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Primary structure and neuronal effects of α-bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia*

(high pressure liquid chromatography/autotransmitter/egg-laying behavior/co-transmitter/long-term inhibition)

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ABSTRACT **A discharge of impulse activity in a group of neuroendocrine cells, the bag cells, produces several types of prolonged responses in various identified neurons of the abdominal ganglion of *Aplysia*. Two excitatory responses are almost certainly mediated by egg-laying hormone, but this peptide cannot account for other responses, such as inhibition of left upper quadrant neurons. We report here the isolation from bag cell clusters of three structurally similar peptides, seven, eight, and nine residues long, that are candidate transmitters for mediating bag cell-induced inhibition. They may also serve as autoexcitatory transmitters since the seven-residue peptide produces a slow depolarization of the bag cells similar to that which occurs during bag cell discharge. The amino acid sequence of the largest peptide, termed α-bag cell peptide[1–9], is H-Ala-Pro-Arg-Leu-Arg-Phe-Tyr-Ser-Leu-OH. The other two peptides are identical to α-BCP[1–9] except that they lack the COOH-terminal Ser-Leu or leucine residues. The three peptides inhibit left upper quadrant neurons at relative potencies of 10:30:1 (seven-, eight-, and nine-residue peptides, respectively). Recent molecular genetic analysis shows that both α-BCP[1–9] and egg-laying hormone are encoded by the same bag cell-specific gene. The multiple neuronal effects of bag cells are therefore likely to be mediated by at least two transmitters that are cleaved from a common precursor molecule.**

The marine mollusk *Aplysia* is a convenient experimental system for investigating at the cellular and molecular levels the roles of neuropeptides as neurotransmitters and in the regulation of behavior. Egg laying in *Aplysia* is accompanied by a stereotyped behavioral pattern that lasts for several hours. The behavior pattern is thought to be initiated and controlled by the bag cells, a group of neuroendocrine cells located in the abdominal ganglion. In intact animals, egg laying is invariably preceded by a repetitive impulse discharge of the bag cells (1). In the isolated abdominal ganglion, where the central actions of the bag cells have been most extensively studied, a bag cell discharge produces at least four types of long-lasting excitatory and inhibitory effects on various identified neurons (2–4).

To identify the neurotransmitters mediating these effects, extracts of bag cells have been purified and assayed for activity on various neurons. It has been determined that egg-laying hormone (ELH), a 4,400-dalton peptide that is synthesized and released by bag cells (5–8), is probably the neurotransmitter mediating two types of excitatory neuronal responses, burst augmentation of cell R_{15} and prolonged excitation of left lower quadrant (LLQ) neurons (9–11). However, when perfused into the abdominal ganglion at physiological concentrations, purified ELH has little or no effect on left upper quadrant (LUQ) neurons, cells L_2–L_4, L_6, which are normally inhibited by bag cell discharge (10, 11). This indicates that ELH does not directly or indirectly inhibit these cells and suggests that there is a second neurotransmitter that mediates bag cell-induced inhibition (10–12). Recent molecular genetic analysis shows that a bag cell-specific gene encodes a precursor protein that contains the sequences of ELH and several other potential cleavage products (13, 14). We report here the characterization of three peptides that are structurally related to each other and were isolated from bag cell clusters on their ability to mimic bag cell-induced inhibition. They are encoded on the bag cell gene and are candidates for the second bag cell neurotransmitter.

METHODS

Abdominal ganglia were dissected from large (1–4 kg) sexually mature *Aplysia californica* that were obtained from Pacific Biomarine (Venice, CA) or Sea Life Supply (Sand City, CA) or were collected from Monterey Bay, CA. Ganglia were incubated in 50% isotonic $MgCl_2$/50% *Aplysia* blood that had been passed through a glass fiber filter (Millipore AP 25). Bag cell clusters were then surgically removed from the ganglia (15) and stored at −80°C until homogenized.

Sequences were determined by microanalysis on a Beckman 890C sequencer modified according to Wittmann-Liebold (16). The sample was retained in the spinning cup with Polybrene previously cycled with glycylglycine (17), and the phenylthiohydantoin derivatives of amino acids were identified by reversed-phase HPLC (18). Amino acid compositions were determined on 24-hr hydrolysates in 6 M HCl/0.02% 2-mercaptoethanol at 110°C as described by Del Valle and Shively (19). COOH-terminal amino acid analyses were carried out by the method of Jones *et al.* (20).

Intracellular recordings were made from up to four LUQ neurons at a time in the isolated abdominal ganglion, using conventional recording techniques as described (3).

RESULTS

Purification of α-Bag Cell Peptide (α-BCP). Acid-soluble bag cell material was fractionated on a Sephadex G-50 column, which separates proteins in the 0- to 12-kilodalton (kDa) range according to size (Fig. 1*A*). The column eluate was separated into three fractions, each corresponding to one of the major peaks of material detected. The three fractions contained high molecular weight material (>12 kDa), medium molecular weight material (2–12 kDa) including ELH and the acidic bag cell pep-

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Abbreviations: ELH, egg-laying hormone; α-BCP, α-bag cell peptide; LUQ, left upper quadrant; LLQ, left lower quadrant; kDa, kilodalton(s).

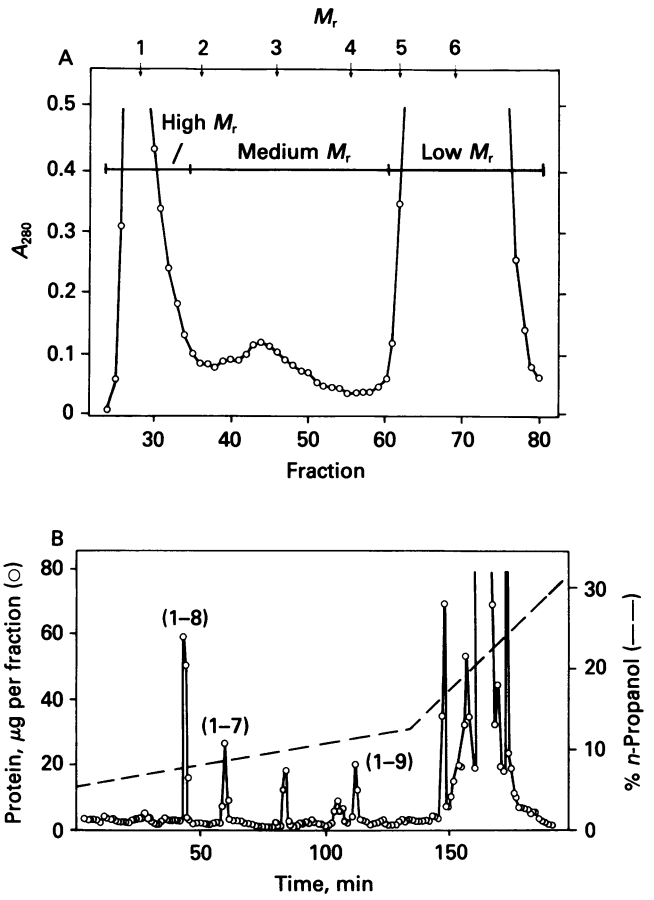

FIG. 1. Purification of α-BCP[1–7], α-BCP[1–8], and α-BCP[1–9] from clusters of bag cells. (*A*) Fractionation of bag cell extract by gel filtration chromatography. One hundred frozen clusters were ground on ice in a glass homogenizer (Kontes Duall 22) in 20 ml of 1.0 M acetic acid/0.1% 2-mercaptoethanol containing phenylmethylsulfonyl fluoride (Sigma) at 300 μg/ml, angiotensin converting enzyme inhibitor (Peninsula Laboratories) at 25 μg/ml, phenylalanylalanine (Vega Chemicals) at 2 mg/ml, and bacitracin (Sigma), ovomucoid (Worthington), lima bean trypsin inhibitor (Worthington), ovoinhibitor (Sigma), leupeptin (Peninsula Laboratories), and antipain (Peninsula Laboratories), each at 250 μg/ml. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C and the supernate was applied to a Sephadex G-50 (superfine) column (2.5 × 30 cm) equilibrated at 4°C with 1.0 M acetic acid and then eluted at a rate of 5 ml/cm²·hr. Fractions of 4 ml were collected and A_{280} values were determined on a Gilford model 240 spectrophotometer. The column was calibrated with the following proteins as molecular weight standards: 1, myoglobin, 17,000; 2, cytochrome *c*, 12,000; 3, aprotinin, 6,000; 4, glucagon, 3,500; 5, bacitracin, 1,400; 6, salts (determined by conductance measurements). (*B*) HPLC purification of α-BCP[1–7], α-BCP[1–8], and α-BCP[1–9] from low molecular weight material. Pooled low molecular weight fractions from a Sephadex G-50 column were applied to a C_{18} reversed-phase HPLC column (4.6 × 250 mm, Supelco). A total volume of 80 ml was applied to the column and the column was washed for 45 min. Then, two linear gradients of *n*-propanol (0–12.5% in 200 min, 12.5–30% in 40 min) in pyridine acetate (0.09 M pyridine/0.5 M acetic acid, pH 4.0) were run. The material eluting in the early part of the first gradient is not shown. The column eluate was monitored by a fluorescamine detection system (21).

tide (6), and low molecular weight material (0–2 kDa including salts), respectively.

The high and medium molecular weight fractions had little inhibitory activity, as assayed on LUQ cells. The medium molecular weight fraction was further purified by HPLC and the resultant fractions were assayed on abdominal ganglion neurons. Again, little inhibitory activity was found although the excitatory activity for R_{15} and LLQ cells was found to purify with ELH as expected (refs. 9–11; unpublished data).

Fractionation of the low molecular weight material by HPLC yielded three peaks of material with inhibitory activity on LUQ cells. They were subsequently identified as α-BCP[1–7], α-BCP[1–8], and α-BCP[1–9] (Fig. 1*B*).

Primary Amino Acid Sequence of α-BCP[1–7] and α-BCP[1–8]. The sequences of α-BCP[1–7] and α-BCP[1–8] were determined by Edman degradation without further purification. The α-BCP[1–7] sample, which was discovered and analyzed first, gave a 31% yield (345/1,100 pmol) of the NH_2-terminal residue (alanine) and an unambiguous sequence of seven amino acids. No residue beyond the seventh was detected. Analysis of α-BCP[1–8] gave essentially identical results for the first seven residues. One additional residue, serine, was found for the eighth residue and no residue beyond the eighth was detected. Thus, the sequence of α-BCP[1–8] was determined to be H-Ala-Pro-Arg-Leu-Arg-Phe-Tyr-Ser-OH.

The amino acid compositions of α-BCP[1–7] and α-BCP[1–8] accounted for all the amino acids expected from their respective sequence data (Table 1). However, the composition data showed significant amounts of other amino acids (e.g., valine) that were not detected in any of the sequence cycles. We therefore tested for the presence of extra COOH-terminal residues by digesting each peptide with carboxypeptidase and identifying the liberated amino acids. The results of these analyses showed that no extra COOH-terminal residues were present in either peptide. Furthermore, the COOH-terminal analyses were in good agreement with the sequence data for the last four residues of each peptide. We interpret these data to mean that some contaminant not available for sequence or COOH-terminal analysis was present in the peptide samples.

To test whether the inhibitory activity on LUQ neurons was accounted for by the peptides for which the sequences had been determined and not any other component of the purified peptide samples, we had α-BCP[1–7] chemically synthesized by Peninsula Laboratories (San Carlos, CA). α-BCP[1–7] was synthesized rather than α-BCP[1–8] or α-BCP[1–9] because its sequence was determined before identification of the larger peptides. The synthesized peptide was more than 96% pure by TLC, paper electrophoresis, and HPLC. Its amino acid composition (data not shown, Peninsula Laboratories) was in perfect agreement with its amino acid sequence. Synthetic and native α-BCP[1–7] behaved identically in three different biochemical tests: (*i*) on reversed-phase HPLC under isocratic conditions at pH 3.0 [0.14 M pyridine/0.5 M formic acid/9% (vol/vol) *n*-propanol; Supelco C_{18} DB column, 4.6 × 250 mm; flow rate, 0.6 ml/min] the two showed identical retention times (35.1 min) when run separately and ran as a single peak when combined;

Table 1. Amino acid compositions

Amino acid	α-BCP[1–7]			α-BCP[1–8]		
	Mol %	Ratio	Sequence	Mol %	Ratio	Sequence
Arg	23.1	2.14	2	18.0	1.78	2
Ser	2.5			9.4	0.93	1
Pro	7.9	0.73	1	11.0	1.09	1
Ala	6.9	0.64	1	10.3	1.02	1
Val	4.8			11.4	1.13	
Leu	13.5	1.25	1	14.1	1.40	1
Tyr	11.0	1.02	1	8.6	0.85	1
Phe	13.3	1.23	1	9.4	0.93	1

α-BCP[1–7] (1.3 μg) and 1.1 μg of α-BCP[1–8] were hydrolyzed separately in 6 M HCl/0.02% 2-mercaptoethanol for 24 hr and analyzed according to the method of De Valle and Shively (19). All amino acids other than those shown above were detected at ≤4.1 mol % each.

Table 2. Yield of peptides from bag cell extract

	μg	nmol	Molar ratio
α-BCP[1–7]	42	46	
α-BCP[1–8]	126	125	
α-BCP[1–9]	36	32	
Total α-BCP	204	203	1.00
ELH	1,325	301	1.48
Acidic peptide	475	161	0.79

Approximately equimolar amounts of three peptides encoded on the ELH/α-BCP precursor were recovered from extracts of bag cell clusters when purified in the presence of protease inhibitors.

(*ii*) similar results were obtained when the peptides were run at pH 6.3 [0.5 M pyridine/17 mM acetic acid/7% (vol/vol) *n*-propanol; retention time, 31.5 min]; and (*iii*) the two peptides comigrated on TLC plates [E. Merck silica gel 60 in butanol/pyridine/acetic acid/water (42:24:4:30), $R_f = 0.35$].

Identification of α-BCP[1–9]. Molecular genetic data shows that a bag cell-specific gene encoding ELH also encodes a nine-residue peptide (13, 14). The first eight residues correspond exactly to α-BCP[1–8] and are followed by a ninth residue, leucine, at the COOH terminus. We had this nine-residue peptide synthesized (Peninsula Laboratories) and found that it comigrated on HPLC with a small peak of material purified from the low molecular weight fraction of the bag cell extract. Comigration occurred under two different conditions, one at pH 2.1 [0.9% trifluoroacetic acid/17% (vol/vol) *n*-propanol; retention time, 33 min] and the other at pH 6.3 [12.5% (vol/vol) *n*-propanol; retention time, 22 min]. This indicates that α-BCP[1–9] is present in bag cell extracts but in small amounts compared with α-BCP[1–8] (Table 2). It is worth noting that approximately equimolar amounts of α-BCP (total of the three forms), ELH, and bag cell acidic peptide (6) were recovered (Table 2). ELH and the acidic peptide were purified from the 2- to 12-kDa material by reversed-phase HPLC (unpublished data).

Neuronal Effects of Native and Synthetic α-BCP. Native and synthetic α-BCP[1–7], native α-BCP[1–8], and synthetic α-BCP[1–9] were tested for inhibitory activity on LUQ neurons. At a given concentration, the membrane hyperpolarization and accompanying decrease in bursting pacemaker activity produced by synthetic and native α-BCP[1–7] were apparently identical (Fig. 2*A*). The dose–response relationships for the two peptides were also apparently identical (Fig. 2*B*). Thus, both the biochemical and the pharmacological results indicate that synthetic and native α-BCP[1–7] are the same. α-BCP[1–8] was

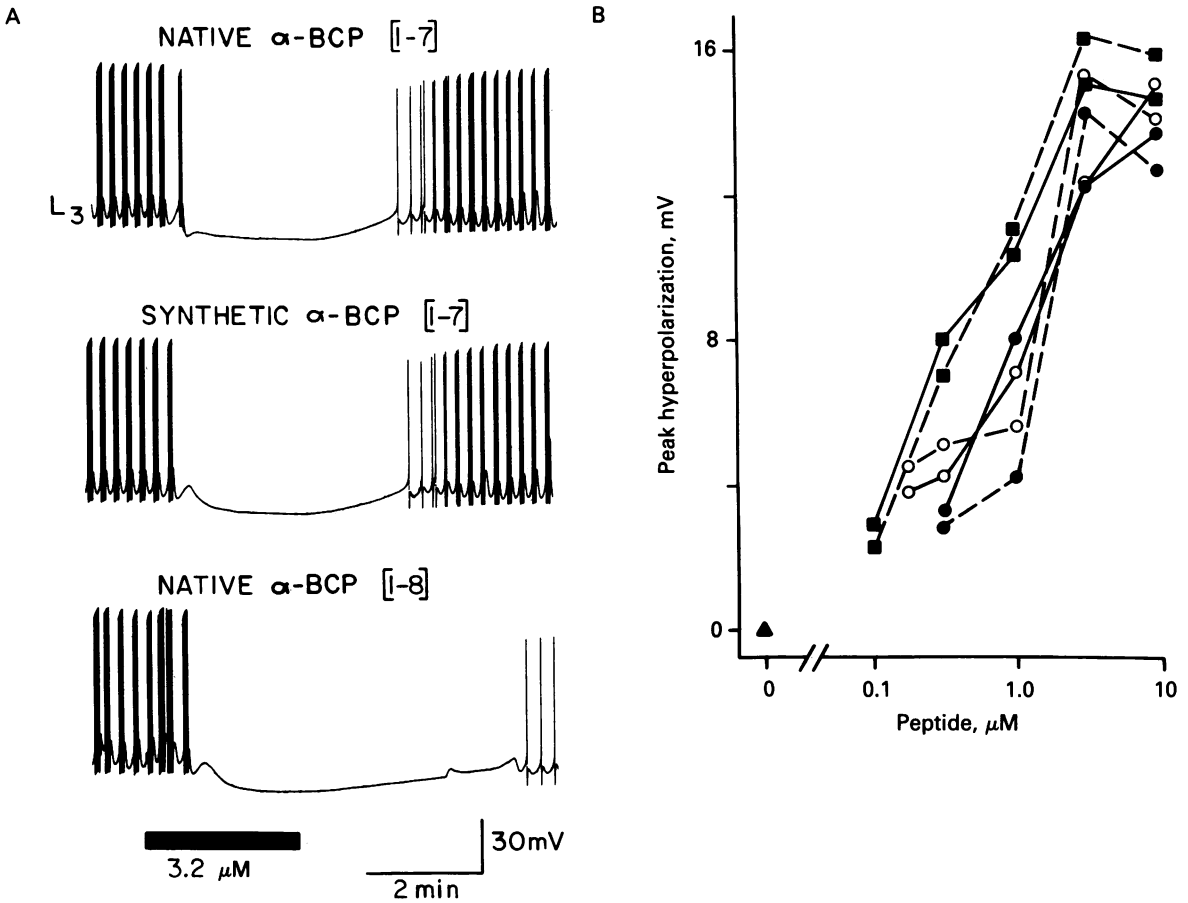

FIG. 2. Inhibitory effects of native and synthetic α-BCP on LUQ neurons. (*A*) Intracellular recording from cell L_3 showing hyperpolarization caused by α-BCP. The 3.2 μM α-BCP solutions were perfused through the abdominal ganglion artery at a rate of 3 μl/min for 2.67 min (bar). Synthetic and native α-BCP[1–7] had identical effects while native α-BCP[1–8] caused a larger longer-lasting hyperpolarization. Bag cell activity was continuously monitored by an extracellular electrode placed on one of the bag cell clusters. Peptides were dissolved in sea water containing lima bean trypsin inhibitor, ovoinhibitor, ovomucoid inhibitor, and bacitracin, each at 250 μg/ml, and arterially perfused (11, 22). (*B*) Dose–response relationship for α-BCP on cells L_3 and L_6 from a single abdominal ganglion. Response is measured as peak hyperpolarization after arterial perfusion of peptide (data from same preparation as in *A*). Synthetic (○) and native (●) α-BCP[1–7] have identical dose–response curves. Native α-BCP[1–8] (■) is about 3 times as potent as α-BCP[1–7].

about 3 times as potent as α-BCP[1–7] (Fig. 3b) and α-BCP[1–9] was about 1/10th as potent as the seven-residue peptide (data not shown). Otherwise all three peptides had the same neuronal effects (Fig. 2). In a desheathed ganglion, pressure application of α-BCP[1–7] by a micropipette placed in close proximity to a LUQ cell showed that the effect on LUQ neurons was direct.

Application of α-BCP[1–7], α-BCP[1–8], and α-BCP[1–9] mimicked several aspects of bag cell-induced inhibition of LUQ neurons, such as the rate of onset of membrane hyperpolarization, the accompanying decrease in bursting pacemaker activity, and the decrease in impulse amplitude. In two of the LUQ cells, L_2 and L_4, the duration of the response to each of the three α-BCPs appeared comparable with the duration of neurally evoked inhibition: the inhibition produced by α-BCP ended a few minutes after arterial perfusion ceased, just as the neurally evoked inhibition ends a few minutes after the last impulse in the bag cell discharge (see figure 13 of ref. 2). Thus, the effects of α-BCP could entirely account for the neurally evoked inhibition in cells L_2 and L_4. By contrast, in the two other LUQ cells, L_3 and L_6, neurally evoked inhibition persists as long as several hours (3, 23), much longer than the response to α-BCP in these cells. One explanation for this difference is that α-BCP may act together with another bag cell transmitter to mediate the prolonged inhibition of cells L_3 and L_6, which has two ionic components (23).

In addition to inhibiting LUQ neurons, arterial perfusion of α-BCP[1–7] excited the bag cells (Fig. 3). In a series of 10 experiments in which a bag cell was monitored intracellularly, application of 1 μM α-BCP[1–7] depolarized the bag cells by 6.2 ± 1.1 mV (mean ± SEM). The magnitude and time course of the depolarization at this concentration was similar to that which occurs preceding and during a bag cell discharge initiated by direct electrical stimulation of the bag cells (figures 4 and 8 of ref. 4). In 10 of 32 preparations under a variety of experimental conditions, application of α-BCP at concentrations ranging from 0.5 μM to 1 mM produced a bag cell discharge during or within 5 min of application. Variability in the occurrence of the discharge is attributable to differences in the excitability of different bag cell preparations (3) and to desensitization of the depolarizing response at high concentrations of α-BCP[1–7] (unpublished data).

These results raise the possibility that α-BCP serves as an excitatory transmitter among the bag cells. According to this view, initial depolarization of the bag cells causes release of α-BCP, which, in turn, causes further depolarization and release. Thus, a positive feedback mechanism may contribute to the sustained depolarization underlying the repetitive discharge of bag cells.

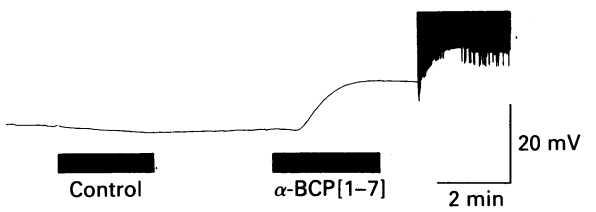

FIG. 3. Excitation of a bag cell by α-BCP[1–7]. Intracellular recording from a bag cell soma. Eight microliters of a control solution (sea water containing protease inhibitors) followed by 8 μl of a solution containing 1 μM synthetic α-BCP[1–7] were each perfused arterially for 2.67 min. α-BCP caused the bag cell to depolarize and later to initiate a burst discharge. An extracellular monitor of the contralateral bag cell cluster showed that the burst discharge began in both clusters at approximately the same time.

DISCUSSION

Our results suggest that there are at least two bag cell transmitters: α-BCP and ELH. α-BCP mediates inhibition of LUQ neurons and excitation of the bag cells, while ELH mediates two types of excitation. The role of α-BCP as a transmitter is supported by the following evidence. (*i*) α-BCP[1–7] and α-BCP[1–8] were the most potent factors purified from the bag cell clusters in inhibition of LUQ neurons and synthetic α-BCP[1–7] was shown to act directly on these neurons. (*ii*) α-BCP[1–9] is encoded on the same bag cell-specific gene as ELH (14). (*iii*) The bag cells very likely synthesize α-BCP because material corresponding to α-BCP[1–7] was radiolabeled when isolated clusters of bag cells were incubated with ^{3}H-labeled amino acids (unpublished data). (*iv*) α-BCP may bind to the same receptors as the endogenous transmitter. After desensitization of the inhibitory response to application of a high concentration of α-BCP[1–7], the inhibition normally produced by a bag cell discharge was greatly reduced or eliminated (24). Other aspects of the postulated transmitter role of α-BCP remain to be studied, such as immunocytochemical localization, release, and identity of ionic mechanism of response.

After determination of the amino acid sequence of α-BCP, Scheller and co-workers (Columbia University) in collaboration with us used recombinant DNA techniques to obtain the entire base sequence of a bag cell-specific gene that encodes a protein precursor for ELH (14). A striking finding is that, in addition to ELH, α-BCP is represented on the precursor. The gene encodes the sequence for α-BCP[1–9] bounded on each end by basic residues, which are presumed cleavage sites during precursor processing. This finding combined with the present data strongly suggests that bag cells use two or more peptide transmitters that are enzymatically cleaved from a common precursor molecule.

It is not clear why α-BCP exists in three neuroactive forms. Although the eight- and seven-residue peptides might simply be artifacts of purification, it is possible that all three forms serve as transmitters. This is suggested by the fact that the nine-residue peptide has much less effect on LUQ cells than the other two peptides. A simple possibility is that the nine-residue peptide is originally cleaved from the precursor molecule and that, before or after release from the bag cells, it is activated by proteolysis to the eight- and seven-residue peptides. The idea that multiple forms of a peptide might function as neurotransmitters is similar to what is thought to occur for peptide hormones acting on peripheral tissue (25).

Although the details of processing and localization of α-BCP in the bag cells remain to be determined, available data are consistent with the view that most if not all bag cells synthesize the same precursor protein that is processed to yield one copy each of α-BCP, ELH, and acidic bag cell peptide. Thus, approximately equimolar amounts of α-BCP, ELH, and the acidic peptide were recovered from bag cell extracts (Table 2), as predicted by the gene sequence. These peptides would be expected to coexist in bag cell processes and terminals, as do the products of pro-opiomelanocortin in anterior pituitary cells (26).

Why is it useful for the bag cells to synthesize two or more transmitters from the same precursor when one transmitter might suffice? One answer to this question can be best understood in the context of the physiological actions of bag cells and their contributions to the regulation of egg-laying behavior. Egg laying is a fixed action pattern that, like stereotyped reproductive behaviors in other animals, is complex and lasts for several hours. It involves alterations in locomotion, head movements, feeding (27), and visceromotor activity (28). These changes are thought to be regulated by transmitters and neurohormones that are

released during the bag cell discharge in order to modify activity for appropriate lengths of time in neuronal circuits mediating the various elements of the behavior pattern (3, 4, 29). Perhaps the present behavior pattern evolved from a more simple one that was regulated by a single peptide transmitter. The present complexity of the behavior might therefore be the result of addition of new peptide transmitter sequences to the precursor as a result of duplication, mutation, or insertion and new neuronal activities brought into play by the effects of the new transmitters. From an evolutionary perspective, the presence of more than one transmitter may have provided a means for increasing the complexity of the behavior pattern.

Available data on the physiological properties of ELH and α-BCP provide another indication of why two neurotransmitters might be more advantageous than one. ELH has prolonged actions lasting up to several hours; it is slowly degraded and in addition to acting on abdominal ganglion neurons (9, 11) is released into the general circulation to act on other parts of the central nervous system (29) and on the ovotestis to release eggs (30). α-BCP, in contrast, has actions lasting 5–20 min, and it is apparently degraded within the ganglion since the inhibitory activity characteristic of α-BCP is not released from the ganglion during bag cell discharge unless protease inhibitors are present (unpublished data). The different durations of action of ELH and α-BCP may be important in coordinating long- and short-lasting events in the behavior pattern associated with egg laying.

It may be especially adaptive that the transmitters occur on a common precursor, since this feature ensures that all the peptide transmitters are synthesized and released together at the initiation of a behavioral episode. The use of a common precursor might in theory still allow a certain degree of plasticity in the system. During development, or as the result of a physiological event, an alteration in the processing of the precursor or posttranslational modification of processed peptides might produce changes in the amounts or activities of released peptides and corresponding changes in electrical signaling.

Although many aspects of egg-laying behavior are controlled by circuitry in other parts of the nervous system, the multiple actions of bag cell discharge on abdominal ganglion neurons serve as a model for how the behavior is regulated. It will therefore be possible to test whether all bag cell actions on ganglionic neurons can be accounted for by peptides processed from the α-BCP/ELH precursor. If true, this would support the hypothesis that for the bag cell system the precursor and not the individual peptides is the key evolutionary determinant of the egg-laying behavior pattern.

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