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Associative conditioning analog selectively increases cAMP levels of tail sensory neurons in *Aplysia*

(synaptic facilitation/serotonin/activity-depependent neuromodulation)

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ABSTRACT **Bilateral clusters of sensory neurons in the pleural ganglia of *Aplysia* contain cells involved in a defensive tail withdrawal reflex. These cells exhibit heterosynaptic facilitation in response to noxious skin stimulation that can be mimicked by the application of serotonin. Recently it has been shown that this facilitation can be selectively amplified by the application of a classical conditioning procedure to individual sensory neurons. We now report that an analog of this classical conditioning paradigm produces a selective amplification of the cAMP content of isolated sensory neuron clusters. The enhancement is achieved within a single trial and appears to be localized to the sensory neurons. These results indicate that a pairing-specific enhancement of cAMP levels may be a biochemical mechanism for associative neuronal modifications and perhaps learning.**

Associative modifications within individual neurons have recently been implicated as mechanisms for associative learning and memory (1–5). A recently developed preparation that offers particular promise for the analysis of biochemical and biophysical modifications underlying neural plasticity is the defensive tail withdrawal reflex in the marine mollusc *Aplysia*. An associative conditioning paradigm applied directly to individual sensory neurons can specifically modify monosynaptic connections between these cells and the motor neurons involved in tail withdrawal (3). This was shown with a differential conditioning procedure that utilized intracellular activation of individual tail sensory cells as the conditioned stimulus (CS) and shock to the skin as the unconditioned stimulus (US). Since the US was delivered outside the receptive fields of the sensory neurons examined, these neurons were not activated by the US. However, they were exposed to diffuse neuromodulatory effects of the US that produced heterosynaptic facilitation of all of the connections of the sensory neurons (3). Paired presentation of the CS and US resulted in a significant enhancement of the excitatory postsynaptic potential (EPSP) amplitude compared to the facilitation produced when the CS and US were separated by 2 min or when the US was presented alone (3). We termed this enhancement "activity-dependent neuromodulation" because the effects of a presumed neuromodulator released by the US are specifically enhanced by spike activity that immediately precedes the US. Although models formally similar to activity-dependent neuromodulation have been proposed previously as candidate mechanisms for associative memory (6–8), these and similar results obtained independently by Hawkins *et al.* (4) in *Aplysia* provided direct experimental evidence for such a phenomenon at the cellular level.

The tail withdrawal is sensitized by the same weak electrical stimulation of the skin used as the US (above), and the sensitization is accompanied by heterosynaptic facilitation of the monosynaptic connections between the sensory and motor neurons (9). This heterosynaptic facilitation can be mimicked by substituting application of serotonin (5-hydroxytryptamine; 5-HT) for the sensitizing electrical stimulus (9), and the 5-HT effects appear to be mediated by cAMP (10–12). Based on the observations that spike activity in the sensory neurons seems to amplify the effects of sensitization (3) and that the effects of 5-HT and sensitization appear to be linked to increased levels of cAMP, we predicted that pairing spike activity with a sensitizing stimulus would lead to an associatively specific enhancement of cAMP levels. As a first step in testing this hypothesis at the biochemical level we further simplified the conditioning procedure and developed a preparation in which we could readily measure cAMP levels. For these experiments we substituted depolarization produced by exposure to high K^+ artificial seawater (ASW) for the spike activity that had been used as the CS and substituted exposure to 5-HT for the tail shock that had been used as the US (3). In this paper we describe the effects of such a conditioning analog on the cAMP levels of isolated clusters of sensory cells that contain the tail sensory neurons. A portion of this work has been reported (42).

MATERIALS AND METHODS

Aplysia californica (150–350 g) were obtained from Marine Specimens Unlimited (Pacific Palisades, CA) and were maintained in ASW at 15°C. Prior to dissection, animals were anesthetized with injections of a volume of isotonic $MgCl_2$ equal to approximately one-half of their body weight. The cell bodies of the sensory neurons that innervate the tail comprise a subset of a distinctive cluster of sensory cells that is found in both the left and right pleural ganglia (13). All of the sensory neurons in this cluster have similar electrophysiological and biophysical properties (12, 13). Both of the pleural ganglia were removed and desheathed. A cluster of about 100 sensory neuron somata was surgically isolated from each pleural ganglion by undercutting the somata with iridectomy sissors. Clusters were then equilibrated for 2 hr in Petri dishes containing ASW (10 mM KCl/460 mM NaCl/55 mM $MgCl_2$/10 mM $CaCl_2$/10 mM Hepes, pH 7.6). These isolated sensory cell clusters were exposed to the various experimental treatments by transferring clusters using 100-μl glass Micropets (Fisher) to dishes containing $\approx$10 ml of the appropriate solutions (cluster plus transfer volume was <20 μl). The phosphodiesterase inhibitor Ro 20-1724 was included at a concentration of 0.1 mM during all experimental manipulations. Intracellular recordings from isolated clusters were obtained with single electrodes by using conventional electrophysiological techniques (13). All experiments were performed at room temperature (20–22°C).

The cAMP content of sensory neuron clusters was ana-

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Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); CS, conditioned stimulus; US, unconditioned stimulus; ASW, artificial seawater; EPSP, excitatory postsynaptic potential.

lyzed by radioimmunoassay according to the methods of Brooker *et al.* (14). Clusters were frozen in 5 mM sodium acetate assay buffer containing 10% trichloroacetic acid immediately following exposure to the experimental protocol. Tissue was homogenized by sonication and centrifuged to remove precipitable protein and the supernatant was assayed for cAMP content. In our hands, the limit of resolution for this assay was 6 fmol of cAMP and our lowest measured sample was 20 fmol. Iodinated succinyl cAMP was prepared from succinyl cAMP tyrosine methyl ester and $Na^{125}I$ (New England Nuclear) by the above published procedures. Antibody to cAMP was a gift from Jeffrey F. Harper.

The protein content of both the sensory neuron clusters and the pleural ganglia was analyzed by using a fluorogenic reagent, fluorescamine (Roche Diagnostics), according to the procedure of Udenfriend *et al.* (15). Samples were lyophilized to dryness and solubilized in 1 M NaOH, and the pH was adjusted to 9.2 with boric acid. Following addition of reagent, sample fluorescence was determined on a Turner spectrofluorimeter (model 430).

These procedures were performed as double-blind experiments. Since a single *Aplysia* has two symmetrical clusters of sensory neurons (13), each animal served as its own control and, unless otherwise indicated, a two-tailed *t* test for non-independent groups was used to analyze the data. Significance levels were 0.05.

5-HT was obtained from Sigma and Ro 20-1724 was a gift from Hoffmann–LaRoche.

RESULTS

Demonstration of a 5-HT-Sensitive Adenylate Cyclase. Although the natural neuromodulator mediating the facilitatory effects of the US has not yet been identified, 5-HT mimics both the heterosynaptic facilitation of the connections of the tail sensory neurons and sensitization of the reflex behavior (9). We therefore used 5-HT as an agonist of the natural neuromodulator. We first examined the ability of 5-HT to increase the cAMP content of the sensory neurons involved in tail withdrawal. Bath application of 0.1 mM 5-HT to isolated clusters for 5 min resulted in an elevation of cAMP levels by 89% ± 18% (mean ± SEM, $n = 12$, $t_{11} = 3.43$, $P < 0.01$) relative to the contralateral controls exposed to ASW alone. These results corroborate previous demonstrations of a 5-HT-induced increase in cAMP synthesis in the siphon sensory neurons (16) and tail sensory neurons (11) and indicate the presence of a 5-HT-sensitive adenylate cyclase in the sensory neuron cluster containing the tail sensory cells (see also ref. 12).

Effect of Conditioning Analog on cAMP Levels of Isolated Sensory Neuron Clusters. We tested the hypothesis that depolarization during spike activity (CS) can enhance cAMP synthesis in response to 5-HT application (US) by measuring cAMP levels in the sensory neurons. Sensory neuron clusters were exposed to a modification of the conditioning paradigm used by Walters and Byrne (3) (Fig. 1, protocol 1). A 5-sec exposure to high-K^+ ASW (340 mM K^+, equimolar substitution for Na^+) was used to depolarize the entire sensory neuron cluster and served as the CS. Intracellular recordings from sensory cells in an isolated cluster demonstrated that the high-K^+ ASW depolarized these cells by ≈40 mV. This degree of depolarization should activate voltage-dependent Ca^{2+} channels in these cells (see *Discussion*) (17). A 15-sec exposure to 5 μM 5-HT served as the US. Both the concentration and duration of exposure to 5-HT were reduced from that used above to insure that the 5-HT effects were submaximal. The concentration used is close to the concentration reported to exert half-maximal effects on cAMP production in isolated sensory cell clusters (43) and in the abdominal ganglion (18). Pairing of the stimuli was accomplished by exposing isolated clusters to high-K^+ ASW (CS) immediately followed by exposure to 5-HT (US). Contralateral clusters received a specifically unpaired presentation with the CS administered 2.5 min prior to presentation of the US. Exposure to high-K^+ ASW in each cluster of sensory cells was balanced with a control exposure to ASW in the contralateral cluster.

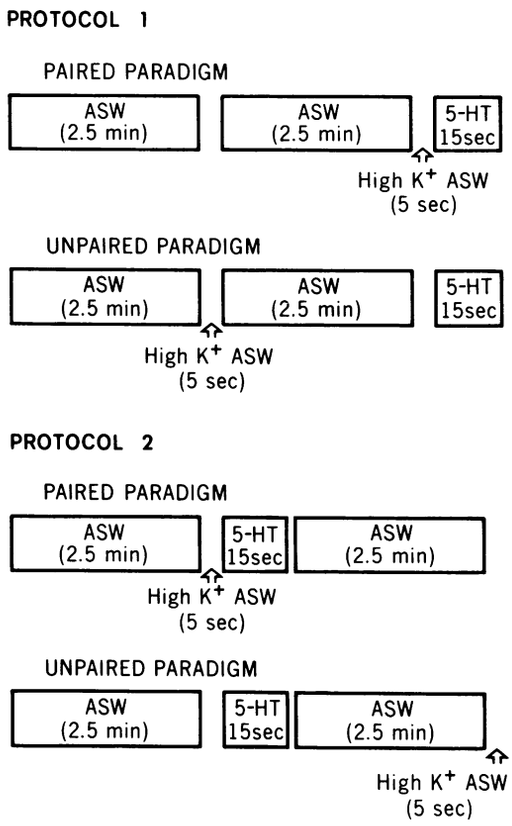

FIG. 1. Schematic representation of the protocols used for the paired and unpaired presentation of CS (5-sec exposure to high-K^+ ASW) and US (15-sec exposure to 5 μM 5-HT). The only difference between the paired and the unpaired paradigms is the timing of the CS relative to the US. Protocol 1, exposure to the CS precedes the US by 2.5 min in the unpaired presentation. Protocol 2, exposure to the CS follows the US by 2.5 min in the unpaired presentation.

The cAMP content of treated tissue was determined by radioimmunoassay, with the cAMP levels normalized to protein content. A *t* test for non-independent groups was used to analyze the results. The ability to compare pairs of clusters within each animal was useful since considerable variability in cAMP levels was observed among animals. Both the experimental and the control groups had equal numbers of clusters from right and left pleural ganglia. We found that the cAMP levels of sensory neuron clusters receiving the paired presentation of the CS and US were significantly elevated ($n = 21$, $t_{20} = 2.90$, $P < 0.01$) relative to the levels seen in the contralateral control clusters receiving the unpaired paradigm (Figs. 2*A* and 3, protocol 1). These results confirmed those obtained in an initial pilot study ($n = 15$, $t_{14} = 2.63$, $P < 0.02$, data not shown).

The effect of the CS alone or the US alone on cAMP levels was also examined. Following preincubation in ASW for 5 min, one of each pair of clusters was exposed either to a 5-sec pulse of high-K^+ ASW (CS) followed by 15 sec in ASW or to 5 sec in ASW followed by a 15-sec exposure to 5 μM 5-HT (US). Contralateral clusters served as controls and were exposed for an equal time to ASW alone. Only slight increases in cAMP levels were observed in response to either the US ($n = 19$, $t_{18} = 1.72$) or the CS ($n = 18$, $t_{17} = 0.25$)

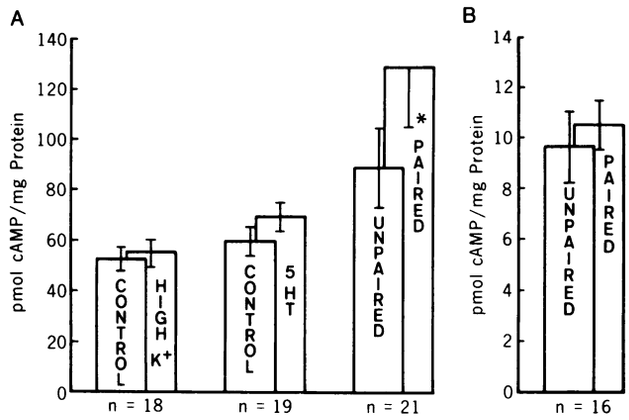

FIG. 2. (*A*) Effects of CS alone (5-sec exposure to high-K^+ ASW), US alone (15-sec exposure to 5 μM 5-HT), and conditioning analog (see Fig. 1, protocol 1) on cAMP levels of tail sensory neurons. Asterisk indicates difference is significant. (*B*) Effects of conditioning analog (Fig. 1, protocol 1) on cAMP levels of pleural ganglia remnants (sensory neuron clusters were removed). Mean (±SEM) cAMP levels are expressed as pmol of cAMP per mg of protein.

alone compared to the ASW control and neither effect was statistically significant (Fig. 2*A*). We did not perform separate experiments to examine whether there were differences between the US (or CS) alone and the paired (or unpaired) CS and US.

To examine whether the pairing effects were also found in other parts of the nervous system, the portions of the desheathed pleural ganglia that remained following removal of the sensory neuron clusters were exposed to an identical conditioning analog. An analysis of the cAMP levels in these pleural ganglia remnants showed no significant difference between the remnants receiving the paired and those receiving unpaired treatments ($n = 16$, $t_{15} = 0.71$, Fig. 2*B*). A similar conditioning analog applied to single, intact abdominal ganglia also revealed no significant difference in cAMP levels when ganglia receiving the paired treatment were compared to ganglia receiving the unpaired treatment ($n = 30$, $t_{28} = 0.21$, t test for independent groups). These results suggest that the pairing-specific effects on cAMP levels may be largely limited to the sensory neurons. However, by analyzing these larger groups of neurons it is possible that increases in cAMP levels occurring in only a few cells were obscured or that the high-K^+ ASW and 5-HT treatments failed to affect all portions of the ganglion.

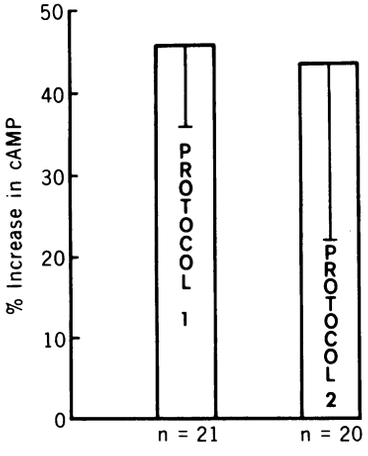

FIG. 3. Similar effects of two different conditioning protocols on cAMP levels. Mean (±SEM) cAMP levels of sensory neuron clusters receiving the paired stimuli expressed as a percent of the contralateral control (unpaired stimuli). Paired presentation of CS and US with either protocol 1 (raw data shown in Fig. 2*A*) or protocol 2 resulted in increases (47% and 44%, respectively) in the cAMP levels.

Order Effects of the CS and US. It was conceivable that the relative enhancement of cAMP levels was not a direct consequence of pairing the CS and US but, instead, was due to an indirect effect of the order of the unpaired presentation of these stimuli. To test this possibility we performed an additional control experiment in which the unpaired presentation of the CS and US was altered so that the US preceded the CS (Fig. 1, protocol 2). The paired paradigm was also altered slightly to compensate for the altered timing sequence. The results confirmed those obtained by using the original paradigm. The paired presentation of CS and US resulted in a significant elevation ($n = 20$, $t_{19} = 3.23$, $P < 0.01$) in the cAMP levels of sensory neuron clusters relative to those of the contralateral clusters receiving the unpaired presentation (Fig. 3, protocol 2). An identical analysis of the remnants of the pleural ganglia again revealed no significant difference between cAMP levels in response to the two treatments ($n = 18$, $t_{17} = 1.14$, data not shown).

DISCUSSION

Our results demonstrate a pairing-specific enhancement of cAMP levels that is produced by a single pairing and that appears to be localized to the sensory neurons. Although the effects observed in the present study were measured in the sensory cell soma, similar effects may also take place in presynaptic terminals, where an increase in cAMP levels could result in enhanced release of neurotransmitter. In both the tail sensory neurons and the extensively studied siphon sensory neurons of *Aplysia* an electrical US applied to either the skin or a nerve produces heterosynaptic facilitation (which contributes to sensitization), perhaps by causing the release of 5-HT or a related neuromodulator, which activates adenylate cyclase in the sensory neurons (9, 11, 12, 19–21) (Fig. 4*A*, based on ref. 21). The cAMP produced by 5-HT activation of adenylate cyclase in turn closes resting K^+ channels (S-channel, ref. 23), presumably by activating one or more protein kinases, which phosphorylate regulatory proteins associated with K^+ channels or the K^+ channel itself (24, 25). Closure of these K^+ channels delays the repolarization of the action potential, allowing additional influx of Ca^{2+} ions and enhanced transmitter release (21, 22). Based on this cellular model for sensitization, Walters and Byrne (3) and Hawkins *et al.* (4) independently proposed activity-dependent neuromodulation as a mechanism for associative conditioning. The model illustrated in Fig. 4*B* is based on the molecular mechanism suggested by these investigators. According to the model, the CS causes depolarization, which enhances Ca^{2+} influx, which, in turn, enhances the synthesis of cAMP in response to a neuromodulator released by the US. Thus, increased cAMP levels might provide a biochemical mechanism for encoding information about the temporal association of separate inputs to these cells. This information would be provided by the proximate and sequential interaction of Ca^{2+} and 5-HT (or related neuromodulators) with the adenylate cyclase complex. The associative memory would be produced by the persistent effects of the enhanced cAMP levels. Evidence supporting such a Ca^{2+} interaction with cAMP is provided by studies of vertebrate brain tissue in which a Ca^{2+}/calmodulin-dependent activation of neurotransmitter-stimulated adenylate cyclase has been demonstrated (26–28). More direct support of this hypothesis is provided by studies of *Drosophila* in which it has been shown that a mutant deficient in associative learning also exhibits a loss of Ca^{2+}/calmodulin sensitivity of the particulate adenylate cyclase (29, 30). Preliminary evidence in-

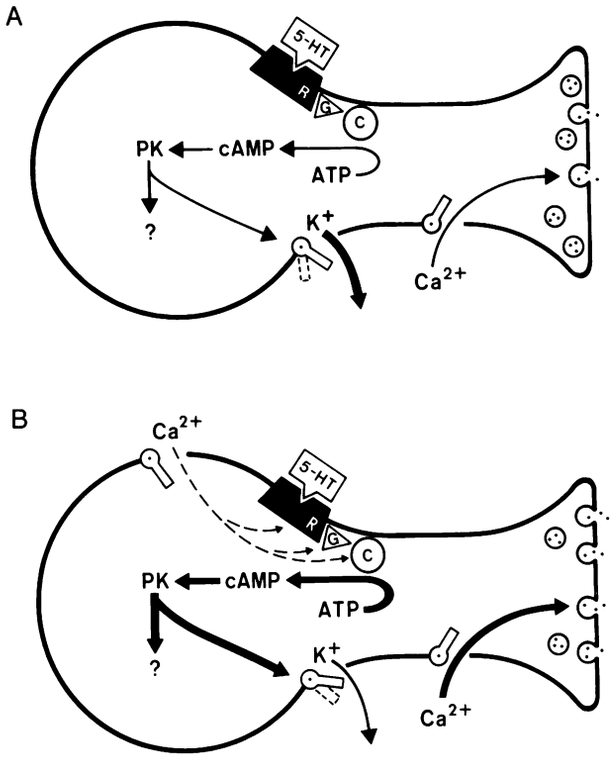

FIG. 4. Model of possible molecular events contributing to heterosynaptic facilitation and activity-dependent neuromodulation. (*A*) Heterosynaptic facilitation (which may account for sensitization). Neurotransmitter (5-HT) binding to receptor (R) activates adenylate cyclase (C) via a regulatory subunit (G). The resulting cAMP activates one or more protein kinases (PK), whose action(s) includes closure of steady-state K^+ channels. Closing K^+ channels results (indirectly) in increased Ca^{2+} influx and increased transmitter release (modified from ref. 21). (*B*) Activity-dependent neuromodulation (which may be a mechanism for associative learning). An initial influx of Ca^{2+} affects one or more components of the adenylate cyclase complex. Subsequent transmitter activation of the cyclase results in an amplification of cAMP production, ultimately resulting in enhanced transmitter release relative to that produced by 5-HT alone. Additional or longer term effects of amplified cAMP production (mediated by increased activation of protein kinases) are also possible (see *Discussion* and refs. 20 and 22).

dicates the presence of a Ca^{2+}-sensitive cyclase in *Aplysia* as well (31).

The results reported here represent an initial attempt to test the molecular mechanism for activity-dependent neuromodulation discussed above. Although our results do not address all aspects of this model, they do support the hypothesis that information regarding temporally contiguous inputs to *Aplysia* sensory cells is encoded by changing cAMP levels. Recently, Abrams *et al.* (32) have shown that spike activity paired with 5-HT increases the cAMP content in siphon sensory neurons of *Aplysia* when compared with application of 5-HT alone. These preparations should allow one to determine the precise temporal relationships of the CS and the US necessary for producing enhanced cAMP synthesis. They should also allow direct tests of the proposed role of Ca^{2+} as the intracellular messenger for the CS (3, 4) and the relative contributions of degradation and synthesis to changing cAMP levels in this tissue (33). In addition, it will be possible to test the hypothesis that enhanced cAMP synthesis due to activity-dependent neuromodulation actually underlies learning since the tail withdrawal reflex is capable of differential classical conditioning (34).

Recently, long-term changes in synaptic transmission and in the excitability of these neurons have been found (35). In view of these persistent effects it is interesting to speculate that the increased cAMP content observed in tail sensory neuron somata is not simply a reflection of events occurring at the synaptic terminals. The fluctuations in the somatic cAMP content might play a role in triggering the long-term changes by acting on cellular elements localized to the soma. Such long-term changes could involve alterations in cell metabolism (e.g., ref. 36) or in genomic regulation and protein synthesis (37–39) brought about via cAMP-mediated phosphorylations (40, 41). Thus, altered somatic cAMP levels may provide a biochemical link between short- and long-term plastic changes in neurons.

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