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Two endogenous neuropeptides modulate the gill and siphon withdrawal reflex in *Aplysia* by presynaptic facilitation involving cAMP-dependent closure of a serotonin-sensitive potassium channel

(behavioral plasticity/synaptic plasticity/neuromodulation/single channel analysis)

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ABSTRACT **We have found that two endogenous neuropeptides in *Aplysia*, the small cardioactive peptides SCP_A and SCP_B, facilitate synaptic transmission from siphon mechanosensory neurons and enhance the defensive withdrawal reflex that these sensory neurons mediate. Single-channel recording revealed that these peptides close a specific K^+ channel, the S channel, which is sensitive to cAMP. Moreover, the peptides increase cAMP levels in these sensory neurons. This reduction in K^+ current slows the repolarization of the action potential in these cells, which increases transmitter release. In these actions, the SCPs resemble both noxious sensitizing stimuli, which enhance the reflex, and serotonin. Bioassay of HPLC fractions of abdominal ganglion extracts and immunocytochemistry indicate that both the SCPs and serotonin are present in the ganglion and are found in processes close to the siphon sensory neurons, suggesting that these transmitters may be involved in behavioral sensitization. Recent evidence suggests that one group of identified facilitatory interneurons, the L_{29} cells, does not appear to contain either the SCPs or serotonin but may use yet another facilitatory transmitter. Thus, it appears that several transmitters can converge to produce presynaptic facilitation in the sensory neurons of the defensive withdrawal reflex. All of the transmitters studied here, the SCPs and serotonin, act via an identical molecular cascade: cAMP-dependent closure of the S-K^+ channel, broadening of the presynaptic action potential, and facilitation of transmitter release.**

Peptide transmitters have been a focus of interest in neurobiology because they often have modulatory effects on behavior (1). However, it has only rarely been possible to relate the behavioral action of a neuropeptide to its cellular action on specific nerve cells (2–4, 38). We here report that two molluscan neuropeptides increase the gain of the gill and siphon withdrawal reflex of *Aplysia* by presynaptically facilitating transmission from the sensory neurons of the reflex pathway. By combining single-channel and biochemical analyses, we have found that this facilitation involves the cAMP-mediated closure of a specific K^+ channel, the recently described S channel (5).

This defensive withdrawal reflex in *Aplysia* is enhanced during two simple forms of learning: sensitization and classical conditioning (6, 7). In each case, a noxious stimulus produces presynaptic facilitation of synaptic transmission from the afferent neurons in the reflex, the mechanosensory neurons in the abdominal ganglion that innervate the siphon, to interneurons and motoneurons (8, 9). Although application of serotonin to the abdominal ganglion stimulates the facilitatory actions of noxious stimuli (10, 11), there is now evidence from the immunocytochemical studies of Ono and McCaman (12) and Kistler *et al.* (13) that at least one set of facilitatory interneurons, the L_{29} cells (14), is not serotonergic. Thus transmitters other than serotonin are involved in sensitization. We were therefore interested in identifying additional transmitters in the *Aplysia* central nervous system that are capable of producing presynaptic facilitation.

We fractionated extracts of abdominal ganglia by HPLC and assayed the resulting fractions for their ability to produce the broadening of the action potential in the siphon sensory neurons that is a concomitant of presynaptic facilitation in these cells (11). We found three predominant peaks of spike-broadening activity: one of these was serotonin; the other two had retention times identical to those of the small cardioactive peptides, SCP_A and SCP_B. These two peptides are 11 and 9 amino acids long, respectively, have similar sequences (15, 16), and appear to be derived from a common precursor (17). The two peptides also have similar actions, and these actions resemble those of serotonin on several peripheral molluscan tissues (18, 19). We therefore began our study of additional facilitatory transmitters by studying these two peptides. We wished to determine whether the peptides broaden the action potential in the sensory neurons via the same molecular cascade that is activated by serotonin: a cAMP-dependent closure of the S-K^+ channel (5, 20–23). In addition, we asked whether the SCPs thereby facilitate synaptic transmission from siphon sensory neurons and enhance the defensive withdrawal reflex.

METHODS

Aplysia californica (100–200 g) were anesthetized by injection of isotonic $MgCl_2$ (100% of body weight). Dissections were done in 50% isotonic $MgCl_2$/50% normal saline to block synaptic transmission. Preparations were then perfused either with normal saline (460 mM NaCl/10 mM KCl/55 mM $MgCl_2$/11 mM $CaCl_2$/10 mM Hepes, pH 7.6) or with high-Ca^{2+}/high-Mg^{2+} saline (212 mM NaCl/10 mM KCl/66 mM $CaCl_2$/165 mM $MgCl_2$/10 mM Hepes, pH 7.6) to reduce the activity of interneurons. In a number of experiments, 50 mM tetraethylammonium (Et_4N^+) chloride was added to the saline.

For experiments on peptide modulation of the gill withdrawal response, the isolated gill, siphon, and mantle shelf were left attached to the abdominal ganglion via the branchial, siphon, and genital nerves. The desheathed abdominal ganglion was isolated in a Vaseline-sealed ring and perfused separately from the peripheral tissues; transmitters were applied only to the ganglion. The separation of the two baths was verified using fast green dye. The siphon was stimulated with 5-msec pulses via implanted silver electrodes.

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Abbreviations: SCP, small cardioactive peptide; Et_4N^+, tetraethylammonium; EPSP, excitatory postsynaptic potential.

Electrophysiological recordings were made from the somata of the siphon sensory neurons in the LE cluster (24). Peptides and serotonin were applied by adding them to the perfusate or by pressure ejecting them onto the sensory neurons from a micropipette. Synaptic facilitation was observed most reliably if application of the peptides was brief.

Single-channel recordings were made from sensory neurons after gigaseals had been obtained using fire-polished patch pipettes filled with normal saline (5). SCP_B was added to the bath, not to the patch pipette.

The levels of cAMP in sensory neuron clusters were determined by RIA (New England Nuclear) and were normalized to the amount of intracellular ATP (the cAMP precursor) to control for differences in the sizes of the clusters. ATP was measured by luciferin–luciferase (Sigma) assay (25). Ganglia were frozen by replacing the bath with propylene glycol/2 M NaCl (1:1) at −30°C and kept at −20°C for 20 min. The clusters were then dissected out at −10 to −20°C (26).

To provide material for bioassays of endogenous facilitatory substances, frozen abdominal ganglia were heated in 0.1 M acetic acid for 10 min at 100°C, homogenized, and centrifuged at 20,000 × *g* for 20 min. The supernatant was fractionated by reversed-phase HPLC on a Dupont C8 column, developed with a gradient of 5–70% acetonitrile in 50 min. The mobile phase contained 0.01 M trifluoroacetic acid. Each fraction, derived from extracts of 30–50 ganglia, was dried, dissolved in 50 μl of saline, and injected into a continuously perfused 100-μl chamber containing the abdominal ganglion. Synthetic SCP_B and Phe-Met-Arg-Phe-NH_2 were obtained from Peninsula Laboratories (Palo Alto, CA) and synthetic SCP_A was from Sequemat (Watertown, CT).

RESULTS

HPLC Reveals That Two Endogenous Peptides and Endogenous Serotonin Broaden the Sensory Neuron Action Potential. To determine which endogenous neurotransmitters in the abdominal ganglion have facilitatory actions on the sensory neurons of the gill and siphon withdrawal reflex, we fractionated extracts of abdominal ganglion and assayed the effects of each fraction on the sensory neurons. Presynaptic facilitation that occurs in these neurons during sensitization is accompanied by the closure of a particular K^+ channel, the S channel (5, 23). This reduces the amount of K^+ current available for repolarizing the action potential and thus increases the duration of the presynaptic action potential (11, 20). We used the broadening of the presynaptic action potential measured in the presence of 50 mM Et_4N^+ as a sensitive index of the presynaptic facilitation response (11). At 50 mM, Et_4N^+ blocks a substantial portion of the K^+ channels that are not affected by facilitatory input and places the burden of repolarizing the action potential on the S channels, making the spike duration very sensitive to changes in the activity of these channels.

We observed three predominant peaks of spike-broadening activity (Fig. 1). The first represents endogenous serotonin. The two later peaks had retention times identical to those of two small *Aplysia* neuropeptides, SCP_A and SCP_B. We would emphasize that these three major peaks do not represent all of the endogenous facilitatory substances. First, there were several smaller peaks of spike broadening activity, which we have not yet characterized (Fig. 1) and which might represent physiologically important facilitatory transmitters. In addition, a number of fractions produced spike narrowing; facilitatory activity could have been masked if it was present in those fractions. Finally, we were unable to assay the fractions eluting in the first 8 min because of the high concentrations of salt from this large amount of tissue.

We also observed a narrowing of the action potential in two fractions with retention times identical to those of two transmitters present in the abdominal ganglion, dopamine (28) and Phe-Met-Art-Phe-NH_2 (ref. 29 and unpublished observations). Synthetic dopamine and Phe-Met-Arg-Phe-NH_2 had similar narrowing effects on the action potential.

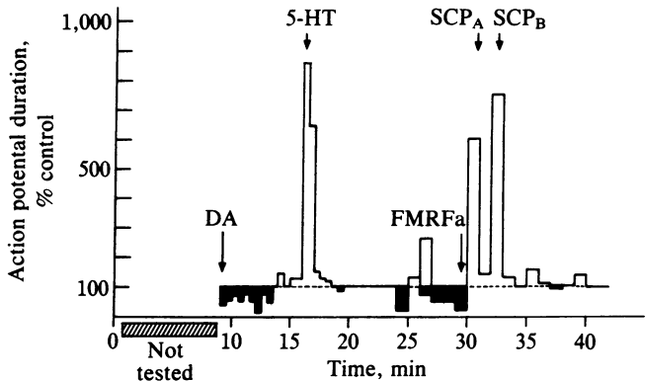

FIG. 1. Effects of HPLC fractions of abdominal ganglia on duration of the action potential in siphon sensory neurons. Action potentials in sensory neurons were recorded at 20-sec intervals in high-Ca^{2+}/Mg^{2+} saline with 50 mM Et_4N^+. Responses >100% represent maximal spike broadening and those <100%, maximal spike narrowing within 2 min after addition of a fraction. Given the stability of the spike duration in these experiments, changes >15% were considered to be significant. The starting material for this fractionation was 50 abdominal ganglia. Each data point represents the response of one sensory neuron to a single fraction; this histogram consists of data acquired from a total of nine sensory neurons in six preparations. Qualitatively similar results were obtained with two other HPLC fractionations. The retention times of dopamine (DA), serotonin (5-HT), Phe-Met-Arg-Phe-NH_2 (FMRFa) and the SCPs are indicated.

We have focused on the three major peaks of facilitatory activity that we observed. Specifically, we have characterized the effects of the SCPs both on the siphon sensory neurons and on the withdrawal reflex that they mediate, and we have compared their actions with the known actions of serotonin. In most of our study we used synthetic SCP_B because this peptide was available at the outset of the study. More recently SCP_A has also been sequenced and synthesized (16) and a number of the critical experiments were repeated with it.

Synthetic SCPs also Increase the Duration of the Presynaptic Action Potential. Because two of the peaks of facilitatory activity from HPLC had retention times equal to those of the neuropeptides SCP_A and SCP_B, we first tested whether the synthetic SCPs increase the duration of the action potential in the siphon sensory neurons. In >30 preparations, in the presence of extracellular Et_4N^+, 1 μM SCP_B broadened the action potentials more than 3-fold. The peptides appear to act directly on the sensory neurons rather than through interneurons since responses to SCP_B were also observed in clusters of sensory neurons surgically isolated from the remainder of the abdominal ganglion (Fig. 2) and in the presence of high-Ca^{2+}/high-Mg^{2+} saline, which raises the spike threshold of interneurons. This response to SCP_B decreased in a concentration-dependent manner, reaching a threshold at <1 nM. In four preparations, 1 nM SCP_B produced a 25 ± 10% (mean ± SD) increase in spike duration. In six experiments in which we tested synthetic SCP_A, we found that this peptide produced similar broadening of the action potential. Moreover, we also found that both peptides caused spike broadening in a second group of mechanosensory neurons, the neurons in the VC cluster in the pleural ganglion, which mediate the tail withdrawal reflex (30).

At low concentrations (<1 μM), SCP_B was more effective than the same concentrations of serotonin. Indeed, the

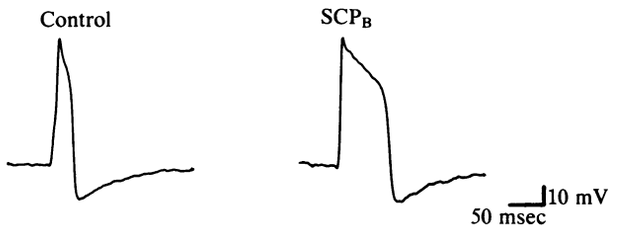

FIG. 2. Effect of 1 μM SCP_B on the action potential of isolated sensory neurons. Action potentials were recorded from a cell in a surgically isolated cluster of siphon sensory neurons in 50 mM Et_4N^+/saline.

threshold for spike broadening was 50-fold higher for serotonin (22) than for SCP_B. However, at higher concentrations, the SCPs and serotonin produced similar maximal effects.

SCP_B Closes the S Channel. Klein *et al.* (23) found that extracellular application of serotonin broadens the action potential in the sensory neurons by decreasing a specific K^+ current called the serotonin-sensitive K^+ current, or I_S. This current is different from three other voltage-sensitive K^+ currents in these neurons (the delayed, I_K; the early, I_A; and the Ca^{2+}-activated, I_C). The S channel has also been identified by single-channel recording and its sensitivity to serotonin has been characterized (5). We have asked whether the S channel is also closed by SCP_B. S channels were selectively recorded by holding the transmembrane potential of the patch near 0 mV, thereby inactivating the early and delayed K^+ channels. Since the cell itself remains at resting potential, the level of intracellular Ca^{2+} is below that required for opening the Ca^{2+}-activated K^+ channel. In seven of nine experiments, SCP_B, at concentrations of 1–100 μM, reduced the number of open channels observed in the patch (Fig. 3). The channels modulated by the peptide had the properties characteristic of S channels (5): the average slope conductance at 0 mV was 52 ± 6 pS (mean ± SD), the channel gating was relatively insensitive to voltage, and the channels did not inactivate. Similarly, the action of SCP_B on the channels was indistinguishable from that of serotonin (5); the peptide caused prolonged, all or none, closure of individual channels and did not affect the conductance or kinetics of channels that remain open.

SCP_B Increases the Level of cAMP. The closure of the S-K^+ channel and broadening of the action potential produced by serotonin result from increased cAMP-dependent protein phosphorylation in the sensory neurons (21, 22). It therefore seemed likely that the effects of SCP_B on the channel might be mediated by an increase in intracellular cAMP levels. To explore this possibility, we measured cAMP levels in sensory neurons exposed to SCP_B. A 60-sec exposure to 100 μM SCP_B resulted in a 2- to 4-fold increase in the level of cAMP in siphon sensory neurons. For example in one experiment the amount of cAMP per sensory neuron cluster (expressed as percentage of ATP) increased from 0.057 ± 0.017% in controls (n = 8) to 0.21 ± 0.051% with SCP_B (n = 10; mean ± SEM; $P < 0.02$, two-tailed t test). This is comparable to the cAMP elevations observed in response to serotonin (21).

The SCPs and Serotonin Activate Different Receptors. Since both the SCPs and serotonin stimulate the adenylate cyclase, we asked whether they act through a common receptor. To answer this question, we took advantage of the fact that in many preparations the response to a maintained concentration of the peptides peaked within 60 sec and then decreased rapidly. In contrast the response to serotonin declines slowly, over 15–20 min (21, 22). In these experiments, we first briefly tested the response of the sensory neuron to serotonin. After the serotonin had been washed out and the action potential had recovered, the cell was continuously exposed to SCP_A. By 6 min, the spike-broadening response to this peptide had decayed substantially. Addition of SCP_B now produced little additional spike broadening. By contrast, the cell remained highly responsive to serotonin (Fig. 4). Thus the rapid desensitization of the response to the SCPs affects the response to serotonin minimally, if at all, indicating that the peptides and serotonin act via independent receptors. On the other hand, our observation that the response to one peptide is greatly decreased when the response to the other is desensitized suggests that the two SCPs may act by means of a common receptor. Although cross-desensitization is not observed between the SCPs and serotonin after brief exposures to these transmitters, it does occur with prolonged (>15 min) exposure to serotonin. Similar heterotrophic desensitization of adenylate cyclase-mediated responses has been observed in other systems (31).

SCP Facilitates Synaptic Transmission from Siphon Sensory Neurons to Motoneurons. Closure of the S-K^+ channel increases the duration of the action potential in the sensory neurons (20, 23). This allows more time for Ca^{2+} to enter the sensory neurons during each spike and may thereby enhance transmitter release (32). Although the effects of a reduction

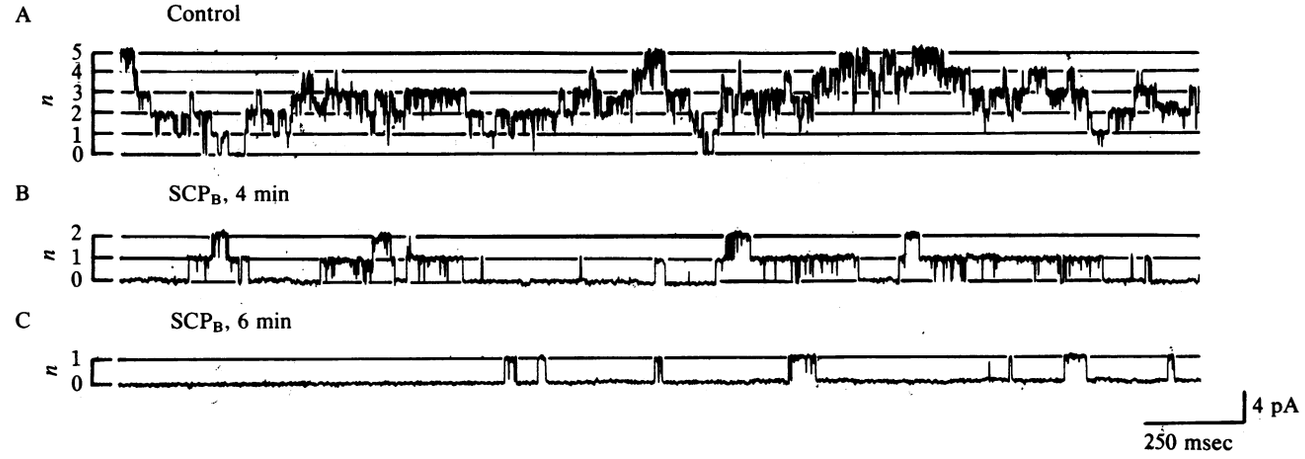

FIG. 3. Effect of SCP_B on single S channels. Patch clamp current was recorded from a siphon sensory neuron, with a transpatch potential of 0 mV. Trace A: current in the absence of peptide. Trace B: current 4 min after addition of 10 μM SCP_B; note that the number of active channels is reduced from five to two. Trace C: current 2 min later; note that one of the two channels remaining in trace B now has closed. All records are representative samples of several minutes of recording. Cell impedance was increased during records obtained in traces B and C. n, Number of open channels.

in the S current are amplified by Et_4N^+, the broadening of the presynaptic action potential by the SCPs can also be observed in normal saline (Fig. 5*A*). The absolute magnitude of these changes in the action potential recorded in the soma is small (≈20%), but they can have profound effects on synaptic transmission. In fact, brief exposure of the abdominal ganglion to SCP_B in normal saline substantially increased the amplitudes of the excitatory postsynaptic potentials (EPSPs) from siphon sensory neurons to siphon motoneurons (Fig. 5*B*).

Centrally Applied SCP Enhances the Gill and Siphon Withdrawal Reflex. The siphon sensory neurons form the afferent limb of the defensive gill and siphon withdrawal reflex. We therefore asked whether the peptide-mediated facilitation of the EPSPs from these sensory neurons would be accompanied by an enhancement of the withdrawal reflex comparable with that which occurs during sensitization (6). To study the effects of centrally applied SCPs on the behavioral response, we elicited the withdrawal reflex at 30-sec intervals with brief electrical stimuli to the siphon skin via implanted electrodes. Possible peripheral effects of the applied transmitters were eliminated by independently perfusing the abdominal ganglion. Brief (<30 sec) application of 0.5 μM concentrations of either of the two SCPs to the abdominal ganglion doubled the amplitude of the gill withdrawal reflex. Higher concentrations of the SCPs produced greater enhancement of the withdrawal reflex, up to 11-fold (Fig. 6 *A* and *B*). Similar enhancement was obtained with serotonin (Fig. 6*C*).

DISCUSSION

We have found that two endogenous neuropeptides, SCP_A and SCP_B, simulate the actions of natural sensitizing stimuli and enhance the defensive withdrawal response in *Aplysia* by facilitating synaptic transmission from siphon sensory neurons.

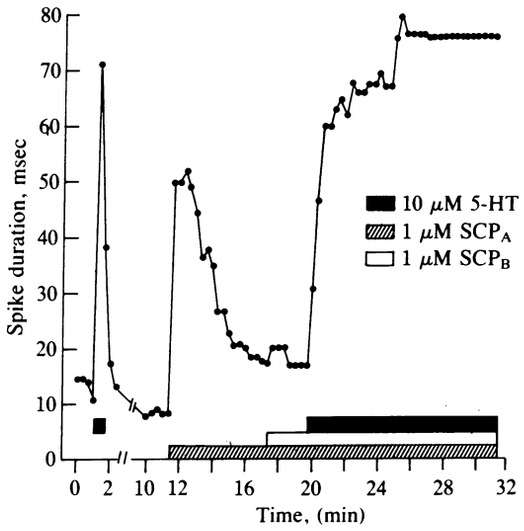

FIG. 4. Effect of desensitization to SCP on the response to serotonin. The action potential in a sensory neuron was recorded at 20-sec intervals in high-Ca^{2+}/Mg^{2+} saline with 50 mM Et_4N^+. The responsiveness to serotonin (5-HT) was first tested with a brief exposure to 10 μM 5-HT. After washout and recovery of the action potential, the preparation was perfused with 1 μM SCP_A for the remainder of the experiment. Once the spike-broadening response to SCP_A had almost completely desensitized, 1 μM SCP_B and then 10 μM 5-HT were added to the perfusion. Note that, although the response to SCP_B was almost entirely eliminated, the response to 5-HT remained comparable with the 5-HT response at the outset of the experiment.

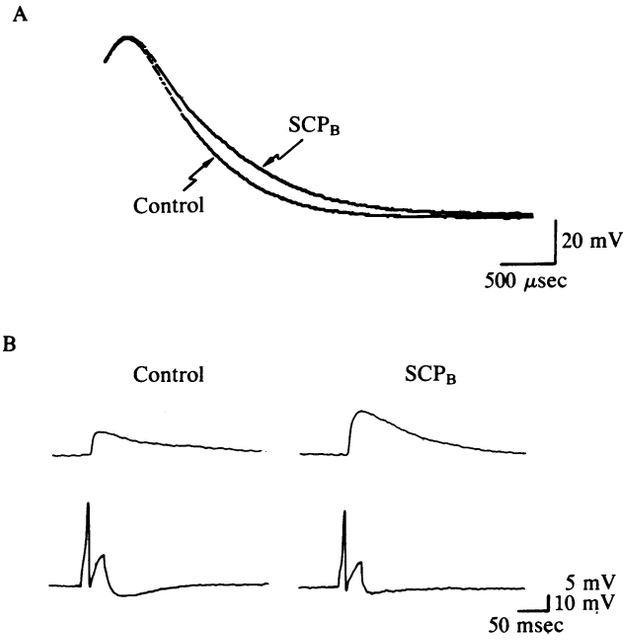

FIG. 5. Effect of SCP_B on the presynaptic action potential and synaptic transmission of siphon sensory neurons. (*A*) Effect of SCP_B (5 μM) on the action potential in a sensory neuron recorded in normal saline. (*B*) Effect of SCP_B on the EPSP from a siphon sensory neuron to a siphon motoneuron. Lower traces: action potential in a presynaptic sensory neuron. Upper traces: synaptic potential in a postsynaptic motoneuron. Recordings made in high Ca^{2+}/Mg^{2+} saline to reduce interneuron activity. The presynaptic cell was stimulated at 30-sec intervals. Immediately after the control EPSP, SCP_B was pressure ejected from a micropipette onto the cluster of sensory neurons, and then, 15 sec later, it was washed out. Note the substantial increase in the amplitude of the next EPSP. The postsynaptic cell was one of a group of recently identified, small siphon motoneurons located just posterior and lateral to the LE cluster of siphon sensory neurons in the abdominal ganglion. The peptide concentration in the micropipette was 250 μM which we estimate was diluted, more than 10-fold in the bath.

Are the SCPs and Serotonin Responsible for Producing Presynaptic Facilitation During Behavioral Sensitization? Both of the SCPs (33) and serotonin (34) are present in the abdominal ganglion. Moreover, there are SCP_B-immunoreactive processes (33) as well as serotonin-immunoreactive processes (12, 13) throughout the region of the neuropil where the siphon sensory neurons arborize; serotonin-immunoreactive processes have also been found by light microscopy to termi-

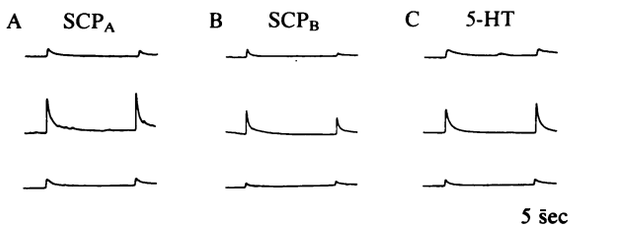

FIG. 6. Effects of 10 μM SCP_A (*A*), 1 μM SCP_B (*B*), and 10 μM serotonin (*C*) on the gill withdrawal reflex to siphon stimulation. The reflex was elicited with an electrical stimulus to the siphon skin. The traces are the voltage output of a photocell located beneath the gill. Upper traces: withdrawal response immediately prior to perfusion of the transmitter onto the abdominal ganglion. Middle traces: response at the end of 3–4 min of exposure to the transmitter. Lower traces: response several minutes after the transmitter had been washed out. 5-HT, serotonin.

nate directly on the somata of the sensory neurons (13). Combined with the results presented here, these immunocytochemical studies suggest that sensory neurons will be exposed to both the endogenous SCPs and endogenous serotonin, resulting in enhancement of the defensive withdrawal reflex. However, we have no information about which circumstances would result in the release of these transmitters. A more complete understanding of the roles of the SCPs and serotonin in behavioral plasticity of the defensive withdrawal reflex must await the identification and characterization of those peptidergic and serotonergic neurons whose processes project to the siphon sensory neurons. Only two group of facilitator neurons have so far been identified, the L_{28} and L_{29} cells (14), and neither of these contains immunoreactive SCP_B (33) or serotonin (12, 13). Rather the L_{29} cells appear to contain yet a fourth facilitatory transmitter (27).

Multiple Facilitatory Transmitters Converge on the Adenylate Cyclase in the Sensory Neurons. One of the major conclusions of this study is that the SCPs, as well as serotonin and natural sensitizing stimuli, produce presynaptic facilitation by acting on a common molecular cascade: they increase intracellular levels of cAMP, which results in closing of the S-K^+ channels and broadening of the presynaptic action potential. These findings suggest that different facilitatory transmitters act via independent receptors that converge on a common molecular cascade to produce the same modulatory effects on the siphon sensory neurons. The existence of several distinct but parallel modulatory systems that use the SCPs on the one hand and serotonin on the other has also been observed in the feeding system of *Aplysia* by Lloyd *et al.* (19). Based on a different experimental approach from that used here, they also concluded that serotonin and SCP_B act via independent receptors to stimulate cAMP levels in *Aplysia* muscle.

Similarities with Vertebrate Neuropeptide Actions; Parallel Actions of Conventional and Peptide Neurotransmitters. A number of slow actions of vertebrate peptides, such as those of luteinizing hormone-releasing hormone (LHRH) on sympathetic neurons (35, 36) and of substance P on myenteric neurons (37), similarly involve decreases in K^+ conductances. Moreover, both LHRH and acetylcholine modulate the same K^+ current, the M current, in sympathetic neurons (35), much as both serotonin and the SCPs modulate a common K^+ current in *Aplysia* neurons. Similar convergent actions of a conventional and a peptide transmitter are suggested by our observation that both dopamine and Phe-Met-Arg-Phe-NH_2 act to shorten the presynaptic action potential in the sensory neurons and reduce synaptic transmission (unpublished results), although we do not yet know whether the two transmitters produce this presynaptic inhibition by modulation of the same channel.

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