The Journal of Neuroscience, November 1987, 7(11): 3623–3632

Alpha Bag Cell Peptide Directly Modulates the Excitability of the Neurons That Release It

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Brief electrical or hormonal stimulation of the bag cell neurons of *Aplysia* triggers a long-lasting discharge during which alpha bag cell peptide (α-BCP) and other neuropeptides are released from the cells. We have carried out experiments, using both intact abdominal ganglia and isolated neurons, demonstrating that α-BCP acts directly on the bag cell neurons to influence cAMP levels and voltage-dependent potassium currents.

Exposure of the bag cell neurons within intact ganglia to α-BCP, at concentrations greater than 1 nM, inhibited an ongoing discharge. α-BCP also significantly reduced both basal and forskolin-stimulated levels of cAMP in bag cell clusters. The inhibition of the discharge by α-BCP could be prevented and reversed by pharmacological elevation of intracellular cAMP levels.

Immunohistochemical staining of neurons maintained in cell culture showed that all isolated bag cell neurons exhibit immunoreactivity with antisera against α-BCP. Application of the adenylate cyclase activator forskolin to such isolated cells, in the presence of a phosphodiesterase inhibitor, attenuates the amplitude of the delayed voltage-dependent outward currents measured in voltage-clamp experiments. Pretreatment of the cells with α-BCP significantly reduced the ability of forskolin to attenuate these currents, demonstrating that α-BCP acts directly at autoreceptors on bag cell neurons.

Experiments with the isolated cells showed that a second autoreceptor-mediated effect of α-BCP was the enhancement of an inwardly rectifying potassium current that was activated at potentials more negative than −40 mV. The reversal potential and conductance of the current induced by α-BCP were dependent on the external K^+ concentration. This response to α-BCP could be blocked by rubidium, cesium, and barium ions.

Our data demonstrate that α-BCP can exert inhibitory biochemical and electrophysiological actions on the bag cell neurons that release it and suggest that autoreceptors for α-BCP play an important role in the termination of a discharge in the bag cell neurons.

Received Dec. 3, 1986; revised Apr. 16, 1987; accepted Apr. 23, 1987.

We thank Dr. William F. DeGrado and the E. I. DuPont Company for invaluable assistance in the synthesis of α-BCP [1-7]. This work was supported by Grants NS-18492, BNS-8202364, and a Klingenstein Award to L.K.K.

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In addition to acting at postsynaptic sites, a neurotransmitter may directly influence the properties of the neuron from which it is released. Autoreceptors have been described for several classical neurotransmitters (Langer, 1981; Roth, 1984; Anderson and Mitchell, 1985), and it has been suggested that the pattern of activity of hypothalamic neurons may be regulated by a neuropeptide, oxytocin, acting at autoreceptors (Freund-Mercier and Richard, 1984; Theodosis, 1985). In this study we have investigated the role of a neuropeptide autoreceptor in the bag cell neurons, a model system of neurons that undergoes prolonged changes in excitability.

The bag cell neurons of *Aplysia* are an electrically coupled network of homogeneous neurons, which ordinarily have relatively negative resting potentials and are electrically silent. Brief electrical or hormonal stimulation triggers a discharge lasting about 30 min (Kupfermann and Kandel, 1970; Dudek and Blankenship, 1977; Heller et al., 1980). During the discharge, the bag cell neurons release several neuropeptides that act at sites throughout the nervous system and at other target tissues to initiate a sequence of reproductive behaviors (Kupfermann, 1970; Pinsker and Dudek, 1977; Branton et al., 1978a, b; Dudek et al., 1979; Mayeri et al., 1979a, b; Stuart et al., 1980; Strumwasser et al., 1981). The peptides released during the discharge include the 36-amino-acid egg-laying hormone (ELH) and several smaller peptides, including alpha bag cell peptide (α-BCP: Ala-Pro-Arg-Leu-Arg-Phe-Tyr-Ser-Leu) (Arch, 1972; Chiu et al., 1979; Scheller et al., 1982, 1983; Rothman et al., 1983; Sigvardt et al., 1986). In this paper we report that α-BCP directly regulates both the electrical and biochemical properties of the bag cell neurons.

Several lines of evidence suggest that the onset of the discharge in the bag cell neurons is linked to an increase in intracellular levels of cAMP. Cyclic AMP levels in clusters of bag cell neurons rise during the first minutes of the discharge. Treatment of bag cell neurons in intact clusters with the adenylate cyclase activator forskolin or with cAMP analogs both triggers discharges and prolongs their duration (Kaczmarek et al., 1978; Kauer and Kaczmarek, 1985). After the end of a discharge, the bag cell neurons enter a 20 hr period of inhibition, known as the refractory period (Kupfermann and Kandel, 1970). During this time, electrical stimulation no longer triggers a discharge (Kauer and Kaczmarek, 1985). Moreover, beginning approximately 1 hr after the end of a normal discharge, elevations of cAMP levels also fail to trigger discharges. There is evidence that calcium entry into the bag cell neurons during the discharge may be involved in initiating the refractory period (Kaczmarek and Kauer, 1983).

The bag cell neurons are anatomically isolated from other neurons within the ganglion. It is therefore possible to culture isolated bag cell neurons, which share many of the electrical

properties of cells in intact ganglia (Kaczmarek et al., 1979; Kaczmarek and Strumwasser, 1981). Exposure of such isolated bag cell neurons to cAMP analogs or to forskolin plus theophylline results in a broadening of action potentials and may cause the cells to fire repetitively. In voltage-clamp experiments, cAMP analogs and forskolin have been shown to reduce the amplitude and alter the kinetics of several voltage-dependent potassium conductances (Kaczmarek and Strumwasser, 1984; Strong, 1984; Strong and Kaczmarek, 1986). Using such isolated bag cell neurons, we have studied the direct effects of α-BCP on the bag cell neurons in the absence of other neurons.

Materials and Methods

Materials. Mature animals (*Aplysia californica,* 150–400 gm) were obtained from Alacrity Marine Biological Services (Redondo Beach, CA) and were maintained in a circulating artificial seawater system at 14°C.

We synthesized α-BCP [1-7] by 2 methods, those of Merrifield (1961) and of Atherton et al. (1978). Synthetic α-BCP was also obtained from Peninsula Laboratories (Belmont, CA), as were α-BCP [1-8] and [1-9]. α-BCP [1-7] was used in all experiments except as noted. The membrane permeant, phosphodiesterase-resistant cyclic AMP analog, N^6-*n*-butyl 8-benzylthio cAMP (BBT-cAMP) was the kind gift of Dr. J. Miller.

Recordings in intact ganglia. Animals were pinned to a dissection tray, and abdominal ganglia together with the pleuroabdominal connective nerves were dissected and placed in an artificial seawater medium (ASW) composed of 460 mM NaCl, 10.4 mM KCl, 55.0 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.8 (Kaczmarek et al., 1978). In some experiments using intact ganglia, protease inhibitors (PI) (soybean trypsin inhibitor, egg white trypsin inhibitor, and bacitracin, 250 μg/ml each) were added to ASW to minimize proteolytic degradation of the exogenous peptides. Control experiments demonstrated that the presence of protease inhibitors by itself did not alter either the duration of the discharge or cAMP levels in the bag cell clusters.

Durations of discharges were monitored either by extracellular recording alone or by combined intracellular/extracellular recording. Ganglia were placed in a recording chamber containing 10 ml ASW/PI, which was maintained at 14°C without perfusion. A suction electrode for stimulation of afterdischarges was placed on the connective nerve about 1 cm from the bag cell clusters. A recording suction electrode was placed over the rostral end of the bag cell clusters. For intracellular recordings from bag cell neurons, the connective tissue over the clusters was first softened by preincubation of the ganglia for 2 hr in ASW containing 1 mg/ml collagenase, 0.5 mg/ml elastase (Kaczmarek et al., 1982; Kauer and Kaczmarek, 1985). For experiments with pertussis toxin-treated cells, abdominal ganglia with the pleuroabdominal connective nerves were first incubated for 20 hr at 23°C in modified Eagle's medium (MEM; see below) containing 5 μg/ml pertussis toxin (List Biochemicals, Campbell, CA). The ganglia were then rinsed in ASW/PI for 30 min before being placed in the recording chamber, which contained 10 ml fresh ASW/PI. Control clusters for the experiments with pertussis toxin were also preincubated in the same way for 20 hr at 23°C without the toxin.

Afterdischarges were stimulated by a train of stimulus pulses (20 V, 2.5 msec, 6 Hz, 5 sec) to the stimulating electrode. Peptides were dissolved in 20 μl ASW/PI and were ejected locally through polyethylene tubing placed less than 1 mm from the bag cell cluster. This method of peptide delivery minimized peptide degradation.

Cyclic AMP measurements. For measurements of cAMP levels in clusters of bag cell neurons following treatment with α-BCP, bag cell clusters were rapidly dissected from the abdominal ganglion and the pleuroabdominal connective nerves were cut at their junction with the clusters. The clusters were then homogenized in 6% trichloroacetic acid at 0°C. Aliquots of homogenate were removed for protein determination (Lowry et al., 1951) and cAMP concentrations were measured using a cAMP ^{125}I radioimmunoassay kit (New England Nuclear, Boston, MA).

Preparation of isolated neurons. Bag cell neurons were isolated from other neurons and maintained in cell culture as described previously (Kaczmarek et al., 1979). The culture medium (MEM) contained 460 mM NaCl, 10.4 mM KCl, 27 mM $MgCl_2$, 28 mM $MgSO_4$, 11 mM $CaCl_2$, 15 mM HEPES, 1 mg/ml glucose, together with essential and nonessential amino acids, vitamins, glutamine, penicillin, and streptomycin (Gibco) (Kaczmarek and Strumwasser, 1981). No protease inhibitors were used in experiments with isolated neurons.

Electrophysiological experiments using cultured bag cell neurons were carried out at 22°C. To avoid the development of extensive neuritic processes, isolated cells that had been in culture for no longer than 2 d were used for all the 2-microelectrode voltage-clamp experiments. Peptides were ejected onto the somata of cultured neurons from a micropipette positioned close to the cell.

Immunocytochemistry. For preparation of antisera to α-BCP, 0.5 mg α-BCP [1-7] was first incubated with 0.5 mg BSA and 2.5 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide HCl in 100 μl of distilled water for 3 hr at room temperature, and then dialyzed extensively against distilled water. Antisera to the conjugated complex were raised in rabbits using the method of Vaitukaitis et al. (1971). Immunofluorescent staining of primary cultures of bag cell neurons was carried out as described by Chiu and Strumwasser (1981).

Computer simulation of the inwardly rectifying current. Voltage responses (V) of a cell to imposed hyperpolarizing current (I_0) were simulated in a model that included only a leakage conductance (g_L) and an inwardly rectifying potassium conductance (g_R). To generate the traces shown in Figure 6*B*, the parameters for the leakage current and capacitance were set to match the experimentally obtained traces from the neuron in Figure 6*A*. Membrane voltage responses were simulated by integration of the equation,

$$C\,dV/dt = g_R[1/(1 + e^{\eta(V - V_R)})]\,(E_K - V) + g_L(E_L - V).$$

In the simulation of Figure 6*B*, $C = 0.5$, $\eta = 0.38$, $V_R = -70$, $E_K = -90$, $g_L = 0.003$, $E_L = -61.5$, $V(0) = -61.5$, and g_R varied.

Statistics. All data are expressed as means ± SEM. Values of p are given for the unpaired 2-tailed Student's t test.

Results

Alpha BCP inhibits the bag cell discharge but does not produce refractoriness

Brief electrical stimulation of the pleuroabdominal connective nerve in the isolated abdominal ganglion triggers a 30 min discharge of the bag cell neurons similar to that observed *in vivo* (Pinsker and Dudek, 1977). We found that application of α-BCP (1 μM) over the bag cell neurons after the onset of a discharge caused premature termination of the discharge (Fig. 1). Three physiologically active forms of α-BCP have been purified from bag cell extracts—α-BCP [1-7], α-BCP [1-8], and α-BCP [1-9] (Rothman et al., 1983), and it has been suggested that the [1-8] and [1-7] forms are products of carboxypeptidase A-like cleavage of α-BCP [1-9] (Sigvardt et al., 1986). We found that each of these 3 forms of α-BCP is effective at inhibiting the discharge at 1 μM. Table 1 shows that α-BCP [1-7] significantly inhibits the discharge at concentrations as low as 1 nM.

At the end of a normal discharge, further electrical stimulation generally fails to trigger a second long-lasting discharge (Kaczmarek and Kauer, 1983). Electrical stimulation was also ineffective in triggering further discharges after the application of α-BCP had caused discharges to end prematurely, and the cells entered the prolonged refractory period that normally follows a discharge. However, exposure to α-BCP alone was not sufficient to cause refractoriness, since pressure-ejection of α-BCP over the bag cell clusters 30–90 min prior to electrical stimulation of a discharge had no effect on discharge duration.

α-BCP decreases basal and stimulated levels of cAMP in bag cell neurons

Because of the role of cAMP in the maintenance of the discharge, we tested the effects of α-BCP on cAMP levels in bag cell neurons. We found that the inhibition of discharge caused by α-BCP is correlated with a reduction in cAMP levels in clusters of bag cell neurons (Fig. 2). We also observed that brief exposure to α-BCP reduced basal levels of cAMP in unstimulated bag

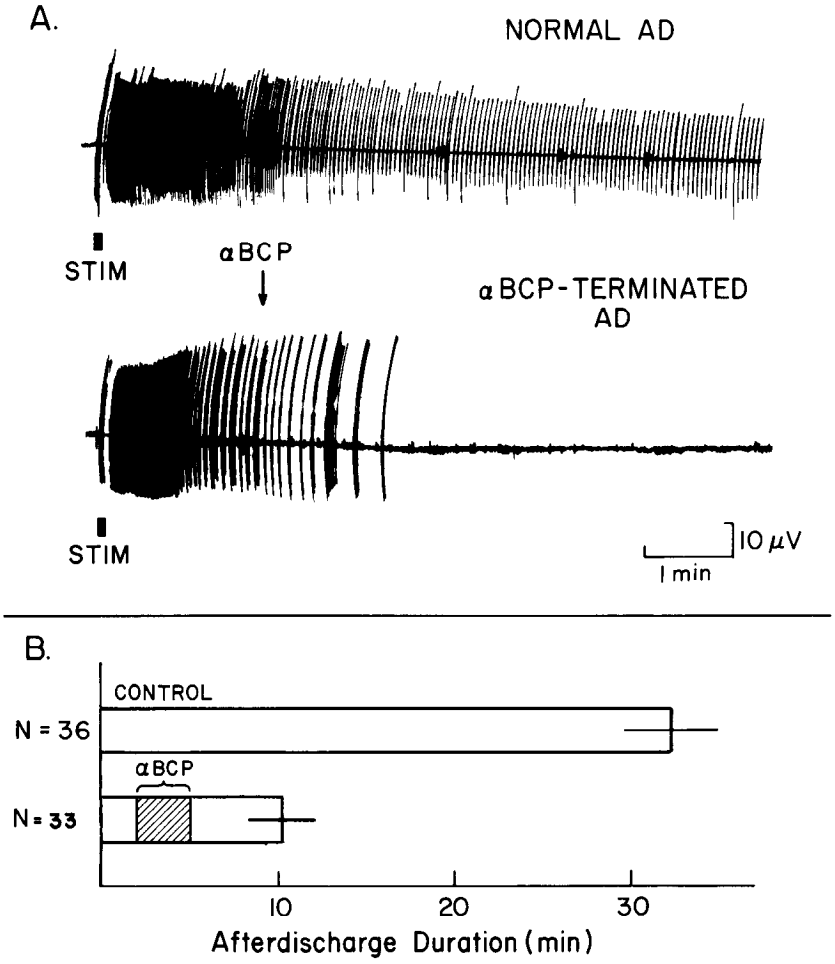

Figure 1. Brief exposure to α-BCP causes termination of an ongoing discharge. *A*, Extracellular records of the onset of a normal electrically stimulated discharge (*AD*) (*upper trace*) and of a discharge inhibited by ejection of 20 μl 1 μM α-BCP over each bag cell cluster of an intact ganglion 2 min after stimulation of a discharge (*lower trace*). *B*, Mean duration of normal discharges and mean duration of discharge following exposure to 1 μM α-BCP 2–5 min after the onset of an electrically stimulated discharge. α-BCP causes a significant inhibition of discharge duration ($p < 0.001$). Ejection of ASW/PI over bag cell clusters had no effect on discharge duration.

cell neurons by approximately 50% ($p < 0.03$). In addition, brief pretreatment with α-BCP without electrical stimulation inhibited the increase in cAMP stimulated by the adenylate cyclase activator forskolin in the presence of theophylline. The end of a normal discharge is associated with a similar attenuation of the action of forskolin (Fig. 2) (Kauer and Kaczmarek, 1985). These data suggest that endogenously released α-BCP may depress the ability of the cells to elevate cAMP levels at the end of the discharge.

Inhibition of the discharge is prevented and reversed by elevation of cAMP levels

If the mechanism by which α-BCP causes termination of a bag cell discharge is related to its ability to decrease cAMP levels, one would expect that α-BCP would not terminate the discharge under conditions in which cAMP levels had been pharmacologically elevated. Figure 3 shows that application of α-BCP over bag cell clusters no longer inhibited the discharge in the presence of the membrane-permeant, phosphodiesterase-resistant cAMP analog BBT-cAMP or following cAMP elevation using forskolin/theophylline. (Although α-BCP decreases cAMP levels in the presence of forskolin/theophylline, cAMP concentrations in this condition are still higher than those measured during a discharge.)

Pharmacological elevation of cAMP levels in the bag cell neurons not only prevented the inhibitory effects of α-BCP, but also reversed them. When discharges had been terminated using α-BCP, they restarted within minutes following the subsequent addition of 50 μM forskolin with 1 mM theophylline to the bath. The mean duration of such reinstated discharges was 18.6 ± 2.8 min ($n = 8$). These data suggest that the reduction of cAMP levels caused by exposure to α-BCP may be sufficient to account for the inhibition of discharge.

In several other systems in which agonists produce a decrease in cAMP levels, the receptors have been shown to be coupled to adenylate cyclase or to a nucleotide phosphodiesterase through a GTP-binding protein (Elks et al., 1983; Katada et al., 1984).

Table 1. Dose-response relationship for inhibition of discharge by α-BCP

Conc. of α-BCP	Duration (min)	*n*	$p <$
0	32.3 ± 2.7	36	
1 μM	10.3 ± 1.9	33	0.001
100 nM	13.8 ± 5.5	4	0.02
10 nM	19.3 ± 6.3	6	0.10
1 nM	12.8 ± 2.4	5	0.01
0.1 nM	46.7 ± 24.2	3	n.s.
0.01 nM	32.8 ± 7.7	4	n.s.

Bag cell neurons within the ganglion were electrically stimulated to discharge. At 2 min after the onset of the discharge, 20 μl of α-BCP, at the concentrations shown, was ejected over each bag cell cluster.

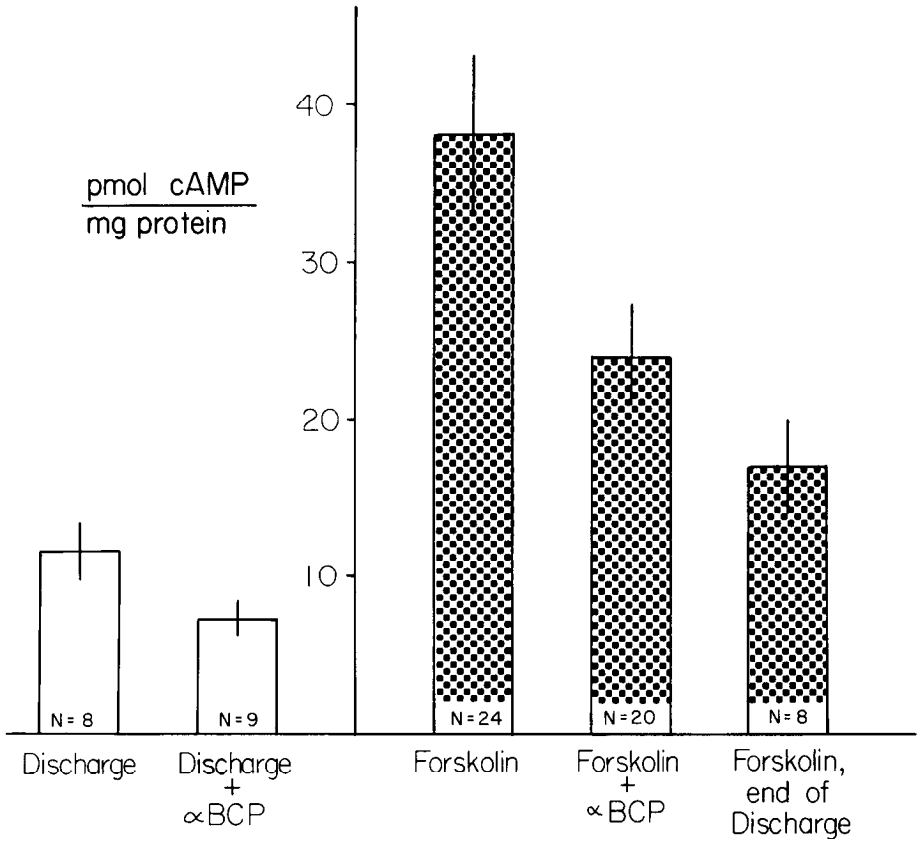

Figure 2. Brief exposure to α-BCP reduces both discharge-stimulated and forskolin-stimulated cAMP levels in clusters of bag cell neurons. Data from *Discharge* experiments were obtained from bag cell clusters dissected from the intact ganglion 7 min after the electrical stimulation of a discharge, with or without application of 20 μl 1 μM α-BCP (*Discharge + α-BCP*) over each bag cell cluster 2 min after the onset of the discharge. Treatment with α-BCP had not yet produced inhibition of discharge at the time of dissection. *Forskolin* data were obtained from whole ganglia incubated for 10 min with 50 μM forskolin and 1 mM theophylline in the bathing ASW/PI. For *Forskolin + α-BCP,* 20 μl of 1 μM α-BCP was ejected over each bag cell cluster 10 min prior to addition of forskolin and theophylline. *Forskolin, end of discharge,* forskolin and theophylline were added 10 min after the end of a full-length, electrically stimulated discharge. Cyclic AMP levels were measured in each bag cell cluster by radioimmunoassay as described.

In such cases the effects of the agonists have been blocked by treatment of the cells with pertussis toxin (PTX), an agent that catalyzes the ADP-ribosylation of a GTP-binding protein in many tissues, including *Aplysia* nervous tissue (Critz et al., 1986). We therefore tested the effects of α-BCP on discharge duration following treatment of the cells with PTX. Although we did not assess the entry of PTX into *Aplysia* neurons, we found that pretreatment with PTX significantly reversed the inhibitory effects of α-BCP on the discharge. The mean durations of discharge following application of 1 μM α-BCP at 2 min following stimulation were 10.3 ± 1.9 min for control clusters (n = 33) and 19.5 ± 1.7 min for PTX-treated clusters (n = 8; $p < 0.03$). These data suggest that the inhibition of the discharge by α-BCP involves a GTP-binding protein that may modulate the activity of either adenylate cyclase or a phosphodiesterase in these neurons.

Immunostaining of isolated bag cell neurons in cell culture

We have carried out experiments using isolated bag cell neurons in cell culture to test whether the effects of α-BCP on the excitability of the bag cell neurons occur through its actions on other neurons in abdominal ganglia or directly through an au-

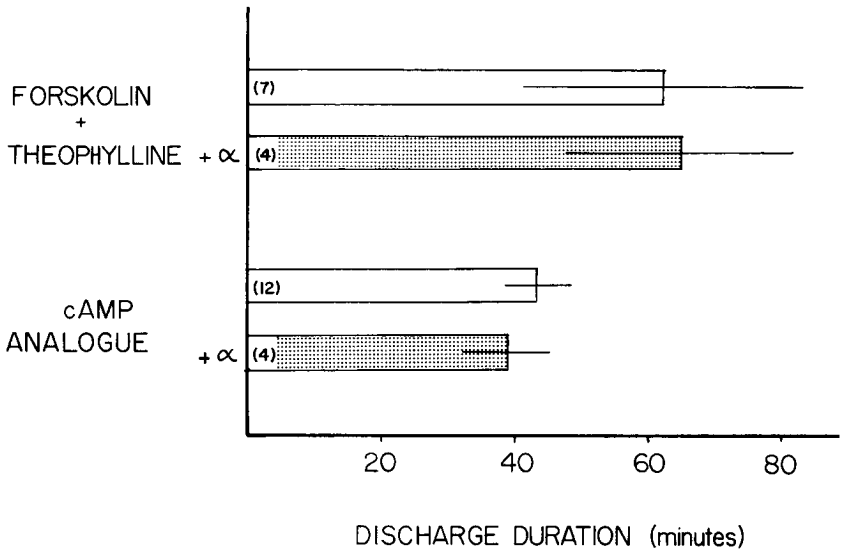

Figure 3. Elevation of cAMP prevents the inhibition of the discharge by α-BCP. Durations of electrically stimulated discharges were measured under 2 conditions: (1) *Forskolin + Theophylline,* where 50 μM forskolin and 1 mM theophylline were added to the bathing ASW/PI 10 min prior to stimulation, and were present throughout the discharge; and (2) *cAMP analogue,* where 500 μM N^6-*n*-butyl-8-benzylthio cAMP was added to the bath 30 min prior to stimulation. *Shading* represents experiments in which 20 μl of 1 μM α-BCP was applied over each cluster of bag cell neurons within the ganglion 2 min into the discharge.

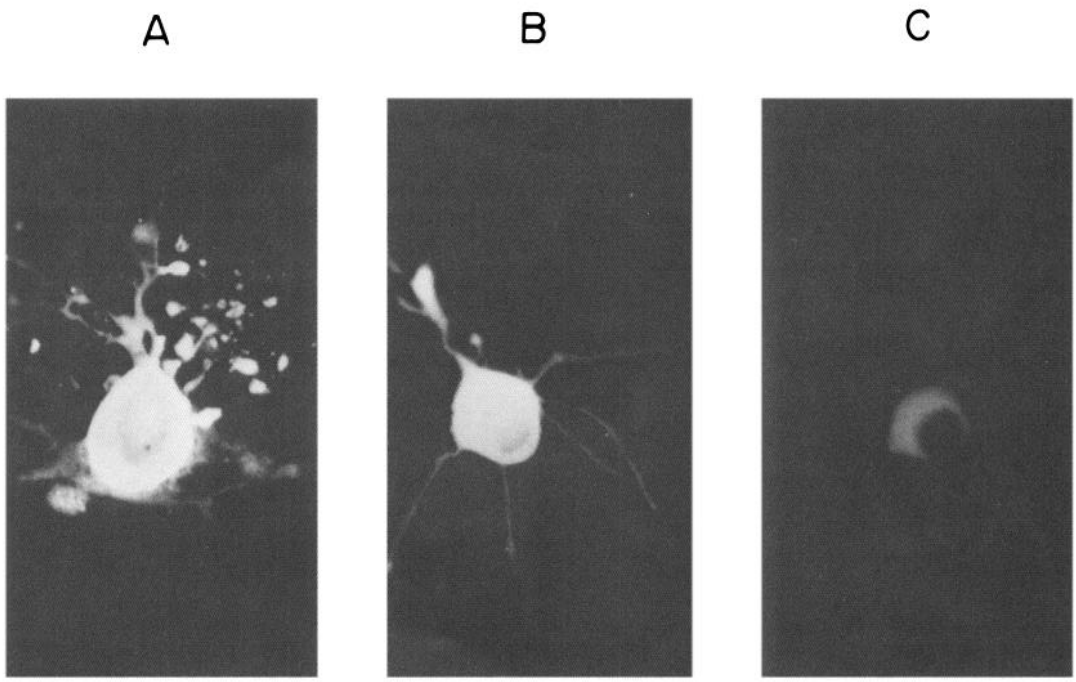

Figure 4. *A, B,* Immunofluorescent staining of isolated cultured bag cell neurons using antisera against α-BCP. *C,* Background staining of an isolated bag cell neuron using preimmune serum.

toreceptor. Such cultured neurons have been shown to immunoreact specifically with an antisera against ELH (Chiu and Strumwasser, 1981). Figure 4 shows that bag cell neurons in cell culture are also specifically stained by antisera directed against α-BCP. Both the cell bodies and neurites of all neurons from a bag cell cluster stained with this antisera, while other neurons from the abdominal ganglion were not stained. As has been reported for antisera against ELH, we found that a few unidentified cells in the cerebral ganglia appeared to be stained with anti-α-BCP antisera. Although we cannot exclude the possibility that the anti-α-BCP antisera cross-react with the peptides that are homologous to α-BCP and derived from the same protein precursor (Scheller et al., 1983), we conclude that isolated bag cell neurons in culture, like bag cell neurons within the ganglion, contain α-BCP or its precursor protein.

α-BCP antagonizes the electrophysiological effects of forskolin on isolated bag cell neurons

Previous work has shown that exposure of isolated bag cell neurons to forskolin plus theophylline or to cAMP analogs decreases the amplitude of delayed potassium currents evoked by depolarization (Kaczmarek and Strumwasser, 1984; Strong and Kaczmarek, 1986). As shown above, α-BCP attenuates the stimulation of cAMP levels by forskolin in intact clusters of bag cell neurons. If α-BCP acts directly on the bag cell neurons, the peptide would be expected to antagonize the effects of forskolin on the delayed outward currents. We therefore examined the effects of α-BCP on the delayed outward currents measured in isolated bag cell neurons using a 2-microelectrode voltage-clamp. We found no significant effects of α-BCP alone on the amplitude of the delayed outward currents in normal medium. However, when forskolin and theophylline were added 10 min after ejection of 1 μM α-BCP over the soma of an isolated neuron, the subsequent attenuation of the delayed outward currents was significantly reduced (Fig. 5*A*). Figure 5*B* shows the mean time course of the attenuation of delayed outward currents following exposure to forskolin plus theophylline. The closed circles represent the attenuation of these currents in control medium. The open circles show that pretreatment with α-BCP significantly reduced the attenuation of these currents at 10 and 15 min after the addition of forskolin plus theophylline. Biochemical measurements in intact clusters of bag cells showed that 10 min after α-BCP, the ability of forskolin to elevate cAMP levels was reduced by approximately 40% (see Fig. 2).

α-BCP hyperpolarizes bag cell neurons and alters the response to hyperpolarizing current

We found 2 additional effects of α-BCP on the voltage responses of isolated cultured bag cell neurons. First, in 32 of 48 cells, ejection of α-BCP (1 μM) over the soma caused a rapid, transient (10–20 sec) hyperpolarization. This hyperpolarization was usually 2–5 mV in amplitude, but in some cases was as large as −35 mV (mean amplitude, 9.4 ± 1.7 mV). This response to α-BCP appears to result for the most part from a transient increase of a potassium current, since the response reversed polarity at about −90 mV, was accompanied by a conductance increase, and was blocked by 10 mM Cs^+ in the bath.

A second effect of α-BCP, which could be seen clearly in isolated bag cell neurons with high input resistances (>~280 MΩ; 7 of 24 cells), is illustrated in Figure 6*A*. The trace at the top of figure shows the normal response of the membrane potential to a hyperpolarizing current pulse. After exposure of the cell to α-BCP, the voltage response to the same hyperpolarizing current pulse became highly nonlinear. In particular, the response became more rapid in onset, and repolarization at the end of the pulse was markedly slowed. Unlike the transient hyperpolarization of the membrane potential described above, this effect of α-BCP was neither immediate nor transient, but

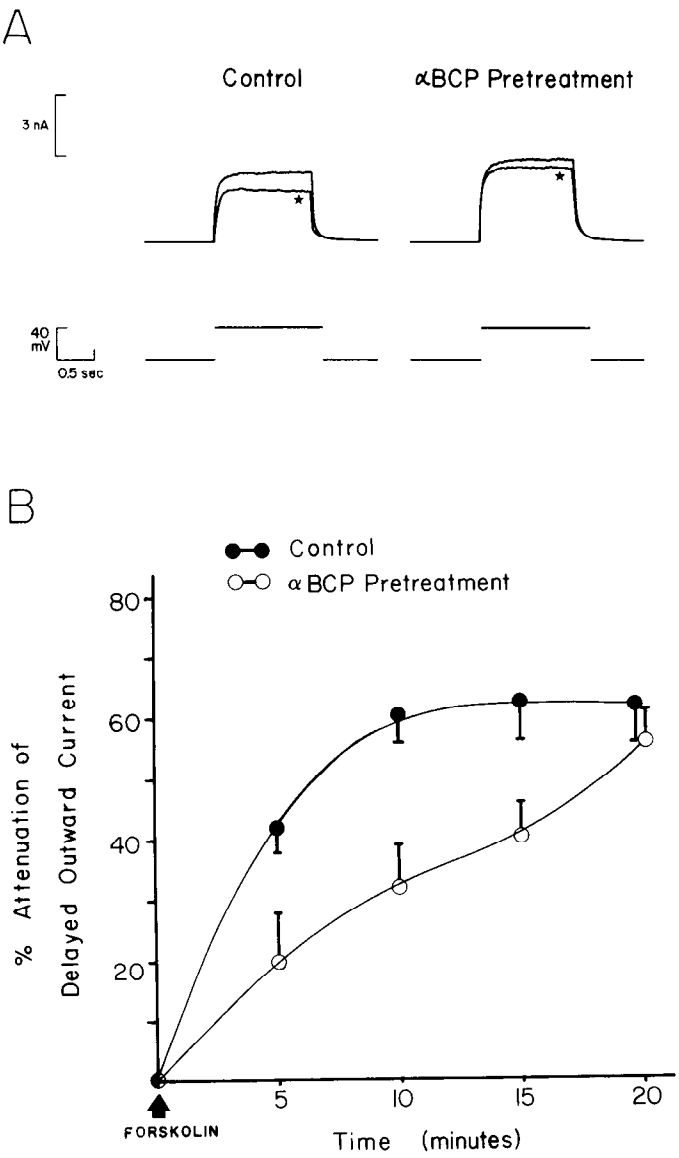

Figure 5. α-BCP reduces the attenuation of delayed outward currents by forskolin and theophylline. *A*, Outward currents evoked in isolated voltage-clamped bag cell neurons by depolarization, from a holding potential of −40 mV, to 15 mV for 1.5 sec. In the *right-hand panel*, 4 μl of 10 μM α-BCP was ejected near the neuron 10 min prior to addition to the bath of 50 μM forskolin and 1 mM theophylline. *Upper traces* show currents before exposure to forskolin/theophylline; *lower traces* (marked by *asterisk*) show currents 10 min after forskolin/theophylline. *B*, Time course of the attenuation of outward currents by forskolin/theophylline, with and without pretreatment with α-BCP. Peak outward current evoked by 1.5 sec depolarizations from −40 mV was measured at 5 min intervals after addition of forskolin/theophylline. *Closed symbols*, no pretreatment ($n = 6$). *Open symbols*, pressure-ejection of 4 μl of 10 μM α-BCP 10 min prior to addition of forskolin/theophylline ($n = 12$). Significance: at 10 min, $p < 0.01$; at 15 min, $p < 0.05$. Test potentials for both experimental and control groups were chosen to elicit at least 3 nA peak outward current before addition of forskolin/theophylline and ranged from −15 to +5 mV. The voltage-dependence of the delayed outward currents was unaffected by treatment with α-BCP alone.

gradually increased over a period of minutes following exposure to α-BCP.

Alpha-BCP enhances an inwardly rectifying potassium current in isolated bag cell neurons

Two-electrode voltage-clamp studies confirmed that ejection of α-BCP over an isolated bag cell neuron alters the steady-state I–V relations at voltages more negative than −40 mV. Figure 7*A* shows I–V relations for a typical isolated neuron before and after exposure to α-BCP. The mean current evoked by α-BCP in a control medium is shown in Figure 7*C*. This α-BCP-induced current resembles the inwardly rectifying potassium current characterized in other cells (Katz, 1949; Hagiwara and Takahashi, 1974; Hagiwara et al., 1978; Benson and Levitan, 1983; Breitwieser and Szabo, 1985; Pfaffinger et al., 1985; Stanfield et al., 1985). As expected for a predominantly potassium-selective current, the measured reversal potential of the induced current (~−89 mV) is near E_K in media containing 10.4 mM K^+, and is shifted to a more positive value (~−50 mV) on elevation of external K^+ to 40 mM (Fig. 7*B*). In addition, the conductance of the α-BCP-induced current was increased on elevation of the external K^+ concentration. In common with inwardly rectifying potassium currents in other systems, the current enhanced by α-BCP was blocked by 10 mM Rb^+, 10 mM Cs^+, and 2 mM Ba^{2+} (but not by 100 mM tetraethylammonium). Figure 7*C* demonstrates that α-BCP applied to an isolated neuron in the presence of 10 mM Rb^+ had no effect on the steady-state I–V relations.

The increase in the inwardly rectifying potassium current could be observed 1 min following exposure of a cell to α-BCP, and I–V relations returned to control following a 15 min washout period. The time course for enhancement of the inwardly rectifying potassium current by α-BCP was generally similar to that for the alteration in the voltage response to hyperpolarization (shown in Fig. 6*A*).

Figure 6*B* shows voltage traces from a computer simulation that demonstrates that the enhancement of an inwardly rectifying potassium current can account for the alteration of the voltage responses to hyperpolarizing current pulses in α-BCP-treated neurons. The top trace shows linear responses to hyperpolarizing current in the absence of all voltage-dependent conductances. In the lower traces, the conductance (g_R) of an inwardly rectifying current is progressively increased.

To test the hypothesis that α-BCP enhances the inwardly rectifying K^+ current as a result of reductions in cAMP levels, we tested the effects of cAMP on this current in isolated bag cell neurons. If a reduction of cAMP were responsible for the enhancement of this current, it would be expected that elevations of cAMP could reduce its amplitude. In contrast, however, we found that elevation of cAMP levels using either 50 μM forskolin with 1 mM theophylline or 0.5 mM BBT-cAMP enhanced the inwardly rectifying potassium current. It is therefore unlikely that α-BCP enhances this current through its actions on cAMP levels.

Discussion

We have found that α-BCP has direct effects on the biochemical and electrical properties of the bag cell neurons from which it is released. This peptide lowers cAMP levels in both electrically stimulated and unstimulated bag cell neurons and attenuates the ability of forskolin to elevate cAMP levels. Exposure of

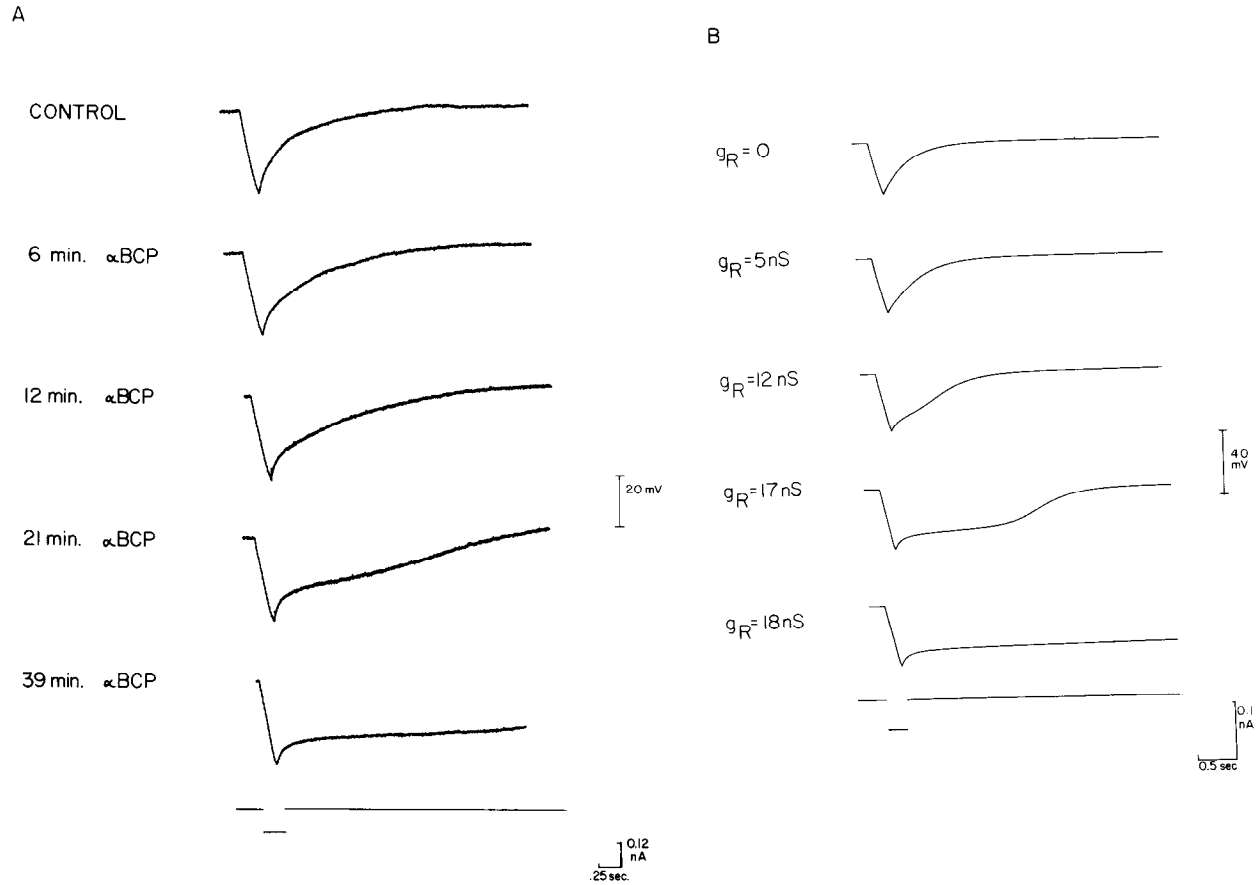

Figure 6. *A*, α-BCP alters the voltage response to a hyperpolarizing current pulse. Single microelectrode voltage recordings of the response of an isolated bag cell neuron to a current pulse of −0.12 nA for 200 msec, before and after ejection of 4 μl of 1 μM α-BCP over the neuron. *B*, Computer simulations of the effect of alterations in the amplitude of an inwardly rectifying potassium conductance (g_R) on voltage responses to hyperpolarizing current. Simulations were carried out using the equation described in Materials and Methods. *Top trace*, voltage response to a current pulse of −0.12 nA in a model cell with $g_R = 0$. *Lower traces*, voltage responses to the same current pulse, with progressive increases in the value of g_R.

intact bag cell clusters to α-BCP prematurely terminates an ongoing discharge. The termination of the discharge can be prevented and reversed by treatments that elevate intracellular cAMP levels, indicating that the decrease in cAMP may be sufficient to explain the peptide-induced inhibition of repetitive firing.

A reduction of the ability of forskolin to elevate cAMP levels, similar to that induced by α-BCP, can be measured in bag cell clusters within 10 min of the end of a normal discharge. This suggests that the actions of α-BCP, released from the bag cell neurons following stimulation of a discharge, may contribute to the normal termination of the discharge. However, although α-BCP terminates ongoing discharges, exposure of unstimulated cells to this peptide alone did not induce the prolonged refractory state that normally follows afterdischarges. This finding supports earlier studies indicating that the termination of the discharge and the onset of refractoriness may result from independent processes (Kauer and Kaczmarek, 1985).

Our studies with isolated cells in culture indicate that the effects of α-BCP on cAMP levels are likely to occur directly through autoreceptors on bag cell neurons. Although we have not yet characterized the mechanism by which α-BCP lowers cAMP levels, the results of experiments using PTX are consistent with the involvement of receptor-coupled GTP-binding protein, as has been described in several other systems in which agonists lower cAMP levels (Elks et al., 1983; Katada et al., 1984). In addition to its effects on cAMP levels, α-BCP also enhances an inwardly rectifying potassium current in isolated bag cell neurons, changing the way the cell responds to imposed hyperpolarizations. The linkage of an agonist-induced increase in inwardly rectifying potassium current with a decrease in cAMP levels may be rather widespread. For example, activation of muscarinic receptors in cardiac cells also evokes the dual effect of an increase in an inwardly rectifying potassium current and a decrease in cAMP levels. In this case, as with the bag cell neurons, the increase in potassium current is apparently not causally linked to the change in cAMP, but has been suggested as occurring through a more direct effect of a GTP-binding protein on the potassium channel (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985).

It is not yet clear what role the hyperpolarization evoked by α-BCP, or the increase of the inwardly rectifying potassium current by this peptide, plays in the regulation of repetitive firing in the bag cell neurons. In neuron R15 of the abdominal ganglion of *Aplysia*, the enhancement of an inwardly rectifying potassium current by serotonin intensifies rhythmic bursting by increasing the interburst hyperpolarizations (Lotshaw et al., 1986). A similar effect may underlie the rhythmic bursting of action poten-

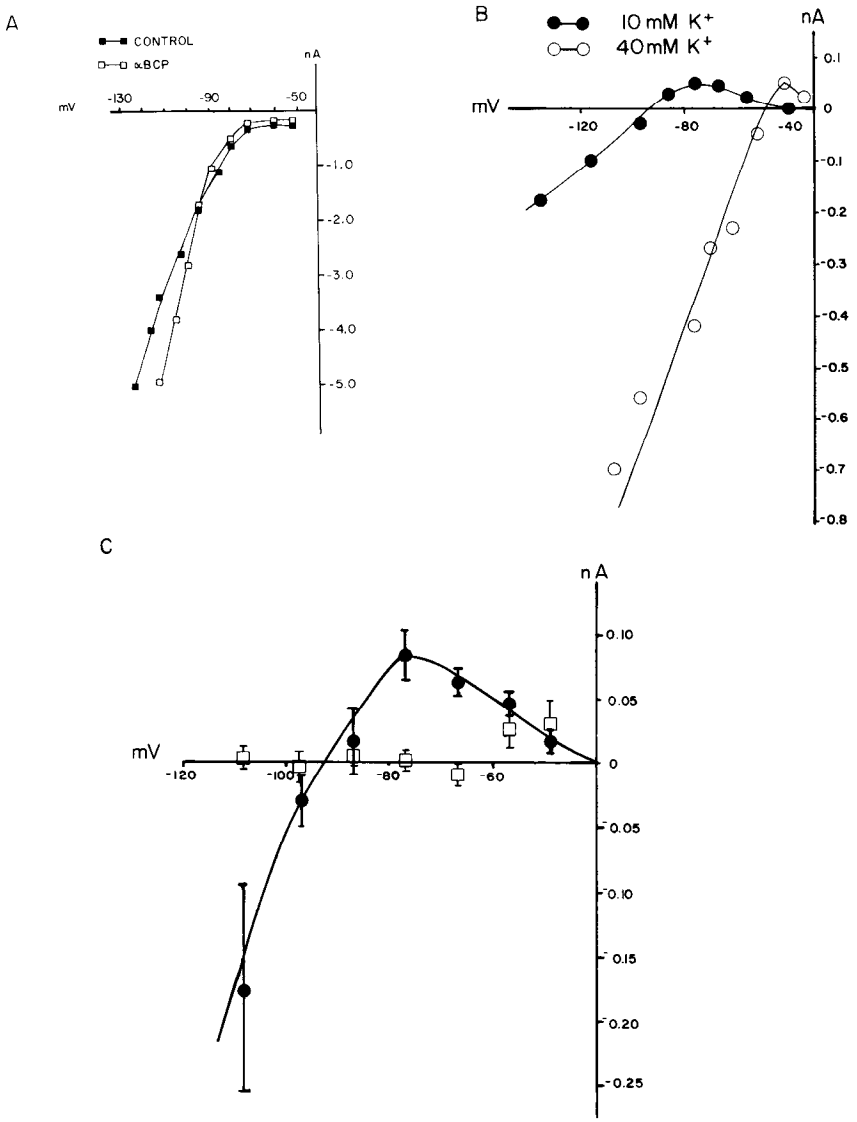

Figure 7. α-BCP enhances an inwardly rectifying K^+ current. *A*, Steady-state currents measured under voltage-clamp during hyperpolarizing steps from −40 mV, before and 10 min after application of 10 μl of 10 μM α-BCP over an isolated neuron. *B*, Effect of α-BCP at 2 different external K^+ concentrations. The plots show α-BCP-induced currents, determined by subtracting control currents from currents after application of α-BCP. Steady-state currents were measured during hyperpolarizing steps from a holding potential of −25 mV. The *closed circles* show the current induced 10 min after application of 1 μM α-BCP in 10 mM K^+. After returning the external medium to normal ASW, the external K^+ concentration was raised to 40 mM. The *open circles* show the current evoked 10 min after reexposure of the cell to the peptide in the high-K^+ medium. *C*, Mean current induced by α-BCP under control conditions and in the presence of rubidium ions. The *closed circles* show the mean current induced by α-BCP, measured within 20 min of exposure to α-BCP (each point represents 7–12 experiments). The *open squares* show the mean current induced by application of α-BCP with 10 mM Rb^+ in the extracellular medium throughout the experiment ($n = 6$).

tials that was sometimes seen after application of α-BCP to bag cell neurons in the intact ganglion prior to the α-BCP-induced termination of the discharge (for example, see Fig. 1*A*). Our data suggest, however, that the increase in this current does not bring about the end of the discharge, since an elevation of cAMP levels also increases this current, yet prolongs discharges and prevents their inhibition by α-BCP.

In addition to the effects that we have described, it is likely that α-BCP also has other, direct or indirect, effects on the bag cell neurons in intact ganglia. Rothman et al. (1983) reported that application of α-BCP (0.5 μM to 1 mM) through a cannula in the abdominal artery resulted in a depolarization of bag cell neurons that could sometimes trigger an afterdischarge. We also sometimes observed small depolarizations when α-BCP was applied over bag cell neurons in intact ganglia, but α-BCP never triggered discharges when applied to unstimulated clusters and we did not observe depolarizations in response to α-BCP in isolated neurons. However, preliminary experiments have suggested that bag cell neurons may be more excitable in abdominal ganglia taken from animals that have been injected with $MgCl_2$ as an anesthetic during dissection (K. Loechner and L. K. Kaczmarek, unpublished observations). This technique was used by Rothman et al. (1983), but not in the present experiments. Thus it is possible that α-BCP has multiple regulatory effects on the bag cell system, some of which may be related to the physiological state of the animal. In addition, it is possible that some effects of α-BCP may occur only at neurites distal to the somata, or may be mediated through other neurons in the abdominal ganglion.

The bag cell neurons also release 2 other peptides, β-BCP and γ-BCP, both of which share a sequence of 4 amino acid residues in common with α-BCP (Scheller et al., 1983). Preliminary evidence suggests that β-BCP and γ-BCP may also have direct autoreceptor-mediated actions on the bag cell neurons (Brown and Mayeri, 1986; K. Loechner, J. A. Kauer, and L. K. Kaczmarek, unpublished observations). It is likely that the coordinate action of all 3 peptides regulates electrical and biochemical parameters of the bag cell neurons during and after an evoked discharge. An unequivocal demonstration of the role that these various peptides play in shaping the electrical properties of the bag cell neurons may require the use of specific antagonists to each of the released peptides. It is an intriguing possibility that

alterations in the rate of synthesis (Berry and Arch, 1981; Bruehl and Berry, 1985) or of processing of the peptides destined for release from the bag cell neurons may indirectly exert a profound influence on the electrical behavior of these neurons.

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