The Bag Cells of *Aplysia* as a Multitransmitter System: Identification of Alpha Bag Cell Peptide as a Second Neurotransmitter

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The bag cell neurons of the marine mollusk, *Aplysia*, are a putative multitransmitter system that utilizes two or more peptide transmitters derived from a common precursor protein. Two putative transmitters are egg-laying hormone (ELH), a 36 amino acid peptide that induces egg laying and mediates bag cell-induced excitatory effects on certain abdominal ganglion neurons, and α-bag cell peptide (αBCP), which mimics bag cell-induced inhibition of the left upper quadrant (LUQ) neurons and the depolarization of the bag cells that occurs during the bag cell burst discharge. αBCP was previously purified from bag cell extracts in three neuroactive forms: αBCP(1-9), a nine amino acid peptide encoded on the ELH/BCP precursor protein, and two NH₂-terminal fragments, αBCP(1-8) and αBCP(1-7). Analyzing bag cell-induced inhibition of LUQ neurons, we report here that αBCP fulfills the main criteria for transmitter identification: (1) stimulation of individual bag cells produces inhibition of the neurons; (2) inhibitory activity is present in releasate collected following an elicited bag cell burst discharge in the presence of protease inhibitors; (3) αBCP(1-9) and αBCP(1-8) are detected in the releasate in the presence of protease inhibitors; (4) αBCP is rapidly inactivated after release, as indicated by the lack of detectable αBCP or inhibitory activity in the releasate in the absence of protease inhibitors, and by the increase in potency of the arterially perfused peptide in the presence of protease inhibitors; (5) αBCP and the endogenous released transmitter produce apparently identical changes in membrane conductance; (6) bag cell-induced inhibition is reduced or abolished following desensitization of the inhibitory response by long-term application of high concentrations of αBCP.

The results provide additional evidence that the bag cells are a multitransmitter system and also suggest that many of the physiological properties of αBCP-mediated neurotransmission differ from those of ELH. First, unlike ELH, αBCP is rapidly inactivated after release. Second, αBCP(1-9) may be activated by carboxypeptidase cleavage since αBCP(1-8) and αBCP(1-7) are 30 and 10 x as potent, respectively, as αBCP(1-9). Third, the inhibitory action of αBCP on its targets has a more rapid onset and a shorter time course than the excitatory actions of ELH. Thus, αBCP may diffuse to less distant targets than ELH and serve to regulate the more rapidly occurring neural events underlying egg-laying behavior.

Studies in both vertebrates and invertebrates suggest that many types of neurons contain more than one biologically active peptide that are thought to function as neurohormones or neurotransmitters (Erichsen et al., 1982; Hunt et al., 1981; Lundberg et al., 1982; Rothman et al., 1983a; Schultzberg et al., 1980; Watson et al., 1978). The bag cells of *Aplysia* are a convenient system for investigating the role of peptidergic neurons in the processing of information in the CNS. A bag cell-specific gene encodes a precursor protein that contains the sequences for egg-laying hormone (ELH), α-bag cell peptide (αBCP), acidic peptide (AP), β-, γ-, and δBCP, and several other peptides (Scheller et al., 1983a). There is strong evidence that ELH, a 4400 Da peptide, mediates two types of bag cell-induced excitatory responses in abdominal ganglion neurons (Branton et al., 1978; Mayeri and Rothman, 1982a; Mayeri et al., 1983), but does not mediate other bag cell-induced responses. αBCP is a candidate as a second bag cell transmitter for mediating the inhibitory responses (Rothman et al., 1983a).

There are neurons in other parts of the CNS that are immunoreactive for ELH (Chiu and Strumwasser, 1981), and ELH may mediate effects on other central neurons, most notably identified buccal ganglion neurons (Stuart and Strumwasser, 1980). Bag cell-induced responses on a subpopulation of abdominal ganglion neurons provide a convenient preparation for understanding how various bag cell peptides act. Bag cell-induced inhibition occurs in the left upper quadrant (LUQ) neurons, the right upper quadrant white cells R3-R14, and in L14A-C, L10, and other identified and unidentified neurons (Mayeri et al., 1979a, b). The LUQ neurons, which were used previously as an assay system in the isolation and sequencing of αBCP, were used in the present study to further elucidate the role of αBCP. The suggestion that αBCP is a neurotransmitter is based on the following evidence: (1) Three neuroactive forms of αBCP have been isolated from bag cell extracts: αBCP(1-9), (1-8), and (1-7) (Rothman et al., 1983a). The sequence of αBCP(1-9) is Ala-Pro-Arg-Leu-Arg-Phe-Tyr-Ser-Leu; αBCP(1-8) and αBCP(1-7) are NH₂-terminal fragments lacking the COOH-terminal leucine and serylleucine residues, respectively. (2) The three forms of αBCP mimic inhibition of LUQ neurons and depolarization of the bag cells. Although they differ in their relative potencies, the three forms otherwise produce apparently identical effects on nerve cells. αBCP(1-7), purified from bag cell extracts, and synthetic αBCP(1-7) have identical inhibitory actions on LUQ neurons at a given concentration (Rothman et al., 1983a). (3) αBCP acts directly on LUQ neurons (Rothman et al., 1983a). (4) αBCP(1-9) is encoded on the bag cell gene between basic residues that represent cleavage sites (Scheller et al., 1983a). (5) Immunohistochemical studies using affinity-purified antibodies to αBCP(1-9) show that the somata of the left and right upper quadrant neurons are surrounded by an extensive network of αBCP(1-9)-immunoreactive bag cell processes.
that terminate in the ganglionic sheath and are coextensive with bag cell processes immunoreactive for ELH (Puls et al., 1985).

We present here further evidence that αBCP fulfills the major criteria for identification as a transmitter and provide additional documentation of the physiological characteristics of αBCP. We find that the physiological characteristics of αBCP differ in important ways from those of ELH and provide a rationale for why it is functionally useful for a group of neurons to use more than one transmitter derived from a common precursor.

Materials and Methods

Electrophysiology and arterial perfusion of peptides

Experiments were carried out on over 100 preparations of Aplysia californica obtained from Sea Life Supply (Sand City, CA). The results of each of the physiological experiments described below were obtained on at least three different preparations. Electrophysiology and arterial perfusion of peptides were performed as described previously (Mayeri and Rothman, 1982b; Mayeri et al., 1979a, b, 1985). In brief, an abdominal ganglion was dissected from animals weighing 400-1500 gm and pinned in a 1 ml capacity chamber. The preparation was steadily superfused with locally obtained seawater containing 10 mM HEPES (pH 7.6) and 1 gm/liter dextrose at a rate of 30 ml/hr. In experiments using low Ca++ high Mg++ bathing medium, the seawater was replaced with a solution containing 220 mM MgCl₂, 110 mM NaCl, and 10 mM KCl. Experiments were performed at room temperature (19-23°C), which usually did not vary more than 1° in a single experiment. Intracellular recordings were made from up to four neurons simultaneously, using conventional methods. The bag cells were continuously monitored during all experiments by placing an extracellular recording electrode on one or both bag cell clusters.

Solutions of the various peptides to be tested were perfused through the usual artery of the abdominal ganglion at a rate of 3 μl/min by means of a micrometer syringe. The peptides to be tested were dissolved in filtered (0.22 μm pore size), buffered seawater. Unless noted, the perfusion medium also contained a mixture of protease inhibitors (see below). In all experiments, an aliquot of the perfusion medium without the peptides was applied before the test solution both as a control and to preincubate the ganglion in protease inhibitors. In experiments in which protease inhibitors were not used, BSA (250 μg/ml) was added to the perfusion medium as a carrier. Neither the protease inhibitors alone nor the BSA alone affected target neuron activity. αBCP(1-7), (1-8), and (1-9), and β, γ, and δBCP were synthesized by Peninsula Laboratories (Belmont, CA). The serial perfusion experiments were performed as described previously (Mayeri et al., 1985).

Protease inhibitors in the perfusion medium

In most physiological experiments, the perfusion medium contained 100 μM Na ascorbate, 25 μg/ml antizin-convertase enzyme inhibitor (Peninsula Laboratories), and 250 μg/ml of each of the following protease inhibitors: bacitracin (Sigma), ovoinhionin (Sigma; hen egg-white trypsin inhibitor), ovomucoid (Whorthington), lima bean trypsin inhibitor (Sigma), leupeptin (Peninsula Laboratories), antipain (Peninsula Laboratories), and phenylalanine (Vega Chemicals). In some earlier experiments, two of the protease inhibitors, angiotensin converting enzyme inhibitor and phenylalanine, were not included.

In the experiments in which releasate was collected for high-pressure liquid chromatography (HPLC) analysis, the perfusion medium consisted of bathing medium containing 3 mM NaHCO₃ and 62 μg/ml each of leupeptin, antipain, diprotin A (Peninsula Laboratories), serylleucine, leucylarginine, and phenylalanine (Vega Biochemicals, Tucson, AZ).

The two groups of protease inhibitors, which were chosen to protect αBCP from degradation, are known to be effective against several of the major classes of proteases, including aminopeptidases (bacitracin, diprotin A, endopeptidases (ovoinhibitor, ovomucoid inhibitor, leupeptin, antipain), and carboxypeptidases (angiotensin-convertase enzyme inhibitor and the dipeptides).

HPLC analysis of releasate

An abdominal ganglion was placed in a small sealed chamber of 200 μl dead volume. The caudal artery was cannulated and bag cell activity was monitored with two extracellular electrodes, one on each cluster. The two electrodes also served as stimulating electrodes (Mayeri et al., 1985). The ganglion was perfused through the cannula and superfused by means of a second tube entering the chamber. The perfusion and superfusion rates were each 250 μl/hr. Releasate (combined perfusate-superfusate) was led out of the chamber and collected at 0°C. Control releasate was collected for 30 min prior to initiation of a bag cell burst discharge; bag cell burst releasate was collected for 30 min following burst initiation. Releasates were stored at -20°C. For analysis, control releasates from six ganglia were pooled to yield approximately 7 ml; bag cell burst releasates (eight bag cell bursts in six ganglia) were also pooled to yield a total volume of 7.75 ml.

Releasate was thawed and applied to a reverse phase HPLC column (Supelco, LC₂₅ DB, 4.6 x 250 mm plus 4.6 x 20 mm precolumn) equilibrated in pyridine acetate (0.11 M pyridine, 0.5 M acetic acid), pH 4.0. Flow rate was 0.6 ml/min. Peptides were eluted with a complex gradient of n-propanol in pyridine acetate, pH 4.0 (see Fig. 6D).

Results

Direct bag cell stimulation produces inhibition

During a bag cell burst discharge, the approximately 400 cells within each cluster fire nearly synchronously for about 20 min (Kupfermann and Kandel, 1970), producing prolonged inhibition of the LUQs (Mayeri et al., 1979a). The usual method for triggering this burst is by focal stimulation of the bag cell cluster with a short (1-2 sec) train of electrical pulses via an extracellular electrode placed directly on the surface of either cluster (Mayeri et al., 1979a). The stimulus intensity is carefully adjusted to a level that produces a spike or depolarizing response in the bag cells without producing synaptic potentials or other responses in abdominal ganglion neurons recorded intracellularly. The presence of postsynaptic potentials one-for-one with the stimulus in other ganglion neurons indicates that the stimulus has excited other axons in the connective in addition to bag cell neurons. Although it is probable that this procedure activates only bag cells, it has not been directly demonstrated that the inhibition of the LUQs is due to bag cell activation and not to other neurons that might be activated by the extracellular stimulating electrode.

To test directly for bag cell-induced inhibition, a bag cell and an LUQ neuron were recorded from simultaneously. As shown in Figure 1A, direct depolarization of the bag cell by a train of intracellular depolarizing current pulses (100 msec pulses at 5/sec) resulted in repetitive spike activity in the bag cell and inhibition of the LUQ neuron. The repetitive spike activity was most likely restricted to one or a few bag cells of the entire cluster because no spikes were recorded by an extracellular electrode placed on the cluster (not shown). The inhibition was characterized by hyperpolarization of the cell membrane potential, slowing of the cell’s burstingpacemaker activity, and a decrease in the spike amplitude. When depolarizing current was again injected into the bag cell, as shown in Figure 1B (arrow), it was then sufficient to initiate the bag cell burst discharge in cells of both bag cell clusters; this resulted in a large and prolonged inhibition of L6. The larger amplitude and longer time course of this response presumably reflects a larger quantity of transmitter released during the burst of the entire bag cell population. Intracellular stimulation of a single bag cell after the end of a long bag cell burst (Fig. 1B, right) did not result in further inhibition, apparently because transmitter is no longer released from the bag cells. It did not result from desensitization, since, in other experiments, arterially perfused αBCP applied following the end of a bag cell burst discharge still resulted in inhibition of the LUQs.

The ability to trigger a bag cell burst discharge by intracellular current injection into a single bag cell neuron is most likely the result of two processes acting together: electrical coupling between bag cells, allowing injected current to spread throughout the population (Kupfermann and Kandel, 1970), and autox-
citation by αBCP, in which αBCP released by bag cells further depolarizes them (Rothman et al., 1983a). Activation of a bag cell burst by this method produced the same effects in the LUQs (and other recorded ganglion neurons) as that produced by carefully controlled extracellular focal stimulation (for example, see Fig. 3, Mayeri et al., 1979a), thus confirming the results obtained by the latter method. Not every bag cell, when activated individually, produced an inhibitory effect; bag cells located closer to target cells seemed to be more effective.

Only αBCP produces inhibition of LUQs

αBCP is one of several peptides encoded on the ELH precursor gene (Scheller et al., 1983a). Five of these peptides (ELH, AP, α-, β-, and γBCP) have been isolated from bag cell extracts and identified (ELH, Chiu et al., 1979; AP, Scheller et al., 1983a; α-, β-, and γBCP, Rothman et al., 1983a, 1985b). The amino acid sequences of β- and γBCP are Arg-Leu-Arg-Phe-His and Arg-Leu-Arg-Phe-Asp, respectively. α-, β-, and γBCP have a common sequence of four residues. Because of their structural similarity, it is important to know whether βBCP, γBCP, or the other peptides have an effect on the LUQ neurons. When a solution containing 1 μM each of ELH, AP, β-, γ-, and δ-bag cell peptide was arterially perfused into the abdominal ganglion, there was no effect on the LUQ neurons (Fig. 2A). Furthermore, concentrations of these peptides as high as 100 μM, applied individually, had no effect on LUQs (not shown). When αBCP(1–7) was added to the solution containing the five other bag cell peptides and perfused, the LUQs were inhibited (Fig. 2B). αBCP(1–7), applied alone, had the same effect (for example, see Fig. 3B). Therefore, the inhibition of the LUQs produced by the bag cell discharge is mimicked only by α-bag cell peptide.

It was previously shown that the effects of arterially perfused αBCP on all four LUQ cells ended within a few minutes after the end of application (Rothman et al., 1983a). Thus, the peptide

Figure 1. Inhibition of the bursting pacemaker neuron, L6, produced by direct depolarization of a bag cell. L6 and a bag cell in the left bag cell cluster were recorded simultaneously with intracellular microelectrodes. A, Repeated activation of impulses in a single bag cell by intracellular current injection (bars) produced inhibition of L6. B, Brief intracellular current injection into a single bag cell (at arrow) initiated a burst discharge, which occurred in all the cells of the bag cell cluster and resulted in more prolonged inhibition of L6. Minutes later (at right), repeated activation of impulses in the single bag cell produced no effect on L6.

Figure 2. Of six of the peptides encoded on the bag cell precursor gene, only αBCP produces inhibition of the LUQs. Two LUQ neurons, L2 and L3, were recorded simultaneously. A, Arterial perfusion of a solution containing 1 μM each of β-, γ-, and δBCP, ELH, and AP during the time indicated by the bar had no effect on either neuron. B, Addition of 1 μM αBCP(1–7) to the solution described in A resulted in inhibition similar to that produced by bag cell activation as shown, for example, in Figure 1.
Figure 3. Effects of the presence of protease inhibitors in the perfusate on the potency of arterially perfused αBCP(1-7). A, Percentage decrease in spike rate of an LUQ neuron, L3, versus the concentration of αBCP(1-7) in the perfusion medium. Dashed line, No protease inhibitors in the perfusate; solid line, eight protease inhibitors (see Materials and Methods) added to the perfusate. Without protease inhibitors (no I’s), a concentration between 1.0 and 3.0 μM αBCP(1-7) was necessary to produce the same effect. B, Intracellular record from L3, the activity of which is plotted in A, shows that arterial perfusion of 1 μM αBCP(1-7) without protease inhibitors did not affect the neuron’s activity, whereas perfusion of the same concentration with protease inhibitors added caused a large inhibition. Arterial perfusion of a solution containing only the protease inhibitors had no effect on the cell’s activity (not shown).

mimics all aspects of bag cell-induced inhibition in L2 and L4 and all but the duration of inhibition in cells L3 and L6 (see Discussion). In the present study, we found that the long duration of inhibition was not mimicked by any of the three forms of αBCP applied singly or in combination with other bag cell peptides (β, γ, δBCP, ELH, or AP). It may be that the longer

duration response in L3 and L6 is produced by a second, as yet unidentified, transmitter released by the bag cells that acts together with αBCP to prolong the inhibitory response. In other experiments, with pressure application of αBCP onto L3 from a micropipette placed directly over the soma, we confirmed that the action of αBCP is direct (Rothman et al., 1983a). This indicates that there are receptors for the transmitter on the neuron’s soma.
Inactivation of αBCP can be demonstrated by comparing the potency of the peptide in the presence or absence of protease inhibitors in the perfusate (Fig. 3). The graph in Figure 3A plots the percentage decrease in spike rate of L3 from control levels as a function of αBCP concentration. With protease inhibitors in the perfusate, a concentration of 0.3 μM αBCP(1-7) produced strong inhibition of L3. Without protease inhibitors, an approximately 10-fold higher concentration of peptide was necessary to produce the same effect. Intracellular recordings from the same experiment (Fig. 3B) show the effects on L3 of arterially perfusing 1 μM concentrations of αBCP(1-7) with and without inhibitors. In the presence of protease inhibitors, the cell was strongly inhibited, whereas in their absence, the cell was unaffected, indicating that the peptide had been rapidly inactivated. A similar result was obtained for αBCP(I-9) (not shown); αBCP(I-8) was not tested. These results suggest that there are membrane-bound proteases in the vascular and interstitial spaces of the ganglion that are highly effective in inactivating αBCP.

αBCP(I-7) is about 10 times more potent than αBCP(I-9)

Of the three forms of αBCP, αBCP(I-8) has been shown to be approximately 3x more potent than αBCP(I-7) in producing inhibitory effects on the LUQs, and αBCP(I-9) has been reported to be the least potent (Rothman et al., 1983a). We confirm here that the largest of the peptides, αBCP(I-9), is the least potent form; it is approximately one-tenth as potent as αBCP(I-7) (Fig. 4). The graph in Figure 4A shows the percentage decrease in spike activity in L6 versus the concentration of peptide in the perfusate. The peptides were applied in the presence of protease inhibitors. The effects of αBCP(I-7) at 0.3 and 1.0 μM were equal to or smaller than the effects of αBCP(1-7) at 10-fold lower concentrations. At 0.1 μM (Fig. 4B, from the same experiment), L6 was only slightly inhibited by αBCP(I-9), while at the same concentration of αBCP(I-7), the cell was nearly maximally inhibited. All three forms of αBCP produce apparently identical inhibition.

Inhibitory activity is released during the bag cell burst

If αBCP is released from the bag cells, one might expect to detect it and its activity in releasate (i.e., perfusate plus superfusate) collected from an abdominal ganglion following a bag cell burst discharge. To test for release, an abdominal ganglion was placed in a sealed chamber and simultaneously perfused and superfused, and bag cell activity was monitored with an extracellular electrode. The medium exiting the chamber was fed directly into the artery of a second abdominal ganglion, located in a normal recording chamber, and the activity of neurons was monitored intracellularly. In an earlier study, following a bag cell burst in the first ganglion, releasate produced excitatory responses mediated by ELH in L8 and LC cells and R15 in the second ganglion. However, no effects were seen on target neurons that are normally inhibited (LUQ neurons) or transiently excited (L1, R1) by the bag cells (Mayeri et al., 1985, Fig. 6b).

We reasoned that, if the inhibition and transient excitation were indeed mediated by bag cell peptides, these peptides may have been inactivated by proteolysis before leaving the ganglion. To test this possibility, we repeated the experiment just described, but added eight protease inhibitors to the perfusate. (See Materials and Methods; either BSA or three inhibitors—bacitracin, ovomucoid, and lima bean trypsin inhibitor—were used by Mayeri et al., 1985.) During a 60 min control period before the bag cell discharge, the neurons fired at a steady rate. Six minutes after a discharge was triggered in the first ganglion (Fig. 5), cells L2, L3, and L6 in the assay ganglion were inhibited,
tors, there may have been a higher concentration of transmitter.

Second, in the release experiments, the bag cell transmitters were released into space with a much smaller volume than in single-ganglion experiments. In three of six experiments, however, a bag cell burst discharge was initiated in the second ganglion within 1–3 min after onset of inhibition evoked by releasate from the first ganglion. This result is consistent with the proposal that aBCP and/or other bag cell peptides are excitatory autotransmitters involved in the initiation and maintenance of the burst discharge within each cluster (Rothman et al., 1983a).

**aBCP is released during the bag cell burst**

Release experiments were conducted on six abdominal ganglia in order to determine if aBCP was present in the releasate. Perfusate collected before the bag cell burst in each experiment was pooled, as was the releasate collected in each experiment after the initiation of a bag cell burst discharge. The perfusate contained six protease inhibitors that were compatible with the HPLC analysis (see Materials and Methods). Peptides present in pooled releasate were analyzed by HPLC using a complex gradient of n-propanol used to elute peptides is shown in Figure 5, an extracellular recording showed that no bag cell activity occurred in the second ganglion. In three of six experiments, however, a bag cell burst discharge was initiated in the second ganglion within 1–3 min after onset of inhibition evoked by releasate from the first ganglion. This result is consistent with the proposal that aBCP and/or other bag cell peptides are excitatory autotransmitters involved in the initiation and maintenance of the burst discharge within each cluster (Rothman et al., 1983a).

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Figure 8. Identity of action of αBCP and the endogenously released bag cell inhibitory transmitter. A three-step hyperpolarizing current pulse was periodically applied to LUQ neuron L6. A. Membrane voltage versus steady-state current measured at the end of each step of current. Current-voltage relationships are shown for control conditions and after arterial perfusion of αBCP (solid lines) or control conditions and after an electrically triggered bag cell burst (BCB; dashed lines). The data points for αBCP and BCB were each taken from the three-step current pulse that occurred during the peak of the inhibitory response. The data points for the control are averages from three current pulses just prior to the inhibitory response. The data points for the control membrane potential at 0 nA were taken at the inflection point on the upswing of the membrane potential between two successive action potentials. αBCP and the BCB produced apparently identical changes in the current-voltage relationship, indicating the ionic mechanisms underlying the two responses are identical. B. Intracellular record of L6, from which the membrane potential between two successive action potentials is presented in Figure 6. Analysis of 320 μl of pooled releasate collected following the initiation of the bag cell burst discharge showed at least 13 peaks of material (Fig. 6B). The protease inhibitors in the releasate eluted prior to the segment shown.) The limit of detection was 10 ng.

Analysis of pooled releasate collected following the initiation of the bag cell burst discharge showed at least 13 peaks of material (Fig. 6B). Two peaks eluted at times close to those of the αBCP(1-9) and αBCP(1-8) standards (Fig. 6D), suggesting that these two peptides were present in the releasate. To confirm the identity of these two peaks, 128 ng of each αBCP standard was added to the pooled releasate and analyzed on HPLC. As seen in Figure 6C, the endogenous peptide and exogenous αBCP(1-9) standard eluted as a single peak at 148 min. (The αBCP(1-9) peak occurred 2.5 min earlier in the run shown in Fig. 6B than the corresponding peaks in Fig. 6, B and D because of small variations in the elution profile over such long-lasting gradients. αBCP(1-7) elutes 1 min earlier in Fig. 6C than in Fig. 6D, and the peak eluting at 127 min in Fig. 6B elutes 2 min earlier in Fig. 6C.) The small peak of material thought to be αBCP(1-8) apparently eluted with the peak of the αBCP(1-9) standard, but because the amount of standard was over 8-fold greater than endogenous peptide, precise co-elution could not be unequivocally demonstrated in this analysis. No peak in the pooled releasate (Fig. 6B) eluted close to the time of the αBCP(1-7) standard, indicating that it was not present in the releasate in detectable amounts. Subsequent peaks (data not shown) were identified as AP and ELH on the basis of comigration with AP and ELH, which were purified and identified from bag cell extracts (Mayeri et al., 1985; Rothman et al., 1985b). ELH and AP, but not αBCP(1-9, 1-8, nor 1-7), were detected in releasate from right bag cell cluster (extracellular recording) and LUQ neurons, L2 and L6 (intracellular recordings). Left. Offset of continuous arterial perfusion of 1 mM αBCP(1-7) for 30 min (beginning at lower arrow), which initially caused inhibition of the LUQ cells. Right. After 25 min, with αBCP still present in the perfusate, activity had returned to baseline, indicating that the LUQ cells had become desensitized. An electrically triggered bag cell burst (beginning at upper arrow) resulted in a lack of inhibitory response in L2 and a greatly reduced inhibitory response in L6.
potential. Application of uBCP (upper trace) and stimulation of bag cells, suggesting that the change in the ionic conductances was similar following application of cvBCP and discharge of the bag cells (lower trace) produced similar hyperpolarizing responses. In each case, the current–voltage relationship plotted from the current pulse applied during the peak of the hyperpolarizing response changed from control in an identical manner, and these changes were maintained during the two subsequent current pulses. The changes from control produced by the first (−4 nA) step are the most prominent, though there are also changes for the other two steps. The change in the slope of the I–V curves in each case (Fig. 8A) indicates that there is an increase in membrane conductance with a reversal potential that extrapolates to near −80 mV, the equilibrium potential for potassium. The results are consistent with an earlier study indicating that bag cell-induced inhibition of LUQ cells is caused by a conductance increase to potassium ions plus a second hyperpolarizing mechanism that is relatively independent of membrane conductance (Brownell and Mayeri, 1979).

Desensitization of the bag cell–induced response by prior application of aBCP

At present, an antagonist to the binding of aBCP to cell receptors has not been developed. In lieu of an antagonist, a cross-desensitization experiment was performed to test whether the inhibitory responses to the bag cell transmitter and to applied aBCP are mediated via the same receptor or postreceptor mechanism. If the same receptor mediates both responses, then the bag cell discharge should produce no inhibition when the inhibitory response to aBCP has been desensitized by prior application of aBCP at high concentration. aBCP was therefore applied to the ganglion by continuous arterial perfusion at high concentration (1 mM) for 30 min, initially producing inhibition of the two LUQ neurons (Fig. 9, left traces beginning at lower arrow). After 25 min of application, the spontaneous pacemaker activity of the LUQ neurons was at or near their level prior to application of the peptide, indicating that desensitization had occurred (Fig. 9, right traces). Under these conditions, an electrically triggered bag cell discharge (upper arrow) produced little or no inhibitory response, suggesting that cross-desensitization of the responses had occurred and that the applied aBCP binds to the same receptors or acts through the same postreceptor mechanism as the transmitter released by the bag cells.

It should be noted that there was a slight response to the bag cell burst that remained after desensitization (Fig. 9, right traces). In L6 (and L3), the bag cell burst produced a slight inhibition, whereas in L2 (and L4) there was actually net excitation. One interpretation of this result is that a second transmitter is involved in mediating bag cell effects on L2 and L4 and that the effects of the second transmitter become apparent only after the response to the first has become desensitized (see Discussion).

Inhibition of other neurons by aBCP

The LUQ neurons were used in the present study as convenient cells in which to study the role of aBCP in mediating bag cell–induced inhibition. However, a bag cell discharge inhibits other identified and unidentified neurons in the abdominal ganglion, including R2, the right upper quadrant white cells (R3–R14), the ink gland motor neurons (L14A, B, C), and L10 (Mayeri et al., 1979a, b). Unlike the other cells, L10 is transiently inhibited for 5–10 min and then undergoes prolonged excitation (Mayeri et al., 1979b), which is thought to be mediated by ELH (unpublished observations). Arterial perfusion of the abdominal ganglion with aBCP inhibited L10 (Fig. 10A), R2, and R3–R14 (not shown). Recording from both identified and unidentified neurons, we found that all cells that were inhibited by the bag cells were also inhibited by application of aBCP. This is consistent with the hypothesis that aBCP is the mediator of all bag cell–induced inhibition in the ganglion. In addition, we found that cells that were excited by the bag cells were inhibited by aBCP. This included left lower quadrant (LLQ) neurons and R15 (Fig. 10B). Although we did not examine the concentration

**Figure 10.** Inhibition of various identified cells by arterial perfusion of aBCP(1–7). A, Inhibition of L10. B, Inhibition of an LLQ cell (LB) and R15.

pooled releasate (Fig. 6B), which corresponds to 103 and 21 ng, respectively, per bag cell burst. These amounts are in good agreement with the amounts recovered from purification of releasate. Of the 660 ng of aBCP(1–9) expected, 540 ng were recovered; of the 138 ng of aBCP(1–8) expected, 50 ng were recovered.

These results indicate that some, if not all, aBCP is originally cleaved from the ELH/BCP precursor and released from bag cells as a 9 amino acid peptide. aBCP(1–8) may be released simultaneously with aBCP(1–9). Alternatively, only aBCP(1–9) is released and subsequently converted to aBCP(1–8) and perhaps aBCP(1–7) by carboxypeptidase cleavage after release. Our data do not distinguish between these two possibilities (see Discussion).

**aBCP and the endogenous inhibitory transmitter produce the same conductance change in target neurons**

When arterially perfused at an appropriate concentration, aBCP mimics the time course of onset and the magnitude of hyperpolarization that occurs in LUQ cells in response to a bag cell burst discharge (Rothman et al., 1983a). To test whether the effects of the exogenously applied peptide and the endogenously released transmitter on membrane conductance are identical, the membrane potential response to three levels of imposed hyperpolarization were constructed from the three-step current pulse and represents the steady-state current-voltage relationship plotted from the current pulse applied during the peak of the hyperpolarizing response. The shift in the I–V plot away from the control levels perpolarizing current was measured following perfusion of the membrane potential response to three levels of imposed hyperpolarization that occurs in LUQ cells in response to a bag cell burst discharge (Rothman et al., 1983a). To test whether the effects of the second transmitter become apparent only after the second step current pulse and represents the steady-state current-voltage characteristic of the target cell membrane at the time each current pulse was applied. The I–V plots for aBCP application and bag cell burst were constructed from the three-step current pulse that was applied during the peak of the hyperpolarizing response. The shift in the I–V plot away from the control levels was similar following application of aBCP and discharge of the bag cells, suggesting that the change in the ionic conductances underlying the hyperpolarizing response is the same in both cases. As shown in Figure 8B, under control conditions, the first three current pulses produced stable changes in the membrane potential. Application of aBCP (upper trace) and stimulation of the bag cells (lower trace) produced similar hyperpolarizing responses.
cells, ELH by itself has no effect except at very high concentrations when (uBCP was arterially perfused with ELH. As in an experiment described previously (Scheller et al., 1983b, Fig. 5), we confirmed that arterial perfusion of 1 μM each of ELH, aBCP, and acidic peptide (AP) mimicked prolonged excitation of LLQ cells and slow inhibition of L6 and other LUQ cells. We also found that perfusion of this set of peptides mimicked burst augmentation of R15 and depolarization of the bag cells. Thus, for LUQ cells, the hyperpolarizing effect of aBCP is largely offset by the depolarizing effect of ELH in the first 2.5 min after the start of arterial perfusion, and after aBCP is washed away, prolonged excitation by ELH continues. For L6 and other LUQ cells, ELH by itself has no effect except at very high concentrations (≥100 μM), and the response is entirely due to aBCP. None of the three peptides, when applied singly or together, has an effect on L1 or R1 even at very high concentrations (≥100 μM).

Discussion

aBCP is a bag cell neurotransmitter

The results presented here, together with those previously reported (Rothman et al., 1983a), provide strong evidence that aBCP is a bag cell neurotransmitter, as follows: (1) Synthesis of the transmitter by the bag cells is supported by purification of large amounts of aBCP from bag cell extract (Rothman et al., 1983a) and by the molecular genetic data indicating that a bag cell-specific gene codes for a precursor molecule containing the amino acid sequence of aBCP(1-9) (Scheller et al., 1983a). (2) aBCP(1-9) immunoreactive terminals are in close association with LUQ cell bodies (Pulst et al., 1985). (3) aBCP mimics inhibition of L2 and L4 and all but the duration of inhibition of L3 and L6 (see below). (4) aBCP acts directly on the LUQs to produce the inhibition. (5) The changes in the current–voltage relationship in the LUQs in response to the bag cell burst, and to application of aBCP, are apparently identical, suggesting an identical ionic mechanism of action. The precise underlying ionic mechanisms need to be elucidated by further studies. (6) Inhibitory activity characteristic of aBCP is released during a bag cell discharge, and an amount of aBCP consistent with this activity is present in releasate. The amount of aBCP present in the releasate appears to be sufficient to cause inhibition. More specifically, neurally evoked inhibition can be mimicked by a 10 min arterial perfusion of 15% of the amount of aBCP(1-9) or 2% of the amount of aBCP(1-8) detected in the releasate per bag cell burst discharge. (7) Desensitization of the response of the LUQ neurons to applied aBCP results in reduction or abolition of the response to a subsequently elicited bag cell burst discharge. (8) A mechanism for rapid inactivation (proteolysis) for aBCP exists.

Thus, the key criteria for identification of a substance as a neurotransmitter have been established for aBCP. Although blockade of the postsynaptic response to endogenous and exogenous transmitter was not attempted because of the lack of a specific antagonist, the results of the cross-desensitization experiment suggest that aBCP may bind to the same receptor as the endogenous transmitter.

αBCP and ELH do not mediate all aspects of bag cell-induced responses

aBCP and ELH mimic all but two aspects of known bag cell-induced responses: transient excitation of L1 and R1, for which αBCP is a candidate transmitter (Rothman et al., 1983a), and the long-lasting aspect of inhibition of L3 and L6. Arterially perfused aBCP mimics the onset and amplitude of the inhibitory response produced in all the LUQ neurons by a bag cell burst discharge and mimics the duration of the response in L2 and L4; it does not, however, mimic the longer duration of the bag cell-induced response seen in L3 and L6 (Rothman et al., 1983a; and this study). After the initiation of a bag cell burst, all four neurons show the initial hyperpolarizing response that inhibits all spike activity. L2 and L4 return to the baseline membrane potential and firing rate within 1-2 min after the cessation of the bag cell burst, but L3 and L6 exhibit a prolonged inhibitory phase characterized by a sustained hyperpolarization and decrease in the rate of spontaneous spike activity of these neurons for more than 2 hr.

The simplest hypothesis for the prolonged inhibition of L3 and L6 is that another transmitter, in addition to aBCP, is released by the bag cells and that the combined effects of the two transmitters produce the prolonged inhibition. This second transmitter might act in either of two ways: (1) to potentiate the inhibitory effect of aBCP but have no effect by itself or (2) to act similarly to aBCP but with a more prolonged action. One class of candidates for the second transmitter includes modified forms of aBCP (e.g., amidated, phosphorylated, or cleaved from the precursor in an extended form). Whatever the nature of the second transmitter, the principal transmitter on LUQs and other inhibited cells is very likely to be aBCP, because it is released in such large amounts.

Physiological characteristics of aBCP differ from those of ELH

Compared to ELH, aBCP has a more rapid onset and a shorter duration of action and is inactivated more rapidly. After the start of the bag cell burst, the onset of inhibition in LUQ cells begins within a few seconds and reaches a maximum in about 30 sec (Mayeri et al., 1979a). In contrast, bag cell-induced responses mediated by ELH are much slower, requiring 1-2 min to result in spike activity in LLQs and 10-15 min to reach a maximum in R15 (Mayeri et al., 1979a, b). The rates of onset of the responses are mimicked by arterial perfusion of aBCP and ELH, applied separately or together (Branton et al., 1978; Mayeri et al., 1985; Rothman et al., 1983a; Scheller et al., 1983b). This indicates that, to a considerable degree, the more rapid action of aBCP results from a faster cellular response once the peptide has reached the cell.

The effects of arterially perfused aBCP end 1-2 min after perfusion is stopped, whereas the effects of ELH persist for more than an hour (Branton et al., 1978; Mayeri et al., 1985). This suggests that the continued presence of aBCP near target cells is needed for inhibition to persist for as long as the duration of the bag cell burst, as is the case for inhibition of cells L2 and L4.

aBCP is rapidly inactivated after release, as indicated by the lack of detectable aBCP or inhibitory activity in releasate containing no protease inhibitors, and by the increase in potency of aBCP when arterially perfused in the presence of protease inhibitors. It seems unlikely that a rapid uptake system for the peptides could explain these results, since such systems are not known to be inhibited by protease inhibitors. Although direct evidence of proteolytic cleavage is needed before definitive conclusions can be made, the results suggest that there are extracellular proteases bound to connective tissue and/or cell membranes lining the interstitial and vascular spaces of the ganglion and overlying sheath. According to this hypothesis, these proteases totally inactivate aBCP before the peptide is able to diffuse into the medium surrounding the ganglion, which, in intact animals, corresponds to the general circulation. It is therefore unlikely that aBCP released from bag cells has any action on tissues outside the abdominal ganglion. This is in sharp contrast to ELH, which is relatively resistant to proteolysis, is easily detected in the bathing medium (Mayeri et al., 1983) and is
released into the general circulation in apparently sufficient amounts to act on other neural tissue (Stuart et al., 1980) and on gonadal tissue (Rothman et al., 1983b). Therefore, the relative susceptibilities of aBCP and ELH to degradation by extracellular proteolytic enzymes may be important determinants of the distance over which the two peptides can act.

After release, aBCP may diffuse longer distances than conventional synaptic transmitters

Although aBCP may diffuse a shorter distance than ELH before being inactivated, there are indications that it diffuses a longer distance from release sites to its targets than conventional synaptic transmitters such as ACh, at the vertebrate skeletal neuromuscular junction. First, in contrast to the discrete IPSPs produced in LUQ cells by the cholinergic neuron, L10, there are no discrete PSPs occurring with each bag cell spike; instead, the inhibitory response produced by the bag cells is slow and smoothly graded, as expected should aBCP be released fromaptic transmitters such as ACh, at the vertebrate skeletal neuromuscular junction. Therefore, the relative susceptibilities of olBCP and ELH to degradation by extracellular proteolytic enzymes may be important determinants of the distance over which the two peptides can act.

Selective action of aBCP

Target neurons located in the rostral (LUQ and RUQ cells) and rostroventral (cells L12-L14) regions of the ganglion are inhibited by bag cell activity or by application of aBCP, but are unaffected by ELH at apparently physiological concentrations. On the other hand, the target neurons located in the caudal ganglion (LLQs and R15) are excited by bag cell activity and by application of ELH, but are inhibited when aBCP is applied. What, then, determines the selectivity of action of aBCP on neurons within the ganglion; i.e., which cells are ultimately inhibited by aBCP? The answer to this question is of great importance for understanding the mechanisms of action of aBCP and ELH.

Possible activation of aBCP after release

The presence of aBCP(1–8) in the releasate (Fig. 1) suggests that aBCP is released from the bag cell axons within the ganglion, and selectivity of the response to ELH is thought to be determined by the presence or absence of ELH receptors on neurons (Mayer et al., 1982a; Mayer et al., 1985).

A major factor for determining the selectivity of the various neurons to inhibition by aBCP may be the presence or absence of ELH receptors on individual neurons and the consequent interaction between aBCP and ELH on individual target cells where both receptors are present. Cells without high-affinity ELH receptors (e.g., LU/Q cells) are affected only by aBCP, and therefore inhibited. In cells with both ELH and aBCP receptors, the effects of arterially perfused ELH and aBCP interact to produce the observed effect. For example, in LLQs, the hyperpolarizing effect of aBCP appears to sum with or be occluded by the depolarizing effect of ELH to produce net excitation. Summation of effects may also occur in R15, although more data are needed to determine whether additional interactions, such as occlusion or potentiation of the effects of one peptide by the other, also play a role.

Possible activation of aBCP after release

Of the three neuroactive forms of aBCP, aBCP(1–9) was the prevalent form in the releasate (Fig. 6). aBCP(1–8) was also detected, but not (1–7). This strongly suggests that the (1–9) form is an end product of the processing of the ELH/aBCP precursor protein within the bag cells before release. This conclusion is consistent with the finding that the nine-residue peptide is the prevalent form in bag cell extracts when proteolysis is carefully controlled (B. S. Rothman et al., 1985b). It is also consistent with molecular genetic data, which show that the aBCP(1–9) amino acid sequence that is encoded on the precur sor is flanked by presumed cleavage sites consisting of a single arginine residue on the amino terminus and three arginine residues on the carboxy terminus (Scheller et al., 1983a).

Although the releasate contained only one-fifth as much aBCP(1–8) as (1–9), aBCP(1–8) was 30 X as potent as (1–9) and therefore represented the major portion of inhibitory activity in the releasate. The presence of aBCP(1–8) in the releasate presumable results from carboxypeptidase A-like cleavage of the nine-residue form. There are two sites at which this cleavage might occur: either within the secretory granules before release, or in extracellular space after release. Although more data are needed to resolve the issue, we favor the latter possibility, since intragranular cleavage would require a processing step (cleavage of a carboxy-terminal leucine residue) that is not known to occur in secretory granules (Gainer et al., 1985). The proposed activation of aBCP after release is similar to the activation of the polypeptide hormone, angiotensin II, by converting enzyme, a dipeptidyl carboxypeptidase present in the circulation. The present data provide evidence for similar processing of a neuropeptide in the CNS.

If it occurs after release, the process of activation must occur simultaneously with inactivation. Detection of released aBCP appeared to require the presence of carboxypeptidase inhibitors in the perfusion medium. This is consistent with the hypothesis that aBCP(1–9) is activated to (1–8) and even (1–7) by carboxypeptidase activity and then inactivated by continued carboxypeptidase activity to (1–6) and smaller forms. Inactivation is, in addition, likely to involve proteolysis by aminopeptidases and/or endo-peptidases, since inhibitors of these two classes of proteases were required to detect released aBCP. Since aBCP(1–7) was not detected in the releasate, its contribution as a neurotransmitter remains to be established. However, under the
experimental conditions used, the lack of \( \alpha \)BCP(1–7) in the releasate may have occurred because the rate of its production from (1–9) and (1–8) was much slower than the rate of inactivation of (1–9) and (1–8) by endo- and/or aminopeptidases.

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