Bag Cell Extract Inhibits Tail-Siphon Withdrawal Reflex, Suppresses Long-Term but Not Short-Term Sensitization, and Attenuates Sensory-to-Motor Neuron Synapses in *Aplysia*

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The peptides released from the bag cells (bag cell peptides, BCPs) in *Aplysia californica* are responsible for eliciting or modulating a repertoire of behaviors preceding and accompanying egg laying. Our interest was in determining to what extent behaviors that can occur simultaneously with egg laying are modulated. To address this issue, we examined the effects of an extract (bag cell extract, BCE) that contained BCPs, as well as egg-laying hormone (ELH), an identified BCP, on both the tail-siphon withdrawal reflex and the ability of that reflex to exhibit sensitization, a simple form of nonassociative learning. In addition, we examined the ability of BCE, as well as the individual BCPs, to modulate a component of the circuitry of the reflex. Behavioral experiments indicated that BCE inhibited the baseline withdrawal reflex and suppressed long-term sensitization of the reflex while leaving short-term sensitization unaffected. These effects were not observed in animals receiving an extract of a control ganglion or in animals receiving ELH, and therefore may be attributed to the actions of one or more BCPs, but presumably not to ELH alone. Electrophysiological experiments revealed that BCE also attenuated the monosynaptic EPSP elicited in tail motor neurons by tail sensory neurons. In subsequent experiments in which the BCPs were individually applied, only one BCP, β-BCP, significantly attenuated the EPSPs. This effect was independent of narrowing of the presynaptic action potential. Taken together, these results suggest a novel behavioral modification attributed to BCPs, inhibition of the tail-siphon withdrawal reflex, and suggest a possible locus of action and peptide candidate for this effect. In addition, these experiments raise the possibility that BCPs may exert a long-lasting effect (>24 hr), selectively blocking a simple form of long-term memory.

[*Key words: Aplysia, bag cell peptides, learning and memory, long-term memory, mobilization, motor neuron, sensitization, sensory neuron, short-term memory, synaptic transmission*]

Although considerable progress has been made in understanding the actions of reproductive hormones on reproductive behavior and organs, little is known about the mechanisms by which these and other hormones affect other behaviors. For example, in addition to its classical action on the pituitary gland, luteinizing hormone–releasing hormone has effects not only on reproductive behavior but also on various forms of learning (Mora and Diaz-Veliz, 1985; Nasello et al., 1990; Mora et al., 1991). Due to the difficulty in performing cellular studies on the vertebrate CNS, however, the mechanisms by which this peptide acts remain unknown. An alternative approach is to examine these issues in the relatively simple nervous system of higher invertebrates.

The marine mollusk *Aplysia* may prove to be a particularly advantageous animal for the study of the modulatory effects of reproductive peptides on behavior and learning, given its simple nervous system, behaviors, and forms of learning. Indeed, several behaviors of this animal, as well as the neural circuits that mediate them, have been well characterized (Kandel, 1976, 1979). One such behavior is the tail-siphon withdrawal reflex, which consists of a coordinated set of defensive responses to stimulation of the tail, including withdrawal of the tail and siphon. The tail sensory neurons that mediate this reflex are located in the pleural ganglion. These neurons make monosynaptic excitatory connections with tail motor neurons, located in the pedal ganglion, resulting in activation of the motor neurons and withdrawal of the tail (Walters et al., 1983a). In addition, these same sensory neurons also synapse onto various interneurons in the pleural ganglion (Cleary and Byrne, 1985; Xu et al., 1991; Buonomano et al., 1992), some of which relay input to siphon motor neurons in the abdominal ganglion to produce withdrawal of the siphon (Cleary and Byrne, 1986).

The tail-siphon withdrawal reflex has been used to analyze the neural and molecular mechanisms of a simple form of nonassociative learning, sensitization. Sensitization is the enhancement of a behavioral response to a stimulus delivered to one area of the body after delivery of a strong reinforcing stimulus to the same or another area of the animal's body. The tail-siphon withdrawal reflex exhibits both short-term (minutes) sensitization (Walters et al., 1983b; Byrne et al., 1991) and long-term (days) sensitization (Scholz and Byrne, 1987). Short-term enhancement of the reflex is associated with heterosynaptic facilitation of the monosynaptic connections between the tail sensory neurons and tail motor neurons (Walters et al., 1983b). Using *in vitro* analogs of long-term sensitization training and

classical conditioning, long-term (24 hr) facilitation of these synapses has also been demonstrated (Goldsmith and Byrne, 1989; Buonomano and Byrne, 1990; Byrne et al., 1991).

A family of endogenous peptides that may modulate both the tail-siphon withdrawal reflex and sensitization of the reflex is the bag cell peptides (BCPs). The BCPs are believed to play a key role in the neural control of reproductive behavior of *Aplysia*. Indeed, injection of a crude extract of the nervous system containing BCPs into a mature recipient animal reliably induces egg laying (Kupfermann, 1970) and egg-laying behaviors (Arch and Smock, 1977).

The BCPs, which include α-, β-, and γ-BCP and egg-laying hormone (ELH), are synthesized and released by the bag cell neurons. These neurons are found in two homogeneous clusters of approximately 400 cells each, located in paired, bag-like structures attached to the rostral surface of the abdominal ganglion. The processes of these cells spread in many directions: laterally to the opposite cluster, along and within the more rostral extent of the pleural-abdominal connective, and into the connective tissue surrounding the abdominal ganglion and bag cells (Coggeshall, 1967; Frazier et al., 1967; Haskins et al., 1981; Sossin et al., 1990). The connective tissue sheath that surrounds these two clusters is highly vascularized and constitutes the release site of the BCPs into the circulatory system, as evidenced by the many granule-filled processes that end in the connective tissue sheath (Coggeshall, 1967; Frazier et al., 1967; Haskins et al., 1981; Roubos et al., 1990; Sossin et al., 1990).

Electrophysiological studies have revealed that the bag cells are normally quiescent. However, they periodically enter a prolonged phase of intense and synchronous electrical activity, termed an afterdischarge, which apparently involves all the cells of both clusters (Frazier et al., 1967; Dudek and Blankenship, 1977a,b; Pinsker and Dudek, 1977; Haskins and Blankenship, 1979). In intact animals, egg laying is invariably preceded by an afterdischarge in the bag cells (Pinsker and Dudek, 1977). During the reproductive season (summer and fall), egg laying is triggered in this manner as often as once a day (Pinsker and Parsons, 1985). The BCPs released during the afterdischarge have been identified, and the effects they exert on a variety of target neurons, including neurons in the head ganglia, have been extensively studied (Branton et al., 1978a,b; for reviews, see Mayeri and Rothman, 1985; Arch and Berry, 1989; Conn and Kaczmarek, 1989; Jung and Scheller, 1991). Target neurons in the abdominal ganglion may be affected by the release of BCPs from processes of the bag cells that terminate near these neurons. Effects on more distant targets, which may include the neurons in the pleural-pedal ganglion that mediate the tail-siphon withdrawal reflex, may require sites of local release of the BCPs or the circulatory system to transport the BCPs from the abdominal ganglion to the vicinity of these neurons.

Recently, bag cell-like neurons, which are immunoreactive for at least two BCPs, and functional bag cell neurites have also been found in the pleural and cerebral ganglion (Jahan-Parwar and Fredman, 1976; Chiu and Strumwasser, 1984; Shyamala et al., 1986; Pulst et al., 1988; Brown et al., 1989; Painter et al., 1989; Shope et al., 1991). The proximity of these bag cell-like neurons and bag cell neurites to the neurons that mediate the tail-siphon withdrawal reflex provides a more direct means by which the BCPs could affect the reflex. Indeed, α-BCP inhibits giant cell LP11 of the left pleural ganglion, a cell that is involved in mucous secretion and that is inhibited during a bag cell afterdischarge (Shope et al., 1991). Thus, it is conceivable that

these peptides, which have actions throughout the nervous system, may modulate other behaviors as well.

We examined the effects of an extract prepared from the bag cells (BCE), as well as ELH on both the tail-siphon withdrawal reflex, and the ability of the reflex to exhibit short- and long-term sensitization. In addition, *in vitro* electrophysiological experiments using a known component of the circuitry underlying the reflex were performed to gain insights into the site(s) and mechanism(s) of action of the BCPs. In these experiments the effects of BCE as well as the individual BCPs (α-, β-, and γ-BCP and ELH) were examined. We found that BCE significantly inhibited the baseline withdrawal reflex and significantly suppressed long-term sensitization of the reflex while leaving short-term sensitization unaltered. Since these effects were not observed in animals receiving an extract of the abdominal ganglion minus the bag cells or in animals receiving ELH, they may be attributed to actions of one or more of the BCPs, but presumably not to ELH alone. In addition, BCE significantly attenuated the tail sensory-to-motor neuron connection. Further, application of the individual BCPs revealed that only one BCP, β-BCP, significantly attenuated the EPSPs. This effect was independent of presynaptic spike narrowing.

Aspects of this work have appeared previously in abstract form (Goldsmith and Byrne, 1990, 1991).

Materials and Methods

Animals. Aplysia californica, weighing between 125 and 500 gm, were obtained from Alacrity Marine Biological (Redondo Beach, CA), Marine Specimens Unlimited (Pacific Palisades, CA), Marinus Inc. (Long Beach, CA), Pacific Bio-Marine (Venice, CA), and Sea Life Supply (Sand City, CA). Animals were housed in individual perforated cages within a large aquarium containing aerated artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH) maintained at 15°C. Animals were exposed to a 12 hr/12 hr light/dark cycle, and were fed an amount of dried seaweed sufficient to maintain a constant body weight.

Tissue collection and preparation of solutions. Tissue required to make solutions of bag cell extract (BCE) and abdominal ganglion extract (ABG) used in experiments 1 and 2 (see below) were collected from animals anesthetized by injection of volumes of isotonic $MgCl_2$ equivalent to one-half of their body volume. Donor animals were not tested for sexual maturity (see below); however, primarily larger animals were used and in almost all cases the bag cells appeared to be fully developed. The abdominal ganglia, to which the bag cells are attached, were removed and pinned to the floor of a Sylgard (Dow-Corning)-lined chamber containing a solution of isotonic $MgCl_2$/ASW (50/50, v/v). The bag cell clusters, plus 2–5 mm of the pleural abdominal connectives, were then excised (Kupfermann, 1970). In order to collect the neurons of the abdominal ganglia for the preparation of the ABG, the abdominal ganglia were then squeezed between a pair of forceps, forcing the neurons and neuropil out of the surrounding connective tissue. This method of isolating the neurons of the abdominal ganglion avoids contamination of the ABG with bag cell processes that permeate the connective tissue overlying the ganglion. The isolated bag cell clusters and abdominal ganglia exudate were stored (-90°C) until sufficient tissue had been collected for the preparation of BCE and ABG solutions. ASW, used in the control solution and in the preparation of the experimental solutions, was buffered with 10 mM Tris (Trizma-7.6, Sigma, St. Louis, MO). BCE and ABG were prepared by a modified protocol of that previously described (Strumwasser et al., 1969; Kupfermann, 1970; Arch and Smock, 1977). Briefly, BCE was prepared by thawing previously isolated bag cell clusters and homogenizing them on ice with 1 or 2 ml of buffered ASW for approximately 5 min in a hand-held glass homogenizer. Sufficient buffered ASW was then added to the homogenate to arrive at a final concentration of the BCE solution that was equal to one pair of bag cell clusters per 1.3 ml. This volume of BCE is similar to that used previously and has been demonstrated to produce egg laying consistently (Strumwasser et al., 1969; Kupfermann, 1970; Arch and Smock, 1977; J. R. Goldsmith and J. H. Byrne, unpublished observation). ABG was prepared by thawing abdominal ganglia exudate and following a similar
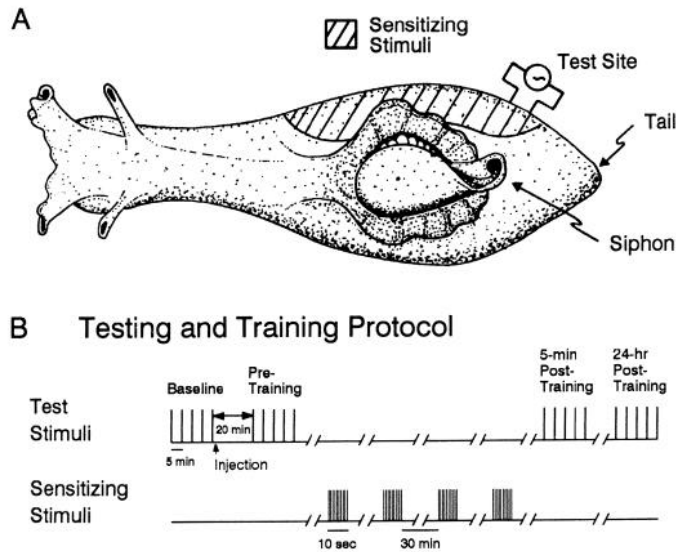
A

Sensitizing Stimuli

Test Site

Tail

Siphon

B   Testing and Training Protocol

5-min Post-Training   24-hr Post-Training

Pre-Training

Baseline

Test Stimuli

20 min

5 min   Injection

Sensitizing Stimuli

10 sec   30 min

Figure 1. Diagram of testing and training protocol for behavioral experiments. *A,* Dorsal view of the animal detailing the area of the body wall that received the sensitizing stimuli and the area of the tail that received the test stimuli. *B,* Testing and training protocol. Testing consisted of four test periods: the baseline, pretraining, 5 min posttraining, and 24 hr posttraining tests. Each of these 20 min test periods consisted of five stimuli delivered to the animal's tail at an ISI of 5 min. The duration of the resulting siphon withdrawal was recorded. Immediately following the last stimulus in the baseline test period, the experimental or control solution was injected (see text) into the hemocoel via the anterior one third of the foot. Training consisted of four blocks of sensitizing stimuli, with each block delivered at an ISI of 30 min to the body wall (same side to which test stimuli were presented). Each training block consisted of a 10 sec train of ten 500 msec, 60 mA AC pulses (1 sec ISI).

homogenization procedure (reaching a similar final concentration, i.e., one abdominal ganglion per 1.3 ml). Synthetic ELH (Peninsula Laboratories, Belmont, CA) was mixed on ice with buffered ASW (15.9 μg ELH/1.3 ml ASW; final concentration, 3 μM). The amount of ELH contained in this volume (2.5 nmol) is similar to that used previously and consistently leads to egg laying (Chiu et al., 1979; Goldsmith and Byrne, unpublished observation). For comparison, the 1.3 ml of BCE contains approximately 4.3–8.9 nmol of ELH (Chiu et al., 1979). The protease inhibitor phenylmethylsulfonyl fluoride (Sigma; 10 mM) was included in the preparation of the ABG and BCE solutions to retard the proteolytic degradation of the peptides contained in the extracts (Chiu et al., 1979) and was therefore also included in the preparation of the ASW and ELH solutions.

For experiments 2–4 (see below), ASW used in the control solution and in the preparation of the experimental solutions was buffered with 15 mM HEPES (Sigma). α-, β-, and γ-BCP, ELH, and arginine vasotocin (ARG-VAS) were obtained from Peninsula Laboratories. BCE was prepared as described above. All solutions were aliquotted and stored at $-90°C$ until use.

Experiment 1: injection of BCE and ELH; in vivo effects on the tail-siphon withdrawal reflex and sensitization of the reflex. All animals used in these experiments were maintained in an aquarium, separate from other animals. In order to ensure that the animals used in this study were reproductively mature (i.e., would lay eggs in response to an injection of BCE), they were given an injection of BCE at least 1 week prior to the beginning of the experiment. Only those animals that laid eggs within a 2 hr period were used. Experiments were carried out during the naturally occurring egg-laying season (June through December). After the 2 hr period had elapsed, all animals that passed the reproductive maturity test were then parapodectomized. The parapodia, paired wing-like extensions of the body wall that surround the mantle organs, were removed by reflecting them away from the mantle and excising along the line where they join the body wall. The parapodectomy and the implantation of electrodes (see below) were performed after the animal was partially anesthetized by placement in ice for several minutes.

One to three days prior to testing, a pair of Teflon-coated wire electrodes (type Ag 5T, Medwire, Mt. Vernon, NY) were implanted on each side of the animal's tail. The wire electrodes were implanted by first inserting a small portion of exposed wire (approximately 1 cm) into the tip of a needle and then inserting the needle into the body wall, leaving the wire implanted in the musculature of the body wall as the needle was withdrawn.

On the day of testing, following a 30 min equilibration period, the threshold for siphon withdrawal was determined. Test stimuli were 30 msec duration AC shocks. The threshold intensity was that which reliably elicited a siphon withdrawal. The side that had the lower threshold was the side used for testing. The other side was not used further. Testing consisted of four 20 min periods, each consisting of five stimuli, delivered to the tail via the implanted electrodes, at an interstimulus interval (ISI) of 5 min (Fig. 1). The intensity of the stimuli was twice that of the threshold intensity. The threshold intensity was generally about 2 mA. The duration of the siphon withdrawal was used as the measure of sensitivity of the animal to the test stimuli, and changes in the duration of the siphon withdrawal as a measure of sensitization and/or treatment effect. The duration of the siphon withdrawal was measured from the start of the contraction to the time the siphon reversed direction and began to relax to its prestimulus position. Although the tail and siphon components of the tail-siphon withdrawal reflex appear to track each other, these experiments were not performed using the tail component of the reflex due to the difficulty in obtaining accurate measurements of tail withdrawal in freely moving animals; however, preliminary experiments were performed using a semi-intact preparation in which the tail withdrawal was also examined (see below). All measures of duration of siphon withdrawal were performed by an observer blind to the experimental manipulations.

The first period of testing (baseline test) was begun 30 min after the determination of threshold. Animals were included in the study only if the mean of the five baseline test values was greater than 1 sec and less than 10 sec. This procedure was followed to ensure that the animals were healthy and not previously sensitized. In addition, animals were not used if spontaneous egg laying had occurred during the previous 24 hr. Immediately following the last stimulus in the baseline test period, 0.9 ml of BCE, ELH, ABG, or vehicle (ASW) was injected into the hemocoel via the anterior one-third of the foot (Strumwasser et al., 1969).

The second period of testing (pretraining test) was begun 20 min after the injection. Immediately following the last stimulus in this period, the training procedure was begun. Training was essentially identical to that used previously (Scholz and Byrne, 1987), and consisted of four blocks of noxious stimuli, delivered once every 30 min to the body wall (same side to which test stimuli were presented) via a hand-held bipolar electrode (Fig. 1). Each training block consisted of a 10 sec train of ten 500 msec, 60 mA AC pulses (1 sec ISI). Training reliably elicited the release of ink and opaline, two defensive secretions released in response to noxious stimuli. The third period of testing (5 min posttraining test) and the fourth period of testing (24 hr posttraining test) were begun 5 min after and 24 hr after the last train of shocks, respectively. All experiments were performed at room temperature (18–23°C), as this temperature falls within the range of temperatures *Aplysia californica* experience during the egg-laying season (Kupfermann and Carew, 1974).

A preliminary set of experiments were performed in which a semi-intact preparation was used to measure the magnitude of the tail component of the reflex withdrawal in response to stimulation of the tail. Animals were anesthetized by injection of volumes of isotonic $MgCl_2$ equivalent to one-half of their body volume. In these experiments, the viscera, body wall, and the majority of the nervous system were dissected from the animal, leaving only the left or right pleural-pedal ganglion, connected to the tail by peripheral nerve 9 (P9). The pleural-pedal ganglion was maintained in a separate chamber from the tail. The preparation was restrained with pins inserted through the most anterior regions of the tail. Wire electrodes used to elicit tail withdrawals were inserted into the tail. Prior to the beginning of each experiment, peripheral motility and reflex responses were restored by perfusing both chambers and the tail with ASW to remove excess $MgCl_2$. Tail withdrawal was measured using a Grass FT.03B force-displacement transducer attached by surgical thread and a stainless steel hook to the tail. The tail withdrawal reflex was tested once every 5 min. Two and one-half minutes after the fifth test, 25 μl of BCE or vehicle (ASW) was applied directly to the bath containing the pleural-pedal ganglion. The final bath concentration of BCPs (contained in BCE) was estimated to
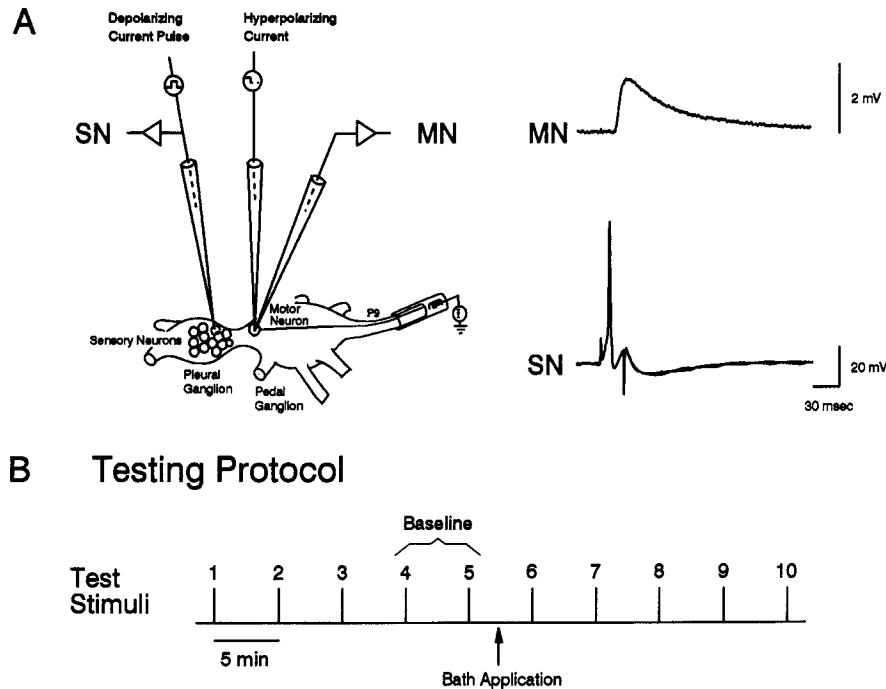
Figure 2. Diagram of the *in vitro* preparation and testing protocol for electrophysiological experiments. *A*, The *in vitro* preparation. Illustrated on the *left panel* is the isolated pleural-pedal ganglion with peripheral nerve P9 mounted in a suction electrode. Tail motor neurons were positively identified by passing current (*I*) through the suction electrode to determine whether the neuron had an axon in the peripheral nerve. A single microelectrode was used to stimulate and record from the sensory neuron (*SN*) in the pleural ganglion, whereas separate electrodes were used to hyperpolarize and record from the motor neuron (*MN*) in the pedal ganglion. The *right panel* illustrates representative traces, obtained during testing, from both a sensory neuron and motor neuron. An action potential elicited in a sensory neuron by a suprathreshold depolarizing pulse produces a short-latency monosynaptic EPSP in the motor neuron. *B*, Testing protocol. Testing was composed of 10 test stimuli (trials) delivered at an ISI of 5 min. Each test consisted of eliciting a single action potential in a tail sensory neuron with a suprathreshold depolarizing current pulse and recording the monosynaptic EPSP elicited in the follower tail motor neuron. Two and one-half minutes following the fifth stimulus, the experimental or control solution was applied to the bath. The value of each trial was normalized to the mean of the two trials preceding the bath application [trials 4 and 5 (baseline)].

be 1 μM. The reflex withdrawal of the tail was then monitored for five more tests.

Experiment 2: bath application of BCE; in vitro effects on synaptic strength. Animals were anesthetized by injection of volumes of isotonic $MgCl_2$ equivalent to one-half of their body volume. The left pleural-pedal ganglion, as well as peripheral nerve P9, were removed and placed in a Sylgard-lined recording chamber containing a solution of isotonic $MgCl_2$/ASW (50/50, v/v). The bulk of the connective tissue overlying the pleural-pedal ganglion was surgically removed. Upon completion of the dissection, the ganglion was firmly pinned and nerve P9 was drawn into a suction electrode (Fig. 2). The 50/50 solution was then exchanged at least five times with ASW to ensure the removal of excess $MgCl_2$.

Tail sensory neurons and motor neurons were impaled with glass capillary microelectrodes (4–6 MΩ) filled with 3 M K-acetate (Fig. 2*A*). Sensory neurons were impaled with a single microelectrode for recording and stimulating. Motor neurons were impaled with two microelectrodes, one for recording and one for current injection. Tail sensory neurons, located in the pleural ganglion, were identified by their location, size, and electrophysiological properties. Tail motor neurons, located in the pedal ganglion, were identified superficially by their location, size, spontaneous EPSPs and IPSPs, and more precisely by their resting membrane potential, synaptic input from the sensory neurons, and antidromic activation by stimulation of nerve P9 (which contains the axon of the motor neuron).

Tail motor neurons were used as the follower cells since it is more feasible to monitor the connections between sensory neurons and their large and readily identifiable follower tail motor neurons than the connections between the sensory neurons and their smaller and more difficult to identify follower interneurons that relay the tail sensory information to siphon motor neurons. It is the monosynaptic connection between the tail sensory neurons and these motor neurons that mediates the tail component of the tail-siphon withdrawal reflex. Plasticity at this

synapse correlates with changes in the tail component of the reflex (Walters et al., 1983b). In addition, preliminary evidence from our laboratory (Y. Xu, L. J. Cleary, and J. H. Byrne, personal communication) indicates that plasticity of the sensory neuron-to-tail motor neuron and sensory neuron-to-interneuron connections are similar. Standard electrophysiological techniques were used to make intracellular recordings from sensory neurons and motor neurons. Experiments were conducted at room temperature (18–23°C).

Testing consisted of 10 test stimuli (trials) delivered at an ISI of 5 min (Fig. 2*B*). Trials 1–5 and 6–10 were analogous to the first two test periods in the behavioral experiments, respectively. Each test consisted of eliciting a single action potential in the sensory neuron with a 30 msec suprathreshold depolarizing pulse and recording the monosynaptic EPSP elicited in the motor neuron. Two and one-half minutes following the fifth stimulus, 25 μl of BCE or vehicle (ASW) was applied to the bath. The final bath concentration of BCPs (contained in BCE) was estimated to be 1 μM. Motor neurons were hyperpolarized by approximately 30 mV during each test stimulus to prevent the EPSP evoked by the sensory neuron from triggering an action potential in the motor neuron. Measurements of input resistance of the motor neuron (while the cell was hyperpolarized) were made by injecting 1 sec, 1 nA hyperpolarizing pulses into the motor neuron.

Experiment 3: bath application of BCPs; in vitro effects on synaptic strength. The procedures of this experiment were identical to those of experiment 2. In addition to examining the effects of α-, β-, and γ-BCP and ELH, arginine vasotocin (ARG-VAS) was included to examine whether it could attenuate sensory-to-motor neuron synapses in the pleural-pedal ganglion as had been previously demonstrated in the abdominal ganglion (Goldberg et al., 1987). Final bath concentrations of all peptides was 1 μM. This concentration has been used previously and leads to autoexcitation of the bag cells (α-, β-, and γ-BCP; Brown and Mayeri, 1989), prolonged excitation of LB and LC cells, and burst augmentation of cell R15 (ELH; Mayeri et al., 1985), as well as to
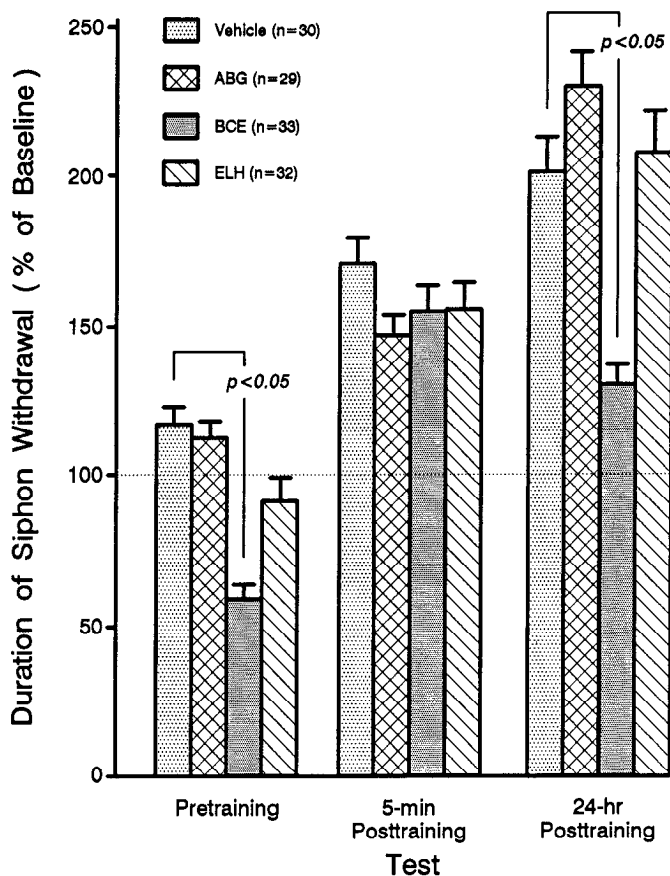
Figure 3. Effects of BCE on the tail-siphon withdrawal reflex. The mean durations (±SEM) of the siphon withdrawal in response to stimulation of the tail for each test period (normalized to the baseline test period) are illustrated. Injection of BCE significantly inhibited the tail-siphon withdrawal reflex (58.9 ± 4.5% of baseline) and significantly suppressed long-term sensitization of the reflex (130.1 ± 6.4% of baseline), while leaving short-term sensitization unaltered.

suppression of sensory-to-motor connections in the abdominal ganglion (ARG-VAS; Goldberg et al., 1987).

Experiment 4: bath application of β-BCP; in vitro effects on synaptic strength and presynaptic spike duration. The procedures of this experiment were identical to those of experiment 2 except for the following. (1) The ganglia were rinsed prior to dissection in a mixture of ASW and isotonic $MgCl_2$ containing 0.5% glutaraldehyde (Sigma) for 50 sec in order to reduce tetraethylammonium chloride (TEA)-induced contractions in the connective tissue sheath (Mirolli and Gorman, 1968; Byrne et al., 1979). (2) The bath contained 100 mM TEA (Eastman Kodak, Rochester, NY) to facilitate detection of changes in duration of the action potential, and low Na^+ (108 mM) to reduce spontaneous synaptic activity. Na^+ was replaced with equimolar amounts of N-methyl-D-glucamine [Sigma; pH adjusted to 7.6 with concentrated hydrochloric acid (Kirk et al., 1988)]. (3) To avoid distortion of the action potential in the sensory neuron by the bridge circuit artifact, a short depolarizing pulse of 1.5 msec was used to elicit the spike. (4) The final bath concentration of β-BCP ranged from 1 to 10 μM.

Data and statistics. Electrophysiological data were stored on a VCR tape recorder, and permanent records were made with a Gould model 2400 pen recorder. The half-amplitude spike duration (duration from the peak of the spike to 50% of its repolarization to the resting potential) in the sensory neuron, and the peak amplitude, slope of the rising phase (measured from a point 20% above baseline to a point 20% below peak amplitude), and time to peak (time from the beginning of the rising phase to the peak of the EPSP) of the EPSP in the motor neuron were measured using a computer program.

In experiment 1, the mean test score in each test period for each animal was calculated and was then normalized to the animal's baseline test score. A logarithmic transformation was then performed on each

normalized value (X), using the equation $X' = \log(X + 1)$ (Howell, 1987). This transformation ensured homogeneity in variances for each group. A two-tailed two-way analysis of variance (ANOVA) with repeated measures on one factor was then performed, followed by two-tailed Dunnett's tests comparing each experimental group to control at each of the three test periods.

In experiments 2–4, the value of each trial was normalized to the mean of the two trials preceding the bath application [trials 4 and 5 (baseline)]. All data, with the exception of measurements of membrane potential, were analyzed using two-way ANOVAs with repeated measures on the normalized values from trials 6–10. Where appropriate, a Dunnett's test was used for post hoc examination of main or simple effects. As per Dunnett (1955; referenced in Zar, 1984), the control group in experiment 3 contained approximately 2.2 times as many experiments as each other group. Changes in membrane potential of the sensory neuron and motor neuron were analyzed by normalizing the resting potential of the neuron at the end of the experiment to the measurement at the beginning of the experiment and then performing a Student's t test or one-way ANOVA to compare the differences between treatments. Two-tailed statistical analyses were performed on the initial experiments examining the effects of BCE. Thereafter, one-tailed analyses were used since the results from the behavioral experiments and the preliminary electrophysiological experiments with bath-applied BCE suggested that the BCPs attenuate synaptic strength. Data in the text and figures are expressed in terms of the mean ± SEM. For all statistical tests a $p < 0.05$ was considered significant.

Results

Experiment 1

The purpose of this experiment was to examine whether the BCPs exert any effects on the tail-siphon withdrawal reflex and short- and long-term sensitization of the reflex. To address this issue, BCE was administered to one group of animals. ELH, which has been demonstrated to elicit egg laying (Stuart et al., 1980; Strumwasser, 1984; Strumwasser et al., 1987), was administered to a second experimental group to determine if it, in the absence of other components of BCE, had any effects on the tail-siphon withdrawal reflex and/or sensitization of the reflex. Another group received an extract of the abdominal ganglion minus the bag cell clusters (ABG). This treatment was necessary since an effect of the BCE could be due to some factor or factors in the BCE unrelated to the BCPs. A control group received injections of the vehicle.

A two-way ANOVA revealed that there was a significant difference in the duration of the siphon withdrawal between treatment groups [$F(3,120) = 3.46$]. In addition, there was also a significant enhancement in the duration of the siphon withdrawal produced by the sensitization training [$F(2,240) = 30.55$; $p < 0.001$]. There was an absence of an interaction effect [$F(6,240) = 1.54$]. The mean duration (±SEM) of the siphon withdrawal in the baseline test was, respectively, 4.1 ± 0.4 sec ($n = 30$), 5.0 ± 0.5 sec ($n = 29$), 4.1 ± 0.4 sec ($n = 33$), and 4.3 ± 0.4 sec ($n = 32$) for the control, ABG, BCE, and ELH groups. The mean duration (±SEM) of siphon withdrawal (mean of each test period normalized to the baseline test period) is illustrated in Figure 3.

BCE significantly inhibited the tail-siphon withdrawal reflex. The mean duration of the siphon withdrawal (measured at the pretraining test) in the BCE group was markedly reduced relative to the baseline test (58.9 ± 4.5%) (Fig. 3). BCE inhibited the siphon withdrawal in each of the trials after the injection, beginning with trial 1 (data not shown). A Dunnett's test revealed that the duration of the siphon withdrawal in the BCE group was significantly different from the duration of the withdrawal in the group that received the vehicle (116.8 ± 5.9% of baseline) [$q(120) = 2.70$]. ABG- and ELH-treated animals were not sig-

nificantly different than control (112.3 ± 5.4% and 91.4 ± 7.9% of baseline, respectively). Egg laying in all BCE- and ELH-treated animals was observed to begin at or after the third trial of the pretraining test period. No egg laying was observed in animals in the ABG or control group.

The baseline tail withdrawal reflex also appeared to be inhibited by BCE in preliminary experiments using a semi-intact preparation in which the tail withdrawal was measured. The tail withdrawal was 86% of baseline after application of ASW ($n = 6$), whereas in experiments in which BCE was applied the withdrawal was 70% of baseline ($n = 7$). These data suggest that BCE may inhibit both components of the tail-siphon withdrawal reflex similarly.

Short-term sensitization was unaffected by any of the experimental treatments. The sensitization training produced a short-term enhancement of the duration of the siphon withdrawal in all groups, which was unaffected by any of the experimental treatments. The degree of enhancement of the duration of the siphon withdrawal (measured at the 5 min posttraining test) in the ABG, BCE, and ELH groups (146.0 ± 6.7%, 154.1 ± 8.1%, and 154.7 ± 8.6% of baseline) was similar to the enhancement observed in the group that received the vehicle (170.4 ± 8.1% of baseline) (Fig. 3). The lack of any effects on short-term sensitization by the ABG, BCE, or ELH treatments was confirmed by a Dunnett's test that revealed no significant differences between any of the experimental treatments and control at the 5 min posttraining test.

BCE significantly suppressed long-term sensitization. The long-term effects of the sensitization training, assessed 24 hr later, consisted of a further enhancement of the duration of the siphon withdrawal in all of the treatment groups except BCE, in which long-term sensitization of the reflex was considerably suppressed (130.1 ± 6.4% of baseline) (Fig. 3). The duration of the siphon withdrawal in the vehicle, ABG, and ELH treatment groups was 201.3 ± 12.2%, 229.6 ± 11.2%, and 207.6 ± 13.0% of baseline, respectively. The long-term enhancement in the duration of siphon withdrawal in the BCE-treated animals was less than the enhancement observed in the 5 min posttraining test period. A Dunnett's test comparing each treatment to control at the 24 hr posttraining test indicated that the suppression of long-term sensitization by the BCE treatment was significant [$q(120) = 2.34$].

The following series of electrophysiological experiments were performed to examine whether the BCPs modulate elements of the circuitry underlying the reflex.

Experiment 2: BCE significantly attenuated synaptic strength

Experiments using an *in vitro* analog of the tail component of the reflex were performed in order to examine whether the circuitry underlying the reflex was a locus of action of the BCE. In these experiments we examined the tail sensory-to-motor neuron connection, which underlies the tail component of the tail-siphon withdrawal reflex. Sensory neurons were stimulated at an ISI of 5 min. The mean amplitude (±SEM) of the baseline EPSP was 5.6 ± 2.0 mV ($n = 3$) and 4.5 ± 1.4 mV ($n = 5$) in the control and BCE experiments, respectively. Homosynaptic depression, a reduction in synaptic strength due to repetitive activation of the sensory neuron, was observed in both the experimental and control group. However, the amplitudes of the EPSPs following bath application of BCE were dramatically attenuated relative to the EPSPs in the experiments in which the vehicle was bath applied (i.e., trial 10: 45.2 ± 3.4% vs 79.3
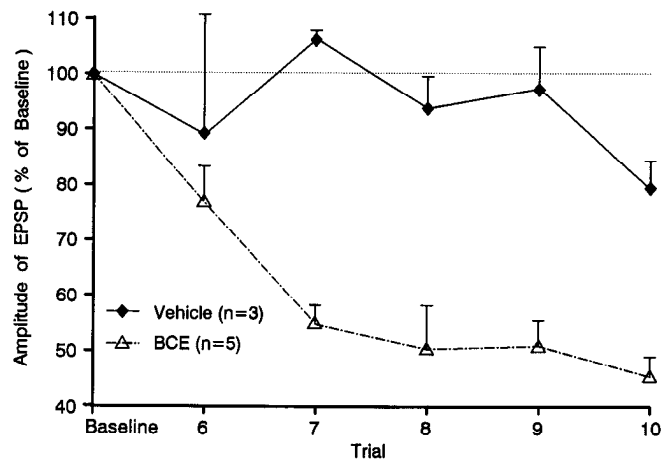

Figure 4. Effects of BCE on the amplitude of the EPSP. The mean amplitudes (±SEM) of the EPSP for trials 6–10 [normalized to baseline (mean of trials 4 and 5)] are illustrated. Bath application of BCE significantly attenuated the amplitude of the EPSP.

± 4.9% of baseline, respectively) (Fig. 4). A two-way ANOVA with repeated measures indicated that the attenuation produced by BCE was significant [$F(1,6) = 44.37; p < 0.005$]. There were no significant differences between treatments in the membrane potential of the sensory neurons ($t_6 = 1.902$) or motor neurons ($t_6 = 0.131$), nor was there any difference between the measurements of input resistance [$F(1,6) = 3.166$]. Thus, BCE significantly attenuates the synaptic strength of the connection between sensory neurons and their follower cells.

In the next series of electrophysiological experiments, the actions of the individual BCPs were examined independently.

Experiment 3: β-BCP significantly attenuated synaptic strength

This series of experiments was designed to determine which of the BCPs contained in the BCE may mediate the modified behavior and attenuated synaptic strength observed after application of BCE, and to provide insight into the possible site(s) and mechanism(s) of action. The mean amplitude (±SEM) of the baseline EPSP in each group was 6.1 ± 1.2 mV (vehicle; $n = 17$), 6.7 ± 1.1 mV (α-BCP; $n = 8$), 3.5 ± 0.7 mV (ARG-VAS; $n = 8$), 7.3 ± 2.5 mV (β-BCP; $n = 8$), 8.3 ± 2.6 mV (ELH; $n = 8$), and 4.4 ± 1.6 mV (γ-BCP; $n = 8$).

In at least one of the experimental groups, β-BCP, the amplitudes of the EPSPs were attenuated beyond that expected from depression alone. Representative traces of EPSPs produced in the motor neuron (MN) by action potentials elicited in the sensory neuron (SN) from an experiment with β-BCP as well as from a control experiment are shown in Figure 5. The change in the amplitude of the EPSP between trials 4 and 5 and trials 9 and 10 in the control experiment (Fig. 5*A1,A2*, respectively) reflects depression of the synapse with repeated stimulation. In contrast, the change in the amplitude of the EPSP over the same period of time in the β-BCP experiment (Fig. 5*B1,B2*, respectively) reflects an attenuation of synaptic strength greater than that expected from depression alone. Summary data from all experiments are shown in Figure 6. For display purposes, β-BCP, the only peptide that produced a significant effect (see below), has been displayed independently (Fig. 6*A*) of the other peptides (Fig. 6*B*).

A two-way ANOVA with repeated measures indicated that

A1 ASW   A2 ASW + Vehicle

MN

SN

B1 ASW   B2 ASW + β-BCP
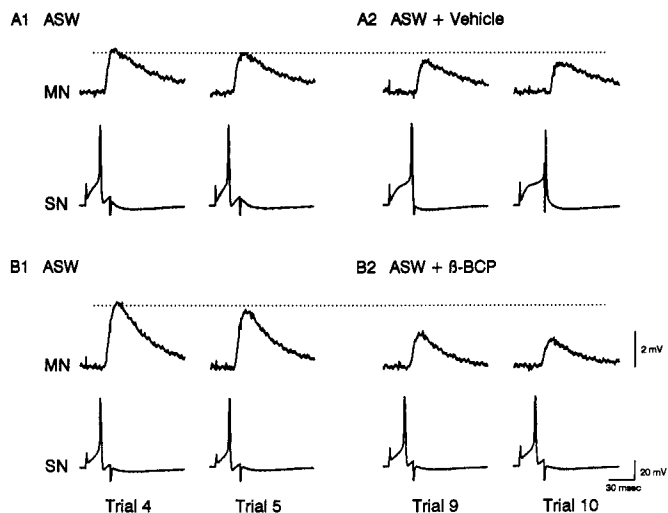
MN

SN

Trial 4   Trial 5   Trial 9   Trial 10

Figure 5. Effect of β-BCP on the amplitude of the EPSP: representative traces. EPSPs produced in the motor neuron (*MN*) by action potentials elicited in the sensory neuron (*SN*) from a control (vehicle) and β-BCP experiment are illustrated. The *broken lines* indicate the amplitude of the baseline EPSP (mean of trials 4 and 5). *A,* In this example from a control experiment, the amplitude of the EPSP in trial 9 and 10 was 70% and 74% of baseline, respectively. This change reflects depression of the synapse with repetitive stimulation. *B,* In this example from a β-BCP experiment, the amplitude of the EPSP in trial 9 and 10 was 53% and 44% of baseline, respectively. This change reflects an attenuation of the synaptic strength produced by β-BCP that is beyond that expected from depression alone.

there was a significant difference between treatments [$F(5,51)$ = 2.86; $p < 0.025$]. A test of main effects (a Dunnett's test comparing the five experimental treatments to control) revealed that only the attenuation produced by β-BCP was significant [$q(51) = 2.70$]. Bath application of α-BCP, γ-BCP, ELH, or ARG-VAS failed to attenuate the connection significantly. By trial 10, the amplitudes of the EPSPs in the β-BCP experiments were attenuated to 39.8 ± 4.6% of baseline whereas the amplitude of the EPSPs in the control experiments were 74.7 ± 6.6% of baseline. There were no significant differences between treatments in the membrane potential of the sensory neuron or motor neuron [$F(5,40) = 0.48$ and $F(5,40) = 0.31$, respectively].

There was, however, a significant decrease in the input resistance of the motor neuron in the β-BCP experiments. A two-way ANOVA with repeated measures performed on the input resistance of the motor neuron revealed a significant interaction effect [$F(20,204) = 2.052$]. Subsequent Dunnett's tests for simple effects indicated that the input resistance measurements for the control and β-BCP experiments were significantly different [$q(51)$ = 2.47] at trial 10 (93.5 ± 6.3% and 75.0 ± 6.0% of baseline, respectively). This decrease in input resistance could contribute to the attenuation of synaptic strength produced by β-BCP at this trial.

In the present experiments, which were conducted in ASW, there did not seem to be any effect of β-BCP on spike duration; however, a decrease in I_{Ca} or an increase in $I_{K,S}$, either of which may be important in the modulation by β-BCP (and cause spike narrowing and thus decreased release of transmitter), would not easily be detected in these experiments.

The next set of experiments specifically examined whether the attenuation of synaptic strength produced by β-BCP could be due, at least in part, to presynaptic spike narrowing.
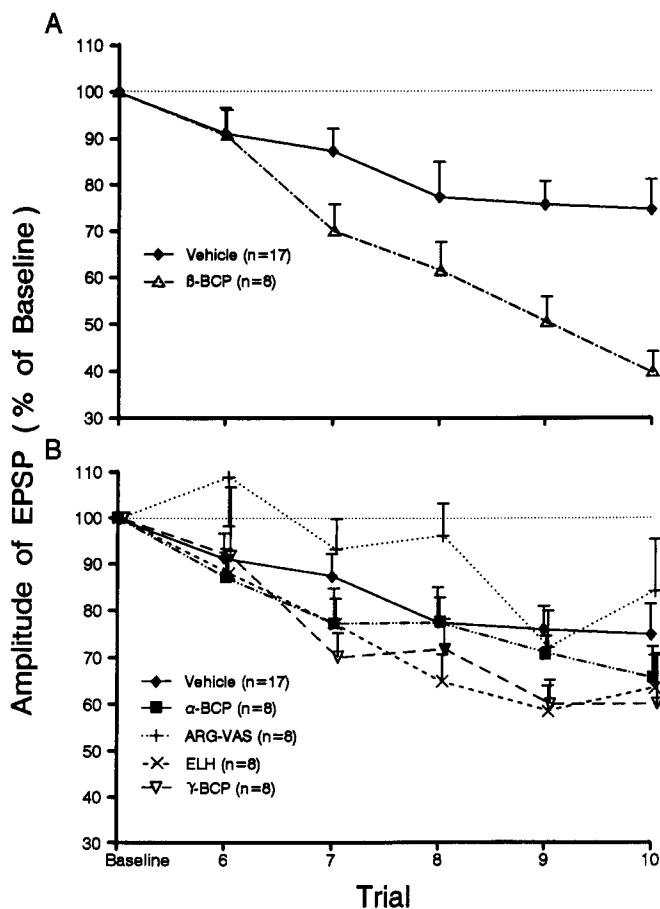
Figure 6. Effects of BCPs on the amplitude of the EPSP. Bath application of β-BCP significantly attenuated the amplitude of the EPSP. For display purposes, β-BCP, the only peptide that produced a significant effect, has been displayed independently (*A*) of those peptides that did not (*B*). β-BCP significantly attenuated the amplitude of the EPSP beginning 7.5 min after (trial 7) its bath application. The mean amplitude (±SEM) of the EPSP for trials 6–10 [normalized to baseline (mean of trials 4 and 5)] are illustrated.

Experiment 4: significant attenuation of synaptic strength produced by β-BCP is not dependent on presynaptic spike narrowing

To address whether I_{Ca} or $I_{K,S}$ may be affected by β-BCP, the present experiments were performed in ASW containing 100 mM TEA and low Na^+. TEA has previously been shown to block both $I_{K,V}$ and $I_{K,Ca}$ in pleural sensory neurons (Baxter and Byrne, 1989; Walsh and Byrne, 1989). Under these circumstances the resultant action potential is significantly broadened and is due primarily to I_{Ca} (for polarization) and $I_{K,S}$ (for repolarization). Moreover, small changes in I_{Ca} or $I_{K,S}$ can have profound effects on the duration of the action potential. Thus, by using this protocol, which facilitates the ability to detect changes in spike duration, we can determine to what extent the modulation of I_{Ca} or $I_{K,S}$ may be correlated with the attenuation of synaptic strength produced by β-BCP.

Representative examples from a control (vehicle) and β-BCP experiment are shown in Figure 7*A.* In this figure the EPSPs produced in the motor neuron (MN) by a TEA-broadened spike elicited in the sensory neuron (SN) are illustrated for the test 2.5 min before (trial 5) and 22.5 min after (trial 10) bath application of vehicle (Fig. 7*A1*) or β-BCP (Fig. 7*A2*). In this
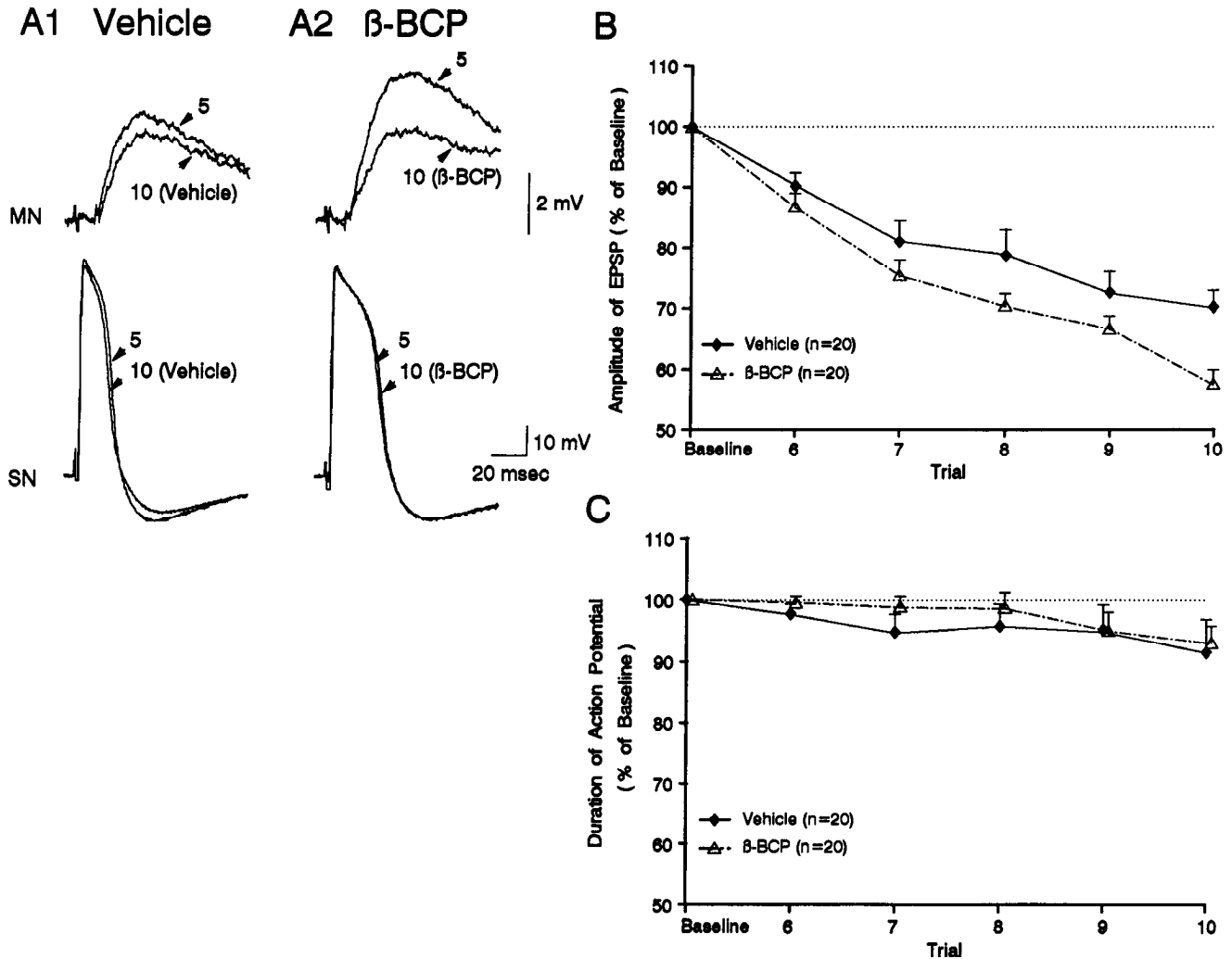
Figure 7. Effect of β-BCP on the amplitude of the EPSP and the duration of the presynaptic action potential. The attenuation of synaptic strength produced by β-BCP is not dependent on presynaptic spike narrowing. *A,* Representative traces of EPSPs produced in the motor neuron (*MN*) by a TEA-broadened action potential elicited in the sensory neuron (*SN*) 2.5 min before (trial 5) and 22.5 min after (trial 10) bath application of vehicle or β-BCP. *A1,* The amplitude of the EPSP 2.5 min prior to application of the vehicle (*5*) was 3.4 mV and depressed to 2.8 mV 22.5 min later [*10 (vehicle)*]. The duration of the spike was 15.6 msec and 13.5 msec, respectively. *A2,* The amplitude of the EPSP 2.5 min prior to application of β-BCP (*5*) was 4.8 mV and was 2.8 mV 22.5 min after adding β-BCP [*10 (β-BCP)*]. The duration of the spike was 22.5 msec and 24.6 msec, respectively. β-BCP, in this example, dramatically attenuated the amplitude of the EPSP even though the duration of the action potential was slightly greater after application of the peptide than before. *B,* β-BCP significantly attenuated the amplitude of the EPSP. The mean amplitudes (±SEM) of the EPSP for trials 6–10 [normalized to baseline (mean of trials 4 and 5)] are illustrated. *C,* The modulation of synaptic strength produced by β-BCP was not accompanied by a significant narrowing of the presynaptic action potential. The mean durations (±SEM) of the action potential for trials 6–10 [normalized to baseline (mean of trials 4 and 5)] are illustrated.

example, β-BCP dramatically attenuated the amplitude of the EPSP even though there was little or no change in the duration of the action potential. Indeed, the duration of the action potential was slightly greater after application of the peptide than before.

Summary data are shown in Figure 7*B*. The mean amplitude (±SEM) of the baseline EPSP in the control and β-BCP experiments was 3.7 ± 0.5 mV (*n* = 20) and 5.8 ± 0.8 mV (*n* = 20), respectively. There was no significant difference between control and β-BCP groups in the mean amplitude of the initial EPSP (trial 1: 6.2 ± 0.9 mV, *n* = 20, and 8.6 ± 1.2 mV, *n* = 20, respectively; t_{38} = 1.620) or in the rate of depression prior to the bath application (trial 5 normalized to trial 1: 58.8 ± 2.7%, *n* = 20, and 64.5 ± 3.1%, *n* = 20, respectively; t_{38} = 1.407). β-BCP produced an attenuation of synaptic strength beginning

2.5 min after bath application that lasted for at least 22.5 min. By trial 10, the amplitudes of the EPSPs in the β-BCP experiments were attenuated to 57.5 ± 2.4% of baseline whereas the amplitudes of the EPSPs in the control experiments, in which the vehicle was applied, were 70.1 ± 2.9% of baseline (Fig. 7*B*). A two-way ANOVA with repeated measures indicated that the attenuation of the amplitude of the EPSP produced by β-BCP was significant [$F(1,38)$ = 5.049].

Interestingly, this modulation of synaptic strength was not accompanied by a pronounced narrowing of the presynaptic action potential [$F(1,38)$ = 0.261] (Fig. 7*C*). Indeed, the duration of the action potential remained relatively constant in both the control and β-BCP experiments, decreasing to 91.6 ± 5.1% and 92.9 ± 2.9% of baseline, respectively, by trial 10 (Fig. 7*C*). The mean duration (±SEM) of the baseline spike was 22.8 ±
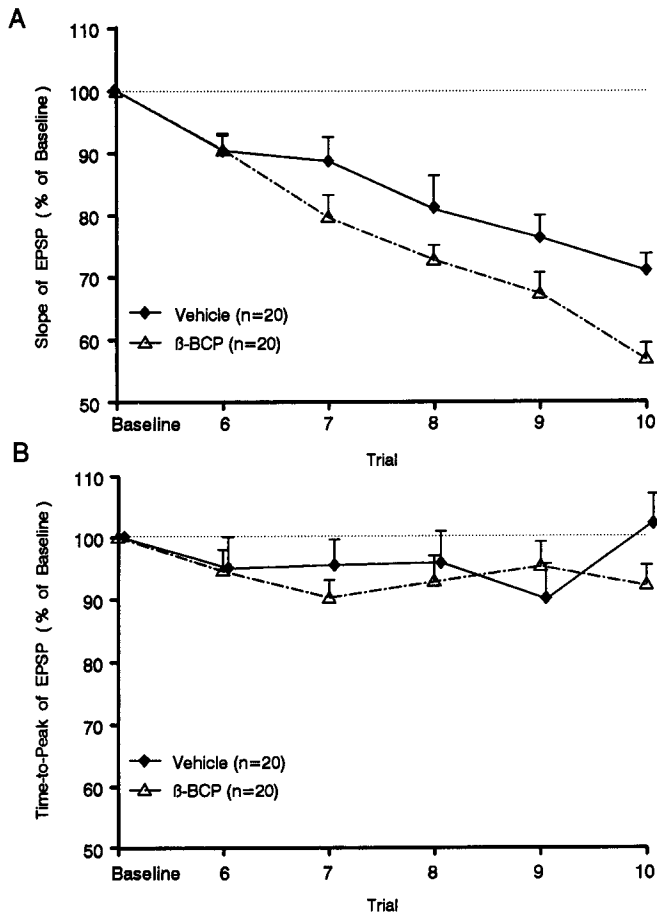
Figure 8. Effect of β-BCP on the slope and time to peak of the EPSP. The mean slope and time to peak of the EPSP for each of the post-bath application trials [normalized to baseline (mean of trials 4 and 5)] are illustrated. The attenuation of synaptic strength produced by β-BCP is accompanied by a significant decrease in slope, but not a significant decrease in time to peak. *A*, β-BCP significantly attenuated the slope of the EPSP. *B*, In contrast to the effect on the slope of the EPSP, β-BCP did not significantly affect the time to peak of the EPSPs.

2.5 msec (vehicle; $n = 20$) and 21.0 ± 1.5 msec (β-BCP; $n = 20$).

Previous empirical and modeling studies on the mechanisms underlying presynaptic facilitation (Hochner et al., 1986; Gingrich et al., 1988) indicate that changes in the duration of the presynaptic action potential are accompanied by changes in the time to peak of the EPSP in the follower neuron, whereas a spike duration–independent (SDI) process(es) (see also Pieroni and Byrne, 1992), such as changes in aspects of the transmitter release mechanisms (e.g., mobilization of transmitter) of the sensory neuron, are accompanied by changes in the slope of the EPSP. Assuming that similar correlations exist for forms of presynaptic plasticity other than facilitation, the shape of the EPSP in the motor neuron was analyzed.

Consistent with the fact that β-BCP did not produce a significant decrease in the duration of the action potential, there was no significant difference between β-BCP and control in the time to peak of the EPSP [$F(1,38) = 0.464$] (Fig. 8*B*). By trial 10, the times to peak of the EPSPs in the β-BCP and control experiments were 92.2 ± 3.2% and 102.5 ± 4.6% of baseline, respectively. In contrast, there was a significant difference between β-BCP and control in the slope of the EPSP [$F(1,38) =$

6.722] (Fig. 8*A*). This effect seemed to parallel the changes in the amplitude of the EPSP. By trial 10, the slopes of the EPSPs in the β-BCP experiments were attenuated to 56.8 ± 2.7% of baseline, whereas the slopes of the EPSPs in the control experiments were 71.0 ± 2.8% of baseline (Fig. 8*A*). This would suggest that β-BCP acts, at least in part, to modulate aspects of the transmitter release mechanisms.

There were no significant differences between treatments in the membrane potential of the sensory neuron [$t_{38} = 1.162$] or motor neuron [$t_{38} = 1.962$] or in the measurements of input resistance [$F(1,38) = 0.077$].

Discussion

BCE inhibits tail-siphon withdrawal reflex

These results suggest that one or more of the peptides contained in the BCE, and released at the time of egg laying, are capable of significantly inhibiting the baseline tail-siphon withdrawal reflex. Thus, in addition to the role the BCPs play in initiating egg laying and in eliciting the behaviors preceding and accompanying egg laying, they may also serve to modulate the animals response to external stimuli during egg laying. Inhibition of this reflex pathway would be consistent with inhibition of reflex release of ink by BCPs. Although this has not been directly tested, the L14 ink motor neurons are inhibited by the bag cells for up to 1 hr, which may temporarily alter the threshold for sensory activation of this reflex during egg laying (Mayeri et al., 1979). In the present experiments, all animals responded to the training protocol with the release of ink and opaline. It was noted (Goldsmith and Byrne, unpublished observations), however, on at least two occasions, that an animal having received an injection of BCE did not respond to the first train of sensitizing stimuli with release of ink. This would suggest that the BCPs, in addition to modulating the reflex withdrawal of the tail and siphon, were also modulating the ink motor neurons.

Since the BCE contains at least five peptides, it is difficult to determine from these experiments alone which peptide or peptides are responsible for this effect. The administration of ELH to one group of animals in this study, however, can be considered as the first step in this direction. That the ELH treatment did not differ significantly from the control treatment would seem to suggest that the effect of the BCE treatment on the baseline siphon withdrawal is not mediated by the actions of ELH alone. It remains a possibility that more than one peptide may be acting in concert to produce the inhibition of the reflex. Further, the finding that the ABG treatment did not differ significantly from the control treatment indicates that the peptide or peptides contained in the BCE that produced this effect are products unique to the bag cells, rather than common constituents of nervous tissue in general.

An important issue is whether the BCPs are acting directly on the tail-siphon circuitry, or the BCP-induced egg laying activates sensory receptors that in turn activate modulatory circuitry for the reflex. For example, it has been found that sensory input from the reproductive tract produced as the egg string moves through the tract may be important in the inhibition of feeding in *Aplysia* (Cobbs and Pinsker, 1982). A similar mechanism is unlikely to produce the inhibition of the tail-siphon withdrawal reflex, however. Support for this is provided by the lack of inhibition in the ELH-treated animals. If feedback from the egg string plays any part in producing the inhibition, the ELH-treated animals, all of which did lay eggs, should have

been affected. In addition, although egg laying began at or after the third trial of the pretraining test period in the BCE group, the inhibition of the reflex was observed to begin with the first trial. Moreover, in semi-intact preparations of the tail in which there is no feedback, preliminary data suggest that BCE may inhibit the tail withdrawal reflex. It should also be noted that the BCPs, in combination and individually (in the case of β-BCP), attenuate the amplitude of EPSPs in the isolated pleural-pedal ganglion. This indicates that the site of action is more than likely the tail-siphon circuitry. Previously, Mackey and Carew (1983) demonstrated that injection of BCE reversibly suppressed 5-HT–triggered locomotion. This effect also is likely to be due to the effects of the extract on neurons in the pleural-pedal ganglion. The mechanism by which the BCPs may exert their inhibitory effect on the tail-siphon withdrawal circuitry is discussed below.

BCE suppresses long-term sensitization

In addition to the effect on the baseline tail-siphon withdrawal reflex, these results suggest that one or more of the peptides contained in the BCE are capable of suppressing long-term sensitization of the reflex. Several of the BCPs are thought to be rapidly degraded and therefore have a limited window of biological activity (Sigvardt et al., 1986; Squire et al., 1991; Owens et al., 1992). However, as these experiments suggest, the BCPs have the capacity to produce effects that are rather long lasting. Indeed, in contrast to previous acute studies of the effects of BCPs, these experiments raise the possibility of a new temporal domain (>24 hr) for the effects of these peptides.

As is true for the inhibition of the baseline siphon withdrawal, the role of ELH in suppressing long-term sensitization remains questionable. If ELH plays any role at all, it is most likely expressed at higher concentrations than used in these experiments and/or is limited to a concerted action with one or more of the other BCPs, as ELH by itself does not suppress long-term sensitization.

The suppression of long-term sensitization is surprising in light of the fact that the memory for short-term sensitization is still intact. If both short- and long-term sensitization were suppressed, one could easily offer several explanations. One or more BCPs might alter the threshold for activation of the facilitatory interneurons in response to the sensitizing stimuli, block the receptor for the facilitatory neurotransmitter, or block the elevation of the second messengers in the sensory neuron necessary for the induction of both the short- and long-term enhancement. An effect on both short- and long-term sensitization does not seem to be the case, however.

An intriguing possibility for the selective effect on long-term sensitization is that the BCPs are acting directly on the processes underlying learning. This is particularly probable considering that there exists a differential effect of the BCE on the short- and long-term forms of sensitization. This leads to the possibility that the BCPs are affecting that part of the mechanism specific to long-term sensitization. Although the mechanisms underlying long-term sensitization have not been fully elucidated, it is known that some of the changes accompanying long-term sensitization are dependent upon new protein synthesis (Castellucci et al., 1989). It is therefore intriguing to speculate that one or more of the BCPs, still active during all or part of the training procedure, interfere with the effect of the neuromodulator, released by sensitizing stimuli, to induce new protein synthesis. Future experiments, using an *in vitro* analog of long-term sensitization training (Goldsmith and Byrne, 1989; Byrne

et al., 1991) to examine the effects of the BCPs, will be necessary to answer this.

An alternative explanation is that both short- and long-term sensitization were suppressed by BCE and that the enhancement observed at the 5 min posttraining test is due, at least in part, to the fact that the intensity of the sensitizing stimuli was so strong that the system was saturated. Thus, even though the peptides in the extract may have been active, their effects are not apparent in the behavior.

β-BCP attenuates the amplitude of the EPSP

The electrophysiological experiments examining the effects of BCE on the sensorimotor synapse revealed that it attenuates the synaptic strength beyond that expected from depression alone. Further, examination of the individual BCPs indicated that only β-BCP significantly attenuates synaptic strength, and does so independently of presynaptic spike narrowing. Thus, the sensory-to-motor synapse may be one locus for the behavioral inhibition of the reflex by BCE, and β-BCP may be the peptide contained in the BCE that is responsible for the inhibition. These studies are the first to indicate that β-BCP may have an inhibitory role. Previously, the only known role for β-BCP was autoexcitation of the bag cells (Brown and Mayeri, 1989).

Neither α-BCP, γ-BCP, ELH, nor ARG-VAS significantly affected the EPSP. The fact that α-BCP produced no effect was interesting given that the majority of the inhibitory effects on neuronal activity in the abdominal ganglia produced by BCE or by activation of the bag cells have been ascribed to the action of α-BCP (Rothman et al., 1983; Rock et al., 1986; Sigvardt et al., 1986). Likewise, the attenuation of sensory-to-motor neuron synapses in the abdominal ganglion produced by ARG-VAS (Goldberg et al., 1987) suggested that it might have similar actions in the pleural-pedal ganglia. Nonetheless, the lack of such an effect by ARG-VAS confirms the specificity of action of β-BCP on this synapse.

The locus of the actions of β-BCP could be presynaptic, postsynaptic, or both. While our study of possible postsynaptic mechanisms was by no means exhaustive, we did not find a significant change in the membrane potential of the motor neuron. There was, however, a small decrease in input resistance produced by β-BCP in the experiments performed in ASW, which became significant at trial 10. A similar effect was not found in the experiments performed in TEA, however. An explanation for this disparity between the two sets of experiments may be that β-BCP increases the motor neurons conductance by increasing the permeability to Na^+, an effect that may have been diminished in experiment 4, which was performed in low Na^+. Thus, the decrease in input resistance observed in experiment 3, although only significant at trial 10, may play a role in the attenuation of synaptic strength produced by β-BCP. Alternatively, β-BCP may have as a target some of the currents that are blocked by TEA. This would help to explain why in the presence of TEA we did not see a decrease in input resistance and by extension why the magnitude of attenuation was not as great in the experiments performed in TEA. Interestingly, application of BCE also did not produce a significant change in the input resistance of the motor neuron. A possible explanation for this finding is that the various BCPs contained in the BCE (some of which have not been tested independently in these studies) have opposing effects on the input resistance of the motor neuron, such that the net effect is difficult to detect. Alternatively, the absence of an effect of BCE may be explained

by differences in the concentrations of the BCPs found in the BCE and those used in the experiments in which the BCPs were applied individually.

With regard to possible presynaptic mechanisms, the effects on synaptic strength do not appear to be due to changes in duration of the action potential or in the resting membrane potential of the sensory neuron. The lack of an effect on spike duration by β-BCP is also consistent with the finding that there was no significant difference between the control and β-BCP experiments in the time to peak of the EPSP. As we are recording only from the soma of the sensory neuron, it remains a possibility that spike narrowing does occur at the terminals of the sensory neuron, where modulation of currents such as I_{Ca} or $I_{K,S}$ may be more consequential. However, as there was no change in the time to peak of the EPSP, which, if present, might reflect a change in the duration of the action potential at the terminals of the sensory neuron, we assume that spike narrowing at the terminals may also be negligible. Again, as mentioned above in the discussion of possible postsynaptic mechanisms, there remains the possibility that β-BCP produces a small amount of narrowing of the action potential and decreases synaptic transmission in part by enhancing $I_{K,V}$. This effect would be abolished in experiments performed in TEA.

Nonetheless, the above results, combined with the fact that there was a significant decrease in slope of the EPSP, support the hypothesis that the attenuation of synaptic strength produced by β-BCP is due, at least in part, to a decrease in the activity of an SDI process (e.g., mobilization of transmitter), rather than to a decrease in spike duration (Hochner et al., 1986; Gingrich et al., 1988; see also Pieroni and Byrne, 1992). Thus, β-BCP–induced attenuation may reside in a presynaptic locus and be due solely to changes in aspects of the transmitter release mechanism. This is in contrast to the actions of FMRFamide, a neuromodulatory peptide believed to contribute to behavioral inhibition of the siphon-withdrawal reflex (Mackey et al., 1987). FMRFamide decreases both the SDI process (Pieroni and Byrne, 1992) and the duration of the action potential (Abrams et al., 1984). In addition to the possible contribution a decrease in input resistance of the motor neuron may make toward the attenuation of synaptic strength produced by β-BCP, other postsynaptic factors may also be of importance. For example, an antagonistic role of β-BCP with the postsynaptic receptors would also be consistent with an attenuated EPSP.

An issue that the present experiments do not address is whether these peptides are acting directly on the pre- and postsynaptic neurons, or indirectly via the activation of inhibitory neurons, the neurotransmitter(s) of which would then effect these changes. However, the experiments performed in low Na$^+$ suggest that the modulation by β-BCP may be a direct effect.

Additionally, it should be pointed out that there is no evidence as yet that the tail-siphon withdrawal reflex or the changes in synaptic strength seen in the isolated nervous system occur after endogenous release of the BCPs (e.g., by stimulation of ELH- or BCP-containing neurons). Indeed, before the role of the BCPs, and in particular β-BCP, can be clearly established, it will be necessary to determine whether injection of β-BCP produces the same behavioral effects as injection of BCE, whether injection of BCE leads to an afterdischarge in the bag cells and thus to the release of additional BCPs, whether the behavioral modifications occur in intact animals during spontaneous egg laying or induction of egg laying by electrical stimulation of bag cells, and whether the discharge of the bag cells and/or other β-BCP-

containing neurons in the *in vitro* preparation leads to changes in the amplitude of the EPSP.

Nonetheless, these experiments, as in previous studies in vertebrate model systems, demonstrate the variety of potential roles peptides may assume in the nervous system. The BCPs have previously been demonstrated to exert effects only on those behaviors that accompany egg laying or that are crucial for egg laying to occur. The present study suggests that these peptides may be important in other roles as well, particularly the inhibition of defensive reflexes. This finding parallels the finding of Advokat (1980) that food stimulation attenuates three defensive behaviors, which include the siphon withdrawal and inking. This is especially meaningful given that feeding and reproductive behaviors appear to be governed by a common arousal system (Ziv et al., 1991). In addition, these experiments suggest that the BCPs have a dramatic effect on a simple form of learning and memory. As we have identified a potential locus of action for at least one of these effects and a peptide that may exert all, or part, of this action, we now have the opportunity to examine in greater detail the mechanisms by which this peptide acts.

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