Common set of proteins in *Aplysia* sensory neurons affected by an in vitro analogue of long-term sensitization training, 5-HT and cAMP

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An in vitro analogue of long-term behavioral training in *Aplysia* was developed to simulate the intensity and timing of shocks delivered to the body wall of animals during sensitization training. The in vitro training analogue consisted of electrical stimulation of peripheral nerves of isolated pleural-pedal ganglia. We found that the in vitro training analogue led to long-term (24 h) changes in the membrane currents of sensory neurons; these changes were similar to those produced by behavioral training. Using two dimensional polyacrylamide gel electrophoresis, we examined early effects on protein synthesis in pleural sensory neurons induced by the training analogue. Incorporation of amino acid into 5 proteins was affected at the end of training. Incorporation of amino acid into some of these proteins was also affected by serotonin (5-HT) and by an analogue of cyclic adenosine monophosphate (cAMP). These results suggest that the effects on protein synthesis that are produced by the in vitro training analogue are mediated, at least in part, by release of serotonin (5-HT) and an increase in the level of cAMP in the sensory neurons.

INTRODUCTION

The mechanisms responsible for the induction, mediation and maintenance of long-term memory have recently begun to be investigated at the biochemical and molecular levels. Several aspects of these mechanisms seem to be well established. Second messengers are involved in the induction phase and changes in synaptic efficacy are involved in the mediation phase. Also, protein synthesis is required in the formation of long-term memory²,³,⁷,⁸,¹⁶,²³,²⁷,⁴¹. These findings have led to the conclusions that proteins, protein synthesis and post-translational modification of proteins such as phosphorylation play important roles in long-term memory. Therefore, a prerequisite for fully elucidating the molecular basis of learning and memory is to identify and characterize the proteins that play important roles as well as to understand the regulation of the proteins and their interactions. One strategy for identifying and characterizing protein components of mechanisms of learning and memory involves taking advantage of features of the invertebrate *Aplysia* in which it is possible to link biochemical events with behavior.

Several defensive reflexes in *Aplysia* exhibit both short-term and long-term sensitization, a simple form of non-associative learning⁹,²⁶. Short-term sensitization lasts minutes and is correlated with modifications of properties of primary sensory neurons which result in increased efficacy of the connections between sensory neurons and interneurons and motor neurons¹¹,¹²,²⁶,⁴³. Interestingly, the sensory neurons are a locus for long-term sensitization as they are for short-term sensitization²⁰,³⁹. Application of 4 blocks of noxious stimuli to one side of the animal over a 1.5 h period produces both long-term (24 h) sensitization of the tail-siphon withdrawal reflex elicited by test stimuli to the trained side and long-term reduction of net outward membrane currents in sensory neurons that innervate the trained side³⁹.

The facilitation contributing to short- and long-term sensitization may utilize some common mechanisms. For example, both short-term and long-term facilitation appear to be induced by serotonin (5-HT) and cyclic adenosine monophosphate (cAMP) and similar membrane channels appear to be involved in mediating short-term and long-term sensitization⁷,⁸,¹⁵,²⁸,³⁴,³⁸,⁴⁰. However, a few differences exist between short-term and long-term facilitation and sensitization. The long-term, but not short-term effects are blocked by inhibitors of protein

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Also, long-term sensitization is associated with the growth and remodeling of synaptic terminals of sensory cells\(^5\). As is the case for long-term biophysical changes in the properties of the sensory neurons, long-term morphological changes are induced by both 5-HT and cAMP\(^22,29,30\).

Although much progress has been made in understanding the loci and some mechanisms of both short-term and long-term sensitization in *Aplysia*, the identification and characterization of specific proteins involved in sensitization have only recently begun. A number of proteins are probably involved in sensitization. Some of these proteins are likely to play regulatory roles in the induction of memory while others must be involved in the expression of memory. Finally, some structural proteins and proteins of metabolic pathways should be involved in the maintenance of memory and growth of the synaptic terminals.

Behavioral training altered incorporation of amino acids into specific proteins from abdominal ganglia 24 h after training of intact animals\(^13\). Furthermore, treatments mimicking behavioral training such as application of 5-HT, forskolin (an activator of adenylate cyclase), and analogues of cAMP induced early as well as late changes in incorporation of amino acids into specific proteins from pleural sensory cells\(^5,19\). In addition to these experiments on protein synthesis, other studies have investigated changes in protein phosphorylation\(^18,42\) and changes in cAMP binding proteins\(^25\). Thus far, only a few of the proteins found in the different studies appear to be the same proteins.

To study further the proteins involved in sensitization, we developed an in vitro analogue of the training procedure to serve as a link between cellular studies on isolated sensory cells and the behavior of the animal. In the analogue, behavioral training was mimicked by activating peripheral nerves that innervate the body wall with the same pattern of shocks delivered to the body wall during sensitization training\(^39\). In the present study we examined the long-term changes in the membrane properties of the pleural sensory neurons induced by electrical stimulation of peripheral nerves of isolated pleural-pedal ganglia and showed that the changes observed were similar to the long-term alterations induced by sensitization training. The analogue training also led to a significant long-term enhancement of the sensory-motor connections\(^24\). Consequently, the in vitro analogue of the training procedure may be used as a bridge to span studies of behavior and of isolated sensory cells. Therefore, we investigated modifications of protein synthesis in pleural sensory neurons induced by electrical stimulation of peripheral nerves of isolated pleural-pedal ganglia. The changes in proteins produced by electrical stimulation were studied and then compared to the effects on proteins produced by 5-HT and an analogue of cAMP.

We were interested in detecting proteins affected in a similar way by the 3 different treatments in order to identify early proteins that might be involved in the induction of sensitization. A preliminary report of aspects of this work has appeared in abstract form\(^31\).

**MATERIALS AND METHODS**

**Voltage clamp procedure**

*P. californica* were obtained from Alacrity Marine Biological (Redondo Beach), Marinus Inc. (Long Beach) and Sea Life Supply (Sand City) and maintained in artificial seawater (ASW, Instant Ocean) at 15 °C for at least 2 days after arrival. The animals were anesthetized by injection of an amount of isotonic MgCl\(_2\) equivalent to approximately one-half of their body volume. Left and right pleural-pedal ganglia with peripheral nerves P7, P8 and P9 attached were removed from the animal and pinned to the floor of a chamber containing 50% isotonic MgCl\(_2\) and 50% ASW. This solution was used to block synaptic transmission during the dissection. Branches of peripheral nerves P7, P8, and P9 were drawn into suction electrodes as illustrated in Fig. 1. One ganglion was then chosen randomly to receive the training and was stimulated over a 1.5 h period. The electrical stimulation consisted of simultaneous presentation to each nerve of four 15 s-duration blocks of stimuli with an interblock interval of 30 min. Each block consisted of ten 500 ms-duration trains with an intertrain interval (onset to onset) of 1.5 s. Each train consisted of thirty-one 3 ms-duration 60 volt pulses with an interpulse interval of 16 ms. After the final stimulation, experimental and control ganglia were placed in organ culture for 24 h as described by Scholz and Byrne\(^40\). Twenty four hours after the stimulation procedure the ganglia were placed in 50% isotonic MgCl\(_2\)/50% ASW and the sheath covering the pleural ganglion was removed. The solution was replaced by ASW and sensory cells in control and experimental pleural ganglia were impaled with two microelectrodes filled with 3 M potassium acetate. In all experiments cells were voltage-clamped at a holding potential of ~50 mV using procedures identical to those described previously\(^39,40\). Briefly, a series of 7 different membrane currents elicited by 300 ms duration pulses to membrane potentials between ~80 and +25 mV in steps of 15 mV were recorded and digitized. For each cluster of sensory neurons, the membrane currents elicited at corresponding potentials were averaged. Thus, the control and experimental families of current responses were obtained representing the current-voltage (I-V) relation of each group of cells. For statistical analysis, the data were reduced so that each control and experimental cluster yielded 2 scores as described previously\(^40\). For example, the net outward current at the end of the 5 depolarizing pulses (~80, ~20, ~10, +10, +25 mV) of the control response family were summed to generate one of the scores for the control cells. The net current at the end of 2 hyperpolarizing pulses (~65, ~80 mV) of the response family were summed to generate the second score for the control cells. The same procedure was used to generate 2 scores for the experimental cells\(^40\). Differences in corresponding scores from all the clusters were tested by a two-tailed paired t-test.

**Treatment of pleural-pedal ganglia and preparation of samples for electrophoresis**

In each experiment, left and right pleural ganglia were isolated from 3 animals and pinned to the floor of 3 ml experimental and control chambers containing 50% isotonic MgCl\(_2\) and 50% buffered filtered seawater (BFSW. seawater containing 30 mM HEPES, 100 μg/ml streptomycin, 1000 U/ml penicillin-G, pH 7.65). One pleural-pedal ganglion from each animal was placed in an experimental group and the contralateral pleural-pedal ganglion was placed in a
Control group. Peripheral nerves were mounted into suction electrodes as described above. The control and experimental chambers were thoroughly rinsed and filled with BF5W. After a 2 h equilibration period, BF5W was replaced with BF5W containing [35S]methionine (0.15 mCi/ml). After 0.5 h in [35S]methionine, the 3 nerves of the experimental ganglia were electrically stimulated simultaneously over a 1.5 h period (Fig. 1). The training procedure was essentially identical to that described above except that the intertrain interval was 1 s rather than 1.5 s. In some experiments serotonin (5-hydroxytryptamine hydrochloride, Sigma) (5 × 10^{-6} M) or CPT-cAMP (8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate, Sigma) (10^{-3} M) was applied to the ganglia for 1.5 h instead of the electric shocks. This concentration of 5-HT approximately doubles the cAMP content of pleural sensory neurons73 CPT-cAMP at 10^{-3} M was chosen for use because it is somewhat beyond the dose (2 × 10^{-4} M) at which the effect of CPT-cAMP on membrane currents of isolated pleural sensory neurons saturates9. In addition, a concentration of 10^{-3} M CPT-cAMP is a common dose used in many other studies. For example, CPT-cAMP was applied at a concentration of 10^{-3} M to isolated pleural-pedal ganglia by Sacktor and Schwartz37. A lower concentration (10^{-4} M) was used by Sweat and Kandel42 on isolated sensory neurons and by Schacher et al.36 on cultured neurons, but in these 2 studies an inhibitor of phosphodiesterase, IBMX, was also applied with CPT-cAMP. Furthermore, in our studies the isolated ganglia were not desheathed, whereas desheathed isolated somata of sensory neurons and cultured neurons were used in most of the previous studies by others. In the experiments using 5-HT and CPT-cAMP, ganglia were also incubated in [35S]methionine throughout the treatment period. The electrical stimulation experiments were performed first, followed by 5-HT experiments and then CPT-cAMP application.

At the end of treatment, control and experimental pleural-pedal ganglia were frozen in 50% BF5W, 50% propylene glycol at −20 °C34,36 and their clusters of sensory neurons were removed while the ganglia remained frozen. Clusters of isolated sensory neurons were placed into 50 μl solubilization buffer consisting of 9.1 M urea, 2% w/v ampholytes, 5% w/v 2-mercaptoethanol, 3% w/v 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Tris base, 5 mM EDTA, 1 mM EGTA, 0.125 mg/ml bacitracin, 0.125 mg/ml trypsin inhibitor, 1.5 mg/ml benzamidine, 0.19 IU/ml aprotinin, 0.175 mg/ml phenylmethylsulfonylfluoride, 40 mM sodium pyrophosphate and 100 mM sodium fluoride. After 2 h at room temperature, the samples of solubilized sensory clusters were stored at −80 °C. The amount of labeled amino acid incorporated into proteins was estimated by measuring the radioactivity in material precipitable by trichloroacetic acid (TCA)45.

**Electrophoresis**

The proteins in samples of sensory neurons were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using procedures similar to O’Farrell35 and reported by Yeung and Esken45 and Esken et al.17,49. Proteins were first separated in a pH gradient of 4–7 established with a mixture of ampholytes (Pharmacia) and then by a 10% polyacrylamide 1% SDS slab gel (Minilab gels; Idea Scientific, Corvallis, OR). Equal amounts of radioactivity from experimental and control samples were loaded onto separate isoelectric focusing gels. Samples from an experiment were always run together on the same batch of isoelectric and separating gels. After fixation with 50% trichloroacetic acid and then 7% acetic acid, gels were equilibrated with Autofluor (National Diagnostics). Experimental and control gels were dried and then placed under a single sheet of Kodak XAR film and kept at −80 °C. Exposure of both experimental and control gels to the same film eliminated the need for us to standardize the gels for different development conditions. Films were exposed to gels for different durations of time to insure that the film was not saturated for certain proteins. Replica gels were often run.

The molecular weights (MW) and isoelectric points (pl) of proteins were determined by running standards along with samples of proteins from sensory neurons. The pl standards used were carbonic anhydrase A from human erythrocytes (pl 6.6), carbonic anhydrase B from bovine erythrocytes (pl 5.8), β lactoglobulin A from bovine milk (pl 5.1) and soybean trypsin inhibitor (pl 4.6). The molecular weight standards were Low Molecular Weight Standards (Bio Rad). After electrophoresis, gels were silver stained (Gelcode, Health Products, Inc.), and then the pls and MWs of proteins were estimated by interpolation.
A. 

**Analysis of fluorographs**

In order to select proteins affected or not affected by the treatments, the fluorographs were first analysed visually by 2 individuals, one of which was ‘blind’. The criteria used to select proteins for analysis were their separation from the surrounding proteins and the consistency of the appearance of the protein between different experiments. Densities of experimental proteins were judged as increased, decreased, or not changed relative to the same protein from the matched control gel. A total of 35 proteins were selected for further analysis. As a second step of the analysis the optical densities (ODs) of these selected proteins were measured using a computerized image analysis system (Technology Resources, Nashville, TN). The fluorographs were scanned, then the backgrounds of images were computed and the integrated OD of each protein was determined automatically. To normalize the integrated ODs for any discrepancies in the amount of radioactivity loaded on the gels, a per cent OD was calculated for each experimental protein by dividing its OD by the summed OD of 8 proteins on the experimental gel that were unaffected by the treatment. A percent OD number also was calculated in a similar manner for proteins on the control gel using the same 8 unchanging proteins as standards. The ratios of the experimental mean percentage of the integrated optical densities over the control (E/C) for the 8 non-changing proteins of the electrical stimulation gels are the following: 1.14, 1.05, 1.05, 0.97, 0.99, 1.00, 0.97, 0.97. The average E/C for the 8 non-changing proteins were equal to 1.02 ± 0.02, 0.97 ± 0.04 and 0.87 ± 0.07 (mean ± S.E.M.) for electrical stimulation, 5-HT and cAMP experiments, respectively. The integrated ODs of the same protein were averaged when they were determined from films exposed to the same gels for different durations or from replica gels of the same sample. This method of quantification depends upon obtaining ODs of proteins from films in which the relationship between disintegrations per min and ODs of the proteins are linear. Fluorographs used for analysis were chosen on visual grounds to satisfy this requirement from the 3 films exposed to the gel for 3 different durations of time. A two-tailed paired t-test was performed to test whether the percentages of the integrated ODs of the experimental protein were significantly different from those of the matched control protein. We report here only the proteins changed significantly (P < 0.05) by any one of the treatments. As a third step of the analysis, the results of quantification were verified visually. The 3 series of experiments using electrical stimulation, 5-HT and CPT-cAMP were performed and analysed separately. The last step of the analysis was the comparison of the effects produced by each treatment.

**RESULTS**

**Effects of nerve stimulation on membrane currents**

To investigate whether an in vitro training analogue of long-term sensitization can serve as a link between the studies on isolated sensory cells and the behavior of the animal, we examined whether long-term changes in membrane current in the sensory cells could be induced by electrical stimulation of peripheral nerves with 4 blocks of shocks over a 1.5 h period. Previously, it was shown that 4 blocks of shocks delivered to an intact animal leads to a long-term (24 h) reduction of net outward currents in the sensory neurons. To mimic the long-term effects of behavioral training on pleural sensory cells, peripheral nerves P7, P8, and P9 which innervate the body wall of the tail, the midbody and the parapodium were stimulated through suction electrodes following procedures similar to those described previously by Walters and Byrne. The pattern of nerve stimulation (4 blocks of shocks over a 1.5 h period, see Fig. 1) was designed to simulate the stimulation used in long-term behavioral training.

The intensity of sensitizing stimuli used in previous behavioral studies was sufficient to elicit inking behavior. Consequently, in pilot experiments we confirmed that electrical shocks of peripheral nerves were effective by performing intracellular recordings from one of the L14 ink gland motor neurons in the abdominal ganglion which was left connected to the pleural ganglion via the pleuroabdominal connectives. Electrical stimulation of the peripheral nerves always produced firing of the nor-

![Fig. 2](image)
### TABLE I

Proteins affected by electrical stimulation of peripheral nerves, by 5-HT and by CPT-cAMP

<table>
<thead>
<tr>
<th>Protein (MW, pl)</th>
<th>Electrical stimulation</th>
<th>5-HT (5 × 10⁻⁶ M)</th>
<th>CPT-cAMP (10⁻³ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VA⁵</td>
<td>E/C⁶</td>
<td>E±S.E.M.⁵</td>
</tr>
<tr>
<td>P9 (43,5.3)</td>
<td>+ 2.0</td>
<td>1.4±0.3*</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>120 (26,4.5)</td>
<td>+</td>
<td>2.1</td>
<td>8.3±2.1*</td>
</tr>
<tr>
<td>65 (54,5.4)</td>
<td>-</td>
<td>0.5</td>
<td>2.3±0.4***</td>
</tr>
<tr>
<td>85 (26,4.7)</td>
<td>-</td>
<td>0.2</td>
<td>1.1±0.8**</td>
</tr>
<tr>
<td>12 (48,5.6)</td>
<td>-</td>
<td>0.7</td>
<td>4.4±1.1*</td>
</tr>
</tbody>
</table>

⁵ effects determined by visual analysis (VA).  +, increase of incorporation of [³⁵S]methionine; - , decrease of incorporation of [³⁵S]methionine; 0, no change of incorporation of [³⁵S]methionine. ⁶ mean percentage of integrated optical densities of an experimental (E±S.E.M.) protein and matched control (C±S.E.M.) relative to the integrated optical densities of a set of unchanging proteins. ⁷ ratio of the means of E and C (E/C). n, number of experiments used for the calculation of the means of E and C. The different n's within a treatment group is due to the absence of some proteins in certain sets of gels. Level of statistically significant difference between the percentages of the experimental and control groups: ***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, non significant. MW: molecular weight (kDa), pl: isoelectric point.

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**Fig. 3.** Current-voltage (I-V) relations of response families. Values plotted are the amplitude of the membrane current at the end of a 300 ms pulse to the indicated potential. A: the averaged I-V relation of cells from the control ganglia (□) or from the stimulated ganglia (●). B: I-V relation of the difference current.

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Mammally silent L₁₄ cell, indicating that the analogue mimicked behavioral sensitizing stimuli that cause the release of ink from the ink gland.

**Nerve stimulation reduced net outward currents in sensory neurons.** The membrane currents in the sensory cells from the stimulated ganglia were compared to the membrane currents in the sensory cells from the opposite non-stimulated control ganglia. The overall average of the net membrane currents for the 3 most depolarized test potentials from sensory neurons in the control ganglia and in the experimental ganglia are shown in Fig. 2A. Over the range of membrane potentials from -35 to +25 mV, the net membrane currents from the stimulated ganglia were significantly smaller than the net membrane currents from the control ganglia (t₀ = 2.97, P < 0.02, n = 10). Although the membrane currents that were elicited at hyperpolarized potentials (-65, -80 mV) were also smaller for the stimulated ganglia than those for the control ganglia, this effect was not statistically significant (t₀ = 1.99). The overall difference currents were determined by subtracting the traces of stimulated cells from the traces of the corresponding control cells (e.g., Fig. 2B). The current that is modified 24 h after nerve stimulation is relatively slow to activate and shows little or no inactivation. The current voltage (I-V) relationship of the net outward currents of the control and the stimulated sensory cells are presented in Fig. 3A. The I-V relation of the difference current is illustrated in Fig. 3B.
These results indicate that the net difference current has a mild voltage dependence and is active over a relatively wide range of membrane potentials. The long-term reduction of the overall membrane current of the sensory neurons produced by electrical stimulation of peripheral nerves is similar to the long-term effect produced by sensitization training in intact animals and by the intracellular injection of cAMP into individual sensory neurons.

Effects of treatments on incorporation of amino acids into proteins

To determine whether the in vitro training analogue affected the synthesis of protein, we examined changes in incorporation of \[^{35}\text{S}\]methionine into proteins of pleural sensory neurons. Groups of 3 experimental and 3 matched control pleural-pedal ganglia were exposed to \[^{35}\text{S}\]methionine for 2 h. One half hour after the label was applied, the peripheral nerves of experimental ganglia were electrically stimulated with 4 blocks of shocks over a 1.5 h period (Fig. 1). In some experiments 5-HT or an analogue of cAMP was applied to the ganglia for 1.5 h instead of nerve stimulation. At the end of the treatment, clusters of sensory cells were removed from the experimental and control ganglia and proteins from sensory cells were separated using 2D-PAGE (see Materials and Methods).

The treatments did not affect the overall incorporation of amino acids into proteins. Barzilai et al. observed increases and decreases in overall \[^{35}\text{S}\]methionine incorporation into proteins at several time points during and after continuous application of 5-HT (1.5 h) to isolated clusters of sensory neurons from pleural ganglia. However, they observed no effects on overall incorporation 1.5 h after the beginning of experimental treatment. The various experimental treatments used in our experiments also did not appear to affect the overall incorporation of \[^{35}\text{S}\]methionine at the end of our 1.5 h experimental treatments. The percentages of TCA precipitable radioactivity of the experimental samples relative to the control samples were 118 ± 44% (Mean ± 95% confidence interval, n = 16) for nerve stimulation, 91 ± 17% (n = 17) for 5-HT and 121 ± 65% (n = 11) for CPT-cAMP.

Nerve stimulation affected the incorporation of labeled amino acids into specific proteins. Incorporation of \[^{35}\text{S}\]methionine into a number of proteins in pleural sensory cells was affected by nerve stimulation. Fig. 4 shows fluorograms of two dimensional gels of labeled proteins from experimental and control clusters of pleural sensory neurons. The effects of treatments on proteins were determined by visual and computer analysis of the gels (see Materials and Methods). Incorporation of \[^{35}\text{S}\]methionine into 3 proteins 65, 85 and 12 was decreased and incorporation into 2 proteins (P9 and 120) was increased by nerve stimulation (Table I). Differences in the number of experiments (n) in which proteins are reported to be observed (Table I) was due to several factors. In some cases we were unable to either identify or quantify the proteins because their patterns were somewhat altered or proteins of interest overlapped with others. In other cases, the proteins did not appear in some gels. Loss of protein might occur during the experimental procedure at several different steps such as during solubilization of tissue or the loading of sample on the gel.

Fig. 4. Electrical stimulation affects incorporation of \[^{35}\text{S}\]methionine into several proteins. Upper: experimental pleural-pedal ganglia from 3 animals were exposed to \[^{35}\text{S}\]methionine for 2 h and to 4 blocks of electrical shocks over a 1.5 h period during the last part of the \[^{35}\text{S}\]methionine incubation period. Lower: matched control pleural-pedal ganglia from the same 3 animals were exposed to \[^{35}\text{S}\]methionine for 2 h but not stimulated. Incorporation of \[^{35}\text{S}\]methionine into specific proteins is proportional to the density of spots on fluorographs of two dimensional gels. Incorporation of label into proteins 65, 85 and 12 was decreased whereas incorporation into P9 and 120 was increased by nerve stimulation. Protein P9, often a faint spot on fluorographs, is located just above another protein which was not affected by nerve stimulation.

5-HT produced similar effects as the in vitro training analogue. Serotonin is believed to be an important transmitter that mediates the effects of sensitizing stimuli. To determine whether 5-HT mimicked the effects of nerve stimulation, the effects produced by 1.5 h of continuous application of 5-HT (5 × 10^{-6} M) on incorporation of amino acid were studied. Incorporation into 3 (P9, 65 and 85) of the 5 proteins that were affected by
the in vitro training analogue were also significantly affected by 5-HT (Table I). In one case, protein 120, the visual analysis of the gels indicated increased incorporation and the quantitative analysis also indicated an increase in incorporation (E/C = 1.4). However, the change in protein 120 produced by 5-HT was not statistically significant. Incorporation of [35S]methionine into protein 12 was not affected by 5-HT although it was decreased by nerve stimulation.

**CPT-cAMP produced similar effects as the in vitro training analogue.** Sensitizing stimuli lead to an elevation of cAMP in sensory neurons7,34 and cAMP can induce long-term electrophysiological and morphological changes associated with long-term sensitization29,30,36,40. Therefore, we compared the effects on proteins of CPT-cAMP with those produced by electrical stimulation and 5-HT. Incorporation of amino acid into 4 proteins (P9, 120, 65 and 85) was affected by CPT-cAMP (Table I). These proteins were affected in a similar way by the in vitro training analogue. Incorporation in protein 12 was not affected by CPT-cAMP.

**DISCUSSION**

To study the involvement of proteins in the modification of pleural sensory cells and the behavior of the animal, we developed an in vitro analogue of behavioral training, which utilized as a stimulus electrical stimulation of peripheral nerves of the pleural-pedal ganglion. The electrical stimulation of peripheral nerves appears to mimic the long-term reduction of net outward currents in sensory neurons produced by sensitization training in the intact animal39 and by the intracellular injection of cAMP into individual sensory neurons40. In addition, electrical stimulation of peripheral nerves leads to long-term enhancement of the sensory-motor connections responsible for mediating the tail withdrawal reflex34. These results indicate that the in vitro analogue of behavioral sensitization training is a useful model with which to explore further the mechanisms of long-term facilitation and particularly, the role of protein synthesis. To identify proteins responsible for sensitization, we examined changes in protein synthesis during a period in which protein synthesis is required for the induction of long-term sensitization.

We found that electrical stimulation of peripheral nerves induced increases or decreases of incorporation of amino acid into 5 proteins in pleural sensory neuron clusters. The effects of electrical stimulation on these 5 proteins were also produced by 5-HT and by CPT-cAMP. The finding that 3 proteins (proteins P9, 65 and 85) affected by electrical stimulation were affected similarly by 2 other different treatments indicates that these effects were produced by the experimental procedures. Protein 120 was also affected by electrical stimulation. Although the effect of 5-HT on protein 120 was not statistically significant, visual analysis indicated that this protein was affected by 5-HT. The effects of cAMP on protein 120 were statistically significant. Therefore, taken together, the results suggest that the experimental procedures also affected protein 120. These observations indicate that proteins P9, 65, 85 and 120 may have an important role in events responsible for the induction of the cellular changes associated with long-term plasticity of the sensory neurons. The results of our experiments also suggest that the effects of electrical stimulation of peripheral nerves on proteins are mediated, at least in part, by release of 5-HT leading to an increase in the level of cAMP in sensory neurons. These results are consistent with other studies showing that the induction of long-term electrical, biophysical and morphological effects can be initiated by application of 5-HT15,22,28 and an elevation of cAMP in sensory neurons29,30,38,40.

Although protein 12 was not affected by CPT-cAMP and 5-HT, the effect on incorporation into this protein by electrical stimulation suggests that electrical stimulation of peripheral nerves may produce release of transmitters other than 5-HT and also involves second messenger cascades other than cAMP37. Alternatively, since protein 12 was not affected by cAMP and 5-HT, effects of electrical stimulation on this protein have not been clearly established. The resolution of this issue will require additional experiments that focus on protein 12.

Thus far, only a few of the proteins found to change in our studies with electrical stimulation have been reported to be modified in other studies. The effects of 5-HT and an analogue of cAMP on the protein P9 has already been reported by Eskin et al.19 who studied the incorporation of [3H]leucine in pleural sensory neurons. The incorporation of leucine into 2 other proteins (P19 and P20) reported by Eskin et al.19 to be decreased by 5-HT and cAMP analogue, was not possible to analyse in the present study. The inability to analyse these proteins was due to the difference of labeled amino acid used in the two studies (leucine vs methionine) and also to a slight difference of protein separation during gel electrophoresis.

It is not surprising that many of the proteins reported changed by Barzilai et al.7 were not similar to those found in the present study. Barzilai et al.5 used experimental treatments to isolated clusters of sensory neurons, whereas the present study involved treatments to isolated ganglia and then removal of the sensory neurons for analysis. Also, the protein solubilization buffers and the two dimensional polyacrylamide gel electrophoresis techniques used in these studies were rather different.
Therefore, it is likely that somewhat different populations of proteins were being studied in the two laboratories. However, the proteins D5 and E10 reported by Barzilai et al.⁵ may be similar to our proteins 85 and 120, respectively.

The events inducing the protein changes produced by electrical stimulation, 5-HT, and CPT-cAMP are unknown so far. Transcriptional events, translational changes or post-translational modifications of proteins could be responsible for the observed changes in proteins. It will be important to determine the mechanisms responsible for the observed changes of proteins. Studies, currently underway, of protein phosphorylation and of expression of mRNA under the same conditions in which we have studied incorporation of amino acids should shed some light on the mechanism of protein changes. Future studies must also be directed towards identifying the cellular function of the proteins changed by the in vitro training analogue. Towards this end, we are presently obtaining amino acid sequences of these proteins.

Our results demonstrate that the in vitro analogue of the training procedure produced early effects on the incorporation of labeled amino acid into proteins. These results together with studies showing that the cellular electrophysiological changes produced by the in vitro training analogue were similar to those produced by behavioral training,⁹ show that the in vitro training analogue may be used as a bridge between cellular studies and the behavior of the animal for the investigation of the molecular events involved in long-term sensitization in Aplysia. The set of early proteins affected by the in vitro training analogue warrants further study as putative components of the mechanisms responsible for the induction of long-term sensitization. One other important issue that must be addressed is the relationship between protein changes observed just after the induction stimulus as those reported here and protein changes occurring at longer intervals after the stimulus.⁵ Recent results of experiments utilizing the in vitro training analogue and then examining protein changes 24 h later have shown that a few of the early changes are present 24 h later but also proteins that were affected 24 h later were not affected just after the stimulus.⁵ Experiments such as these at other time points will help yield an understanding of the relationship between changes of proteins and the induction, mediation and maintenance of long-term sensitization.

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