Effects on protein synthesis produced by pairing depolarization with serotonin, an analogue of associative learning in Aplysia

(associative plasticity/two-dimensional polyacrylamide gel electrophoresis/abdominal ganglia/stringent starvation protein/glutathione S-transferase)

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ABSTRACT A form of associative plasticity in Aplysia, activity-dependent neuromodulation, involves the convergence of neuronal activity and the effects of a modulatory transmitter. To investigate the role of protein synthesis in associative plasticity, we examined the effects of a biochemical analog of activity-dependent neuromodulation on the level of incorporation of labeled amino acid into proteins. To mimic associative training, abdominal ganglia were exposed to paired treatments of a depolarizing agent, elevated potassium, and a modulatory transmitter, serotonin. The effects of elevated potassium and serotonin applied alone were also examined. At least two proteins (nos. 9 and 17) were affected in a nonadditive way by the paired procedure. Incorporation of label into protein 9 was increased by the paired procedure but was not affected by either elevated potassium or serotonin. Incorporation of label into protein 17 was significantly affected by elevated potassium or serotonin, but the effect of the paired procedure was significantly less than the summed effects of elevated potassium and serotonin applied alone. These results indicate that changes in protein synthesis may be important in the induction of associative plasticities. Amino acid sequences of two peptides derived from protein 9 were obtained. Then, a partial cDNA clone for protein 9 was obtained by performing PCR with degenerate primers corresponding to portions of the sequences of the two peptides. The sequence of protein 9 is related to sequences previously reported for a family of genes comprising the stringent starvation protein of Escherichia coli, auxin-induced proteins of plants, and glutathione S-transferases of a number of organisms.

A cellular analogue of classical conditioning produces an enhancement of the connections between sensory neurons and motor neurons in Aplysia (1, 2). Sensory–motor synaptic transmission involved in the tail and siphon withdrawal reflexes of Aplysia is enhanced by pairing spike activity in a sensory neuron (conditioned stimulus) with electrical stimulation of the tail or peripheral nerves (unconditioned stimulus). This type of associative plasticity, called activity-dependent neuromodulation, has been observed in a short-term form lasting minutes (1, 2) and a long-term form lasting 24 hr (3). This short-term associative plasticity in Aplysia is believed to involve the convergent action of two second messengers, Ca2+ and cAMP (4–8). Convergent actions of two second-messenger pathways may also produce the long-term form of the associative plasticity by modifying transcription and protein synthesis. Although the importance of transcription and protein synthesis in some forms of nonassociative plasticity have been well established, their importance in associative plasticity is just beginning to be determined (ref. 9; for review, see refs. 8–14).

In the present study we examined whether an analogue of activity-dependent neuromodulation induced changes in protein synthesis in cells of Aplysia abdominal ganglia. The analogue consisted of exposure of abdominal ganglia to paired treatments of (i) high K+ and (ii) serotonin (5-hydroxytryptamine, 5-HT). Depolarization by high K+ was used to mimic spike activity. 5-HT, which mediates many effects of sensitizing stimuli (ref. 15; for review, see ref. 16), was used to mimic the effects of sensory stimulation. The effects of the paired procedure were compared with the effects of high K+ and 5-HT applied individually. We found that pairing high K+ with 5-HT produced effects on proteins that were different from a summation of the effects produced by each stimulus applied alone. Furthermore, we obtained a partial amino acid sequence and then a partial cDNA clone of one of the proteins affected by the paired treatment.

MATERIALS AND METHODS

Aplysia californica were obtained from Alacrity Marine Biological (Redondo Beach, CA), Marinus (Long Beach, CA), and Sea Life Supply (Sand City, CA) and maintained in artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) at 15°C. Two days or more after their arrival, animals were anesthetized by injection of an amount of isotonic MgCl2 equivalent to approximately half of their body weight. Abdominal ganglia were removed and placed into four separate chambers (three ganglia per chamber) containing filtered seawater [BFSW: artificial seawater containing 30 mM Hepes, streptomycin sulfate (BioWhittaker) (100 µg/ml), and penicillin G (100 units/ml) at pH 7.65]. The ganglia remained in BFSW at 15°C for at least 2 hr before exposure to experimental treatments. Each group of ganglia was exposed to one of the following treatments: (i) high K+ (80 mM KCl) applied alone for 1 hr; (ii) 5-HT (5 µM) applied alone for 2 hr; (iii) high K+ for 1 hr paired with 5-HT for 2 hr, with the two treatments overlapping during the last 0.5 hr of the high K+ treatment and the first 0.5 hr of the 5-HT treatment; or (iv) no treatment. Elevation of the K+ concentration in the medium is a well-established method for producing membrane depolarization of molluscan neurons (6, 17–21). Two hours of training or 5 µM 5-HT are commonly used to elicit long-term plasticity in Aplysia (22–26). The four chambers were thoroughly washed with BFSW at the end of each treatment. The ganglia were metabolically labeled with [3H]leucine (0.1 mCi/ml; 1 mCi = 37 MBq) for 2 hr starting 5 hr after the end of 5-HT treatments or at times of other

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); SSP, stringent starvation protein; GST, glutathione S-transferase; AIP, auxin-induced protein.

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from total RNA extractions with 2× proteinase K, followed by loading onto the gels. To reduce variations, the four experimental and control samples of an experiment were always run on the same batch of isoelectric focusing and SDS/polyacrylamide gels and then exposed to film at the same time. Gels were exposed to films for different times to ensure that fluorographs were not saturated for certain proteins.

The results of each experiment were determined by comparing the treated group with an untreated control group run in the same experiment. The effects of treatments on incorporation of amino acid into specific proteins were first analyzed visually. Densities of experimental proteins on two-dimensional gels were judged as increased, decreased, or not changed relative to the same protein from the control gel. Twelve to 14 proteins that appeared to be affected by at least one of the experimental treatments were selected for further analysis. As a second step, optical densities (ODs) of the selected proteins were determined by computerized image analysis (DNA Proscan, Nashville, TN). To normalize the ODs for any discrepancies in the amount of radioactivity loaded on the gels, a percent OD was calculated for each selected protein by dividing the OD of the selected protein by the summed ODs of 5 proteins that were unaffected by the treatment. An example of percent ODs ± SEM (n = 13) for protein 9 is as follows: 7.73 ± 1.93% (5-HT alone), 8.97 ± 1.94% (high K+ alone), 8.27 ± 1.14% (control), and 12.48 ± 2.27% (paired 5-HT and high K+). To determine whether proteins were significantly affected by one of the treatments, a Wilcoxon ranked sign test was performed between each experimental and control group (26). A Wilcoxon test was also used to determine whether the effect of the paired procedure was significantly different from the sum of the effects of the individual treatments applied alone.

To obtain amino acid sequences of proteins affected by paired treatments, proteins of interest were first purified with preparative two-dimensional gels. Protein purification, digestion with Staphylococcus aureus V8 protease, and peptide sequencing were carried out as described (29) with some modifications (30). Protein 9 was first purified from abdominal ganglia. About 500 μg of protein from abdominal ganglia was loaded on each gel. After Coomassie brilliant blue R-250 staining of the gel, protein 9 was cut out of 26 preparative two-dimensional gels. Protein 9 was digested in situ with S. aureus V8 protease. The peptides derived from protein 9 were separated by SDS/15% PAGE and transferred onto a poly(vinylidene difluoride) membrane (Problott, Applied Biosystems) and stained with Coomassie brilliant blue R-250. Protein 9 was purified a second time from abdominal and pleural-pedal tissue and digested in situ with cyanogen bromide (31). Peptide bands were cut from the Problott membrane and sequenced with a 477A pulsed-liquid protein sequencer (Applied Biosystems). Peptide sequence analysis was performed at the National Center for Biotechnology Information with the BLAST network system (32).

To obtain a partial cDNA clone for protein 9, poly(A)+ mRNA was first isolated from Aplysia abdominal and head ganglia. Total RNA was obtained by homogenization of ganglia in a phenol/SDS mixture at 65°C, followed by two extractions with phenol/chloroform, 1:1 (vol/vol), and then precipitation with 70% ethanol in 0.1 M NaCl. The ethanol precipitate was reconstituted in water and precipitated with 2 M sodium acetate (pH 6.0). Poly(A)+ RNA was isolated from total RNA on an oligo(dT) column (33). cDNA was then synthesized from 7 μg of RNA by using a cDNA synthesis kit (United States Biochemical) with random hexamers as primers. Degenerate primers containing deoxinosine (Genosys, The Woodlands, TX) and corresponding to regions of the two peptides obtained from protein 9 were then designed and synthesized (see Fig. 3).

PCR were performed (Perkin-Elmer/Cetus kit) on 40 ng of Aplysia ganglion cDNA with 10 μM each primer, 10 μl of 10× reaction buffer, 200 μM dNTPs, and 2.5 units of AmpliTaq enzyme in 100 μl. Reaction conditions were optimized as follows: initial (first cycle only) denaturation at 94°C for 2 min, subsequent denaturations at 92°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 1 min. Thirty cycles were run with the PTC-100 programmable thermal controller (MJ Research, Walthertown, MA).

The PCR fragment of interest was cloned into a vector with the TA cloning system (Invitrogen). Plasmid preparations were performed on 10 colonies with Magic Minipreps (Promega). Insert-containing clones were identified by cleavage with EcoRI restriction endonuclease (1 unit/100 ng of DNA). The sequence of the 180-bp insert was verified by dideoxy sequencing (United States Biochemical Sequenase kit); the sequencing reaction products were run on a Sequi-Gen apparatus (Bio-Rad).

RESULTS

In each experiment, groups of three abdominal ganglia were exposed to high K+, 5-HT, paired application of high K+ and 5-HT, or no treatment (Fig. 1). Thirteen independent experiments with 12 animals per experiment were performed. Visual analysis of the gels from these experiments yielded 24 proteins that appeared to be affected by at least one of the experimental treatments. This group of 24 proteins was examined statistically for proteins affected by one of the treatments. One protein, no. 9, was significantly increased by the paired procedure but not by high K+ or 5-HT applied alone (Wilcoxon test). To determine whether the results of the paired application of high K+ and 5-HT could be explained by addition of the effects of high K+ and 5-HT applied alone, the effects on protein synthesis produced by paired high K+ and 5-HT were compared statistically with a summation of the changes produced by high K+ and 5-HT applied alone (Wilcoxon test). For protein 9, the effect of paired application of high K+ and 5-HT was significantly greater than the summed results of high K+ and 5-HT applied alone (Fig. 2A). We did observe some cases—e.g., protein 5—in which the effect of the paired application of high K+ and 5-HT did not differ significantly from the summed results of high K+ and 5-HT applied alone (Fig. 2B).

Another interesting nonadditive type of effect of the paired treatment was observed. For example, protein 17 was significantly affected by high K+ and 5-HT applied alone. However, the effects of paired high K+ and 5-HT were significantly less than the summed effects of high K+ and 5-HT applied alone (Fig. 2C).

More precise study of the role of these proteins in memory formation requires identification of the proteins and determination of their cellular function. To do this, we initiated experiments to obtain a partial amino acid sequence of protein 9. Protein 9 was purified from abdominal and pleural-pedal ganglia by preparative two-dimensional PAGE. Spots of protein 9 were removed from the gels and digested with cyanogen bromide or with S. aureus V8 protease. The peptides were then separated in one-dimensional SDS/polyacrylamide gels, blotted onto poly(vinylidene difluoride) membranes, and stained with Coomassie brilliant blue. A sequence of 36 aa was obtained by cyanogen bromide digestion and another non-overlapping sequence of 37 aa was obtained by V8 protease digestion (Fig. 3A).
To align the two peptides from protein 9 and obtain additional sequence, degenerate primers with deoxyinosines at the points of highest degeneracy were synthesized corresponding to the two peptides and used to amplify cDNA synthesized from poly(A)+ mRNA from Aplysia ganglia. A band of ≈180 bases was obtained and purified from a polyacrylamide gel. After cloning and sequencing, this band was shown to contain sequences that corresponded to the targeted regions of the two peptides. In addition, a sequence of 19 aa between the two peptides was obtained. Therefore, a combined sequence of 92 aa was obtained from protein 9 (Fig. 3A).

The BLAST program (32) was used to compare the 92-aa Aplysia peptide to sequences of known proteins (search was performed on November 20, 1993). This long peptide was found to have 34% identity over an 88-aa region to the SSP of E. coli (34), 43% identity over a 59-aa region to a GST from D. carophyllus (35), and 40% identity over a 46-aa region to an AIP from N. tabacum (36) (Fig. 3A). The similarities between protein 9 and SSP, GST, and AIP are highly significant because the probabilities to obtain such matches by chance are extremely low (Fig. 3B). In addition, the SSPs and GSTs are related (37). An AIP from Nicotiana plumbaginifolia that is very similar to the AIP mentioned above also has significant similarity to the SSP and GST families (38). Finally, the sequence of our peptide from protein 9 was found to have significant similarity to a cosmid encoding a Caenorhabditis elegans protein of unidentified function (39).

**DISCUSSION**

Our finding that paired stimulation produced nonadditive effects on the incorporation of label into proteins suggests that translation, and perhaps transcription as well, may be involved in generating some forms of associative plasticity. Similar conclusions emerged from experiments on PC12 rat pheochromocytoma cells, a neuronal cell line in which cAMP and Ca2+ appear to interact in a nonadditive or synergistic fashion in the regulation of transcription (40, 41). Nonadditive effects of stimuli imply convergence of the inputs at some level of the signaling pathway. In our case, the inputs must converge upon some molecular event of the information processing pathways. The identification of molecular events that may act as coincident detectors in neurons is an important issue (e.g., refs. 7, 42, and 43), and several such detectors (e.g., adenylate cyclases and cAMP response element-binding proteins; refs. 4, 41, and 44) have been under study. The identification of proteins 9 and 17, whose synthesis appears to be governed by such coincident detectors, should allow us to work backwards to identify the mechanisms responsible for coincident detection in Aplysia.

A critical issue raised by our results is the role of these specific proteins in generating behavioral plasticity in Aplysia: Noel et al.
Fig. 2. Changes in incorporation of amino acid into proteins produced by paired high K\(^+\) and 5-HT compared with a summation of the effects of high K\(^+\) and 5-HT applied alone (high K\(^+\) plus 5-HT). Percent differences of integrated ODs of experimental proteins relative to control proteins are shown. (A) Incorporation into protein 9 was increased by paired high K\(^+\) and 5-HT but did not appear to be affected by either high K\(^+\) or 5-HT applied alone. The effect of paired high K\(^+\) and 5-HT was significantly different from the summed effects of high K\(^+\) and 5-HT applied alone. (B) Incorporation into protein 5 was decreased by paired high K\(^+\) and 5-HT or by high K\(^+\) or 5-HT applied alone. The effect of the paired application of high K\(^+\) and 5-HT did not differ significantly from the summed results of high K\(^+\) and 5-HT applied alone. (C) Incorporation into protein 17 was decreased by high K\(^+\) or 5-HT applied alone but was not affected by the paired treatment. Paired high K\(^+\) and 5-HT produced a significantly different effect from the summed effects of high K\(^+\) and 5-HT applied alone. Level of significant difference between the paired high K\(^+\) and 5-HT results and the summed results of high K\(^+\) and 5-HT applied alone: ns, nonsignificant; *, \(P < 0.05\); **, \(P < 0.01\).

It will be important to determine whether the types of pairing specific changes we observed depend on a particular temporal relationship of the two stimuli. Also, it will be important to investigate whether the types of changes we observed occur in neurons known to be responsible for specific types of learning. Protein 9 has not been identified in previous studies examining learning-related changes in proteins in *Aplysia* (13, 22, 25, 27, 29, 31, 45, 46). This is not surprising, however, since those studies examined effects of analogues of nonassociative learning on proteins. Finally, the cellular functions of proteins 9 and 17 need to be determined.

The 92-aa sequence obtained from protein 9 by microsequencing and PCR represents about one-third of the total protein, since protein 9 is about 31 kDa. This derived amino acid sequence had significant similarity to the SSP from *E. coli*, GSTs from a number of organisms, and an AIP from a

![Fig. 3](image)

**Fig. 3.** Comparison of amino acid sequences derived from *Aplysia* protein 9, stringent starvation protein (SSP) of *Escherichia coli* (34), glutathione S-transferase (GST) from *Dianthus caryophyllus* (35), and auxin-induced protein (AIP) from *Nicotiana tabacum* (36). (A) The underlined amino acids in the *Aplysia* peptide sequence are the amino acids obtained by PCR. To the left of this underlined region is the amino acid sequence obtained by cyanogen bromide digestion, while the one to the right of the underlined region is the sequence obtained by *S. aureus* V8 protease digestion. The regions in each peptide to which corresponding degenerate primers were synthesized for PCR are indicated by arrows. The two primers were as follows: 5'-AAC(R) AA(R) CCI-GA(Y) CA(Y) TT(Y) TT(Y) GA-3' (a) and 5'-TG(R) TT(R) AA(R) TAI-ATI-C(KY)-TTC (b), where I is deoxyinosine. To show the similarity among the four peptides, the amino acids which are identical in at least two of the sequences are included in shaded boxes. (B) The percent identities of the *Aplysia* peptide to each of the other three peptides, and the \(P\) values for those sequence identities, were obtained with the BLAST program (32). \(P\) values are probabilities that matches as good or better than those found would occur by chance.
plant. The *E. coli* SSP may interact with RNA polymerase to regulate its function (47). An AIP with similarity to the *E. coli* SSP has been found in *N. plumaginifolia* (38). This plant protein may also have a regulatory effect on transcription. It was proposed that the family of genes coding for this family of plant proteins be called multiple-stimulus response genes (38). GSTs comprise a large family of proteins which are involved in cell protective mechanisms (for review, see ref. 48). Although protein 9 had significant similarity to an SSP, an AIP, and a GST, the function of protein 9 in *Aplysia* remains to be determined. Our results suggest that protein 9 may have a specific regulatory role in events associated with coincident input signals. Further study of protein 9 may allow us to determine how paired treatments alter protein synthesis and how protein synthesis is involved in the induction of associative forms of memory.

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