p[CH_2]ppA (to compete with the ATP) to the complete mixture. Methylated ubiquitin (Ub) was used to block the action of ubiquitin competitively. Neural extracts depleted of proteasomes were added to the reaction mixture containing ATP to test the involvement of proteasomes. Reconstitution of the complete extract was achieved by adding back the 100,000 × *g* pellet containing the proteasomes as described in the text. Values are means ± SEM from three experiments. The amount of R subunits at the start of the incubation is set at 100%.

Formation of High Molecular Weight Conjugates of R Subunits. Conjugation of protein substrates to ubiquitin can lead to a series of components of increasing size that on SDS/PAGE have the appearance of the rungs of a ladder as a result of the sequential conjugation of ubiquitin monomers (M_r, 8600). Multiubiquitinated intermediates are usually present only in small amounts, even when their hydrolysis is inhibited with hemin (30, 32). Accordingly, we observed the formation of a regular ascending series of radioactive components on autoradiograms when recombinant N_4 (which had been photoaffinity-labeled with 8-N_3-[^{32}P]cAMP) was added to rabbit reticulocyte fraction II in the presence of ubiquitin, ATP, and hemin (Fig. 3, lane 2). In this case, we found accumulation of a higher proportion of what might be multiubiquitinated intermediates compared to mono- and diubiquitinated intermediates. On long exposures, however, we observed a regularly spaced "ladder" of intermediates (data not shown), suggesting that under these reaction conditions formation of multiubiquitinated intermediates was favored. Formation of a large proportion of high molecular weight form of ubiquitin conjugates without the appearance of lower

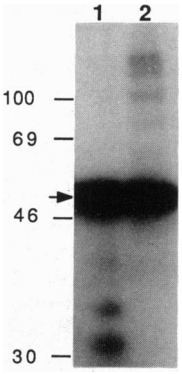

FIG. 3. Formation of high molecular weight components from N_4 in reticulocyte fraction II. Lanes: 1, 8-N_3-[^{32}P]cAMP-labeled N_4 (250 ng); 2, labeled N_4 incubated with fraction II for 1 h in the presence of ubiquitin (2 μg), ATP (5 mM), and hemin (100 μM). On longer exposure of the gel, we observed additional bands immediately above N_4 (arrow) in lane 2 so that the high molecular weight components appeared as a regularly spaced ladder. The two lower bands in lane 1 are either proteolytic products (cleaved at regions other than hinge region) or contaminants present in the preparation of N_4, which become apparent when a large amount of N_4 is applied as sample. Molecular weight markers (×10^{-3}) are indicated at left.

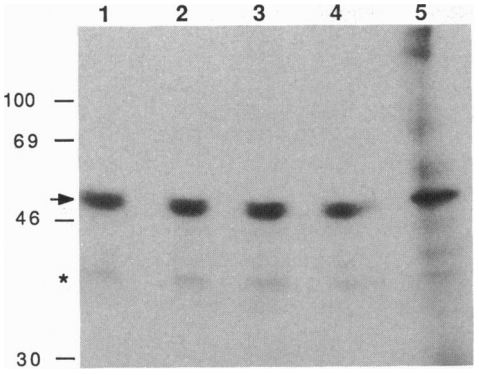

FIG. 4. Ubiquitin- and ATP-dependent formation of high molecular weight components in reticulocyte fraction II. 8-N_3-[^{32}P]cAMP-labeled R_I subunits (100 ng) were added to ATP-depleted fraction II, and the reaction was carried out as indicated. Lanes: 1, sample before the incubation; 2–5, incubation at 37°C for 1 h in the presence of hemin (100 μM); 2, no further addition; 3, ubiquitin (2 μg); 4, ATP (5 mM); 5, ubiquitin (2 μg) plus ATP (5 mM). Asterisk, M_r 37,000 proteolytic product of R_I that results from the cleavage at the hinge region. The two labeled components above this product in lane 5 may be high molecular weight intermediates generated from this peptide by ubiquitination. Molecular weight markers ($\times 10^{-3}$) are indicated at left.

molecular weight intermediates has been observed by Scheffner *et al.* (27) with p53.

Formation of these high molecular weight components is dependent on ubiquitin and ATP. We incubated photoaffinity-labeled vertebrate R_I with reticulocyte fraction II [which is depleted of ubiquitin (29) and of ATP by treatment with apyrase]. In the presence of hemin, the high molecular weight components appeared only in those samples supplemented with ubiquitin and ATP (Fig. 4, lane 5). Neither ubiquitin (Fig. 4, lane 3) nor ATP (Fig. 4, lane 4) alone was sufficient.

The small amounts of high molecular weight components accumulated with R subunits were comparable to those reported for cyclin (35) and phytochrome (36): 3–5% of the initial substrate protein was detectable as ubiquitin conjugates.

DISCUSSION

Selective Degradation of R Subunits. R subunits of PKA are selectively degraded in *Aplysia* sensory neurons within the animal 1 day after training or after treatment of intact isolated ganglia for 2 h with serotonin, a facilitatory neurotransmitter (8, 9). Under these conditions, the most abundant cellular proteins (the tubulins, neurofilament proteins, and actin) are not affected nor are the C subunits of the kinase (4, 8). The 90-min training and the 2-h exposure to serotonin both activate adenylyl cyclase in sensory neurons (1–3), causing the holoenzyme to dissociate (7, 8). This leads to a decrease in R subunits that persists for at least a day. In contrast, treatment of *Aplysia* synaptosomes with permeant analogs of cAMP or with 8-N_3-[^{32}P]cAMP after permeabilization with saponin causes first-order loss of the R subunits. Thus after 24 h, during which the subunits are persistently dissociated in the synaptosomes, only 25% of R subunits remains with no change in the C subunits.

Although *in vitro* the dissociated R subunits are sensitive to a variety of proteases, some of which are present in *Aplysia* nervous tissue, Schwartz and Greenberg (4) failed to show retardation of their degradation in synaptosomes exposed to inhibitors of Ca^{2+}-dependent, serine, acid, or thiol proteases. Earlier work in *Aplysia* sensory neurons strongly suggested that the degradation of R subunits is initiated by the increase in cAMP produced in response to facilitatory neurotransmitters, causing the holoenzyme to dissociate. This has not yet been demonstrated directly, however. Rapid breakdown of free R subunits has also been shown in vertebrate cells (14, 37). Studies in cells in which C subunits are overexpressed indicate that the concentration of R is regulated by the concentration of C subunits (38). In vertebrates, C subunits are believed to protect R subunits because, in the holoenzyme, they mask the hinge region (14), which is particularly susceptible to proteolysis. Nevertheless, the hinge region does not appear to be the site of cleavage *in vivo* at least in *Aplysia* (4).

Bergold *et al.* (10) showed that one neuronal R-subunit isoform of *Aplysia*, N_4, which is closely related to three other neuronal isoforms, to M_1, the major R subunit in muscle, and to the five embryonic isoforms (11–13), contains a domain whose amino acid composition is found in the murine and *Drosophila* R_I homologs. This region, which ends at the phylogenetically conserved pair of dibasic residues (Arg^{93}-Arg^{94}), contains 50% PEST residues (10). Similar domains are present in vertebrate R_{II} subunits (39). Even though no strict correlation between PEST domains and the ubiquitin pathway has been established, Rechsteiner (40) suggested that exposure of cryptic PEST domains might signal degradation.

The following observations implicate the ATP-ubiquitin pathway in the loss of R subunits: (*i*) Degradation of *Aplysia* neuronal R subunits, recombinant N_4, and vertebrate R_I and R_{II} is ATP-dependent in reticulocyte lysates, as is the degradation of R subunits in extracts of *Aplysia* nervous tissue. (*ii*) Breakdown of R subunits is dependent on ubiquitin both in reticulocyte lysates and in the *Aplysia* extracts. (*iii*) Removal by ultracentrifugation of the proteasome from reticulocyte lysates and nervous tissue extracts prevents the degradation of R subunits, and adding back the particulate fraction restores degradation. (*iv*) A series of high molecular weight components—presumably ubiquitin conjugates—appears when recombinant N_4 or R_I is added to reticulocyte fraction II in the presence of ubiquitin, ATP, and hemin. Since the formation of these high molecular weight components requires ubiquitin and is seen only when proteasome function is blocked with hemin, they are likely to represent intermediates in the ATP-dependent proteolytic pathway.

Regulatory Significance of R-Subunit Degradation. For the normal maintenance of cell function and for homeostatic adjustments, it is obviously necessary to maintain a close stoichiometric balance between R and C subunits of PKA, since a great excess of either one would interfere with metabolic regulation and prevent control of the kinase by cAMP. The ratio of these subunits is ≈1:1 in rabbit skeletal muscle, heart, liver, kidney, and brain (41). The mechanisms maintaining parity have not been elucidated fully but are likely to involve cAMP-dependent gene expression and regulated degradation of free R subunits (42).

Another important kind of physiological control involves changing the level of protein phosphorylation to a new set-point, as occurs in long-term facilitation (7). Analogous changes might be expected to occur during development, in response to injury and in other conditions where environmental signals alter cell metabolism for extended periods. As demonstrated in *Aplysia* sensory neurons, small alterations in the ratio of R and C subunits can result in substantial differences in the output of neurotransmitter and, therefore, in the animal's behavior. The selective change in subunit ratio that we observe appears to result from the triggering by cAMP of degradation of R subunits through the ATP-ubiquitin-proteasome-dependent pathway. Regulated degradation of other proteins critical to the control of cell growth (p53, cyclin, c-fos, and c-myc) is believed to use this pathway, based largely on evidence from cell-free studies (43).

The mechanism that degrades R subunits seems to be constitutive and is triggered by prolonged dissociation of the

holoenzyme. While this constitutive process can explain the enhancement of protein phosphorylation in intermediate presynaptic facilitation (8), the long-term maintenance of this state might be expected to involve additional mechanisms, such as acceleration of ubiquitin-mediated degradation of R subunits. This idea is consistent with the requirement for protein synthesis for the increased level of phosphorylation to persist (7) and for the persistent alteration in the R-to-C subunit ratio seen in long-term memory (4, 8). Among these newly synthesized proteins might be factors that target R-subunit proteins for increased ubiquitination or other components of the ATP-ubiquitin-proteasome-dependent pathway that might limit the rate of proteolysis in the naive neuron. There is evidence in the literature for both of these mechanisms. Scheffner *et al.* (27) showed E6 oncoprotein encoded by the human papilloma virus can promote ubiquitin-mediated degradation of p53 in rabbit reticulocyte lysates. In yeast, one of the ubiquitin genes, *UBI4*, is induced in response to heat shock, starvation, and treatment with DNA-damaging agents (44). Also, Mitch *et al.* (45) found increased mRNA levels for ubiquitin and proteasome subunits in rat muscle upon food deprivation and denervation. Presumably these mechanisms are lacking in *Aplysia* muscle where downregulation of R subunits does not occur after prolonged elevation of cAMP (9).

Ubiquitin as well as certain ubiquitin-conjugating factors are induced as part of the cell's heat shock or stress response (16, 17, 46), and in eukaryotic and prokaryotic cells, induction of heat shock proteins leads to enhanced cellular capacity for protein degradation (18, 46). Recently, coordinated increases in mRNA levels for ubiquitin and proteasome subunits and in ubiquitin conjugation have been demonstrated in muscle under several stressful conditions in which general protein degradation rises (45). Furthermore, Jack *et al.* (47) have shown that the ubiquitination of proteins dramatically increases within axons of rat sciatic nerve after crush injury. It has also recently been proposed that injury and memory are parts of the same continuum of responses by an organism to environmental stimuli (48). The degradation of R subunits by the ubiquitin pathway is consistent with the possibility that similar cellular processes might underlie the acquisition of memory and response to stress and injury.

We thank Hagan Bayley and Stephen Cheley for providing purified recombinant N_4, Alice Elste for preparing the figures, and Ken Kirschner for secretarial assistance. We also thank Hagan Bayley, David Hirsh, Irving Kupfermann, and members of the Schwartz lab for reading the manuscript critically. A.N.H. was supported by a fellowship from The Lady Tata Memorial Trust, London, U.K., and J.H.S. was supported by Research Scientist Award MH00921 from the National Institute of Mental Health; this work was supported by Research Grant NS29255 from the National Institutes of Health.

1. Klein, M. & Kandel, E. R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6912–6916.
2. Siegelbaum, S. A., Camardo, J. S. & Kandel, E. R. (1982) *Nature (London)* **299**, 413–417.
3. Kandel, E. R. & Schwartz, J. H. (1982) *Science* **218**, 433–443.
4. Schwartz, J. H. & Greenberg, S. M. (1989) in *Neural Models of Plasticity*, eds. Byrne, J. & Berry, R. (Academic, New York), pp. 46–57.
5. Dudai, Y. (1989) *The Neurobiology of Memory* (Oxford Univ. Press, New York).
6. Montaralo, P. G., Goelet, P., Castellucci, V. F., Morgan, J., Kandel, E. R. & Schacher, R. (1986) *Science* **234**, 1249–1254.
7. Sweatt, J. D. & Kandel, E. R. (1989) *Nature (London)* **339**, 51–54.
8. Greenberg, S. M., Castellucci, V. F., Bayley, H. & Schwartz, J. H. (1987) *Nature (London)* **329**, 62–65.
9. Bergold, P. J., Sweatt, J. D., Winicov, I., Weiss, K. R., Kandel, E. R. & Schwartz, J. H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3788–3791.
10. Bergold, P., Beushausen, S. A., Sacktor, T. C., Cheley, S., Bayley, H. & Schwartz, J. H. (1992) *Neuron* **8**, 387–392.
11. Palazzolo, M., Katz, F., Kennedy, T. E. & Schwartz, J. H. (1989) *J. Neurobiol.* **20**, 746–761.
12. Eppler, C. M., Palazzolo, M. J. & Schwartz, J. H. (1982) *J. Neurosci.* **2**, 1692–1704.
13. Eppler, C. M., Bayley, H., Greenberg, S. M. & Schwartz, J. H. (1986) *J. Cell Biol.* **102**, 320–331.
14. Taylor, S. (1990) *Annu. Rev. Biochem.* **59**, 971–1005.
15. Rogers, S., Wells, R. & Rechsteiner, M. (1986) *Science* **234**, 364–368.
16. Hershko, A. & Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61**, 761–807.
17. Finley, D. & Chau, V. (1991) *Annu. Rev. Cell. Biol.* **7**, 25–69.
18. Goldberg, A. L. (1992) *Eur. J. Biochem.* **203**, 9–23.
19. Goldberg, A. L. & Rock, K. L. (1992) *Nature (London)* **357**, 375–379.
20. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974) *Methods Enzymol.* **38**, 299–308.
21. Weber, W. (1979) *FEBS Lett.* **99**, 62–66.
22. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
23. Etlinger, J. D. & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 54–58.
24. Hershko, A. & Heller, H. (1985) *Biochem. Biophys. Res. Commun.* **128**, 1079–1086.
25. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
26. Kanayama, H., Tamura, T., Ugai, S., Kagawa, S., Tanahashi, N., Yoshimura, T., Tanaka, K. & Ichihara, A. (1992) *Eur. J. Biochem.* **206**, 567–578.
27. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J. & Howley, P. M. (1990) *Cell* **63**, 1129–1136.
28. Ciechanover, A., DiGuiseppe, J. A., Bercovich, B., Orian, A., Richter, J. D., Schwartz, A. L. & Brodeur, G. M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 139–143.
29. Ciechanover, A., Hod, Y. & Hershko, A. (1978) *Biochem. Biophys. Res. Commun.* **81**, 1100–1104.
30. Hough, R., Pratt, G. & Rechsteiner, M. (1987) *J. Biol. Chem.* **262**, 8303–8313.
31. Waxman, L., Fagan, J. M. & Goldberg, A. L. (1987) *J. Biol. Chem.* **262**, 2451–2457.
32. Rechsteiner, M. (1987) *Annu. Rev. Cell. Biol.* **3**, 1–30.
33. Hough, R. F., Pratt, G. W. & Rechsteiner, M. (1988) in *Ubiquitin*, ed. Rechsteiner, M. (Plenum, New York), pp. 101–134.
34. Johnston, N. L. & Cohen, R. E. (1991) *Biochemistry* **30**, 7514–7522.
35. Glotzer, M., Murray, A. W. & Kirschner, M. W. (1991) *Nature (London)* **349**, 132–138.
36. Jabben, M., Shanklin, J. & Vierstra, R. D. (1989) *J. Biol. Chem.* **264**, 4998–5005.
37. Steinberg, R. A. & Agard, D. A. (1981) *J. Biol. Chem.* **256**, 10731–10734.
38. Uhler, M. D. & McKnight, G. S. (1987) *J. Biol. Chem.* **262**, 15202–15207.
39. Scott, J. D., Glaccum, M. B., Zoller, M. J., Uhler, M. D., Helfman, D. M., McKnight, G. S. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5192–5196.
40. Rechsteiner, M. (1990) *Semin. Cell. Biol.* **1**, 433–440.
41. Hofmann, F., Bechtel, P. J. & Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 1441–1447.
42. McKnight, G. S., Clegg, C. H., Uhler, M. D., Chrivia, J. C., Cadd, G. G., Correll, L. A. & Otter, A. D. (1988) *Rec. Prog. Horm. Res.* **44**, 307–333.
43. Rechsteiner, M. (1992) *Cell* **66**, 615–618.
44. Fraser, J., Luu, H. A., Neculcea, J., Thomas, D. Y. & Storms, R. K. (1991) *Curr. Genet.* **20**, 17–23.
45. Mitch, W. E., Medina, R., Greiber, S. & Goldberg, A. L. (1991) *FASEB J.* **5**, A922 (abstr.).
46. Seufert, W. & Jentsch, S. (1990) *EMBO J.* **9**, 543–550.
47. Jack, D. L., Chakraborty, G. & Ingoglia, N. A. (1992) *Neuro Rep.* **3**, 47–50.
48. Walters, E. T., Alizadeh, H. & Castro, G. A. (1991) *Science* **253**, 797–799.