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DISSERTATION

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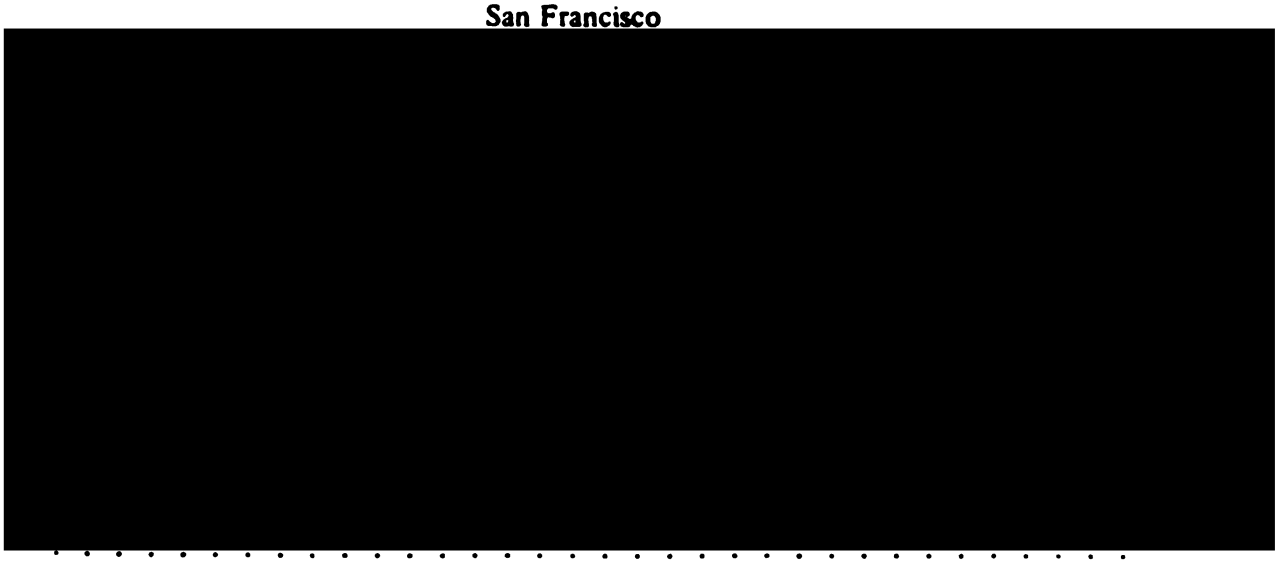

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FUNCTIONS OF NEUROPEPTIDES IN THE CENTRAL NERVOUS SYTEM OF *APLYSIA*

Richard O. Brown

ACKNOWLEDGEMENTS

I want first to thank my advisor, Earl Mayeri, for his unusual humanity, and for providing me the opportunity to conduct research in his laboratory, and training in the profession of science. The complex interplay of positive and negative feedback we enjoyed was an integral part of this work. Earl also allowed me unusual flexibility to follow my nose and pursue a variety of interests inside and outside the laboratory, a freedom I regularly abused, but without which I never could have finished these studies.

This work was enhanced by the insightful criticisms by the other members of my thesis committee, Roger Nicoll, Allan Basbaum, and Robert Zucker, and they deserve special gratitude for their expeditious but thorough reading of the dissertation during these last hectic weeks.

My coworkers in Earl's lab made many essential contributions to this work, and I especially want to acknowledge those of Barry Rothman, Linda Padgett, Susan Kansky, Bill Hopkins, Karen Sigvardt, Dennis Gusman, Amir Dehghan, Stefan Pulst, and Rene Jansen.

I also want to thank the Neurobiology Program at UCSF for providing an academic setting in which I could pursue an education without compromising my values. (And an honorable mention to Josh Sanes, for kindly warning me during an interview that my type is unwelcome in St. Louis.)

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Of course, it's the extracurricular relationships I enjoyed with all these colleagues, and with my fellow students, friends, and family, that I value most. My parents made innumerable contributions to my progress, only the least of which is the financial support that made the first years possible. I also owe special debts to Stuart, Susan, and Lisa, each of whom made me a better person and helped me avoid the pitfalls of complacency and sanity. And last but most of all, it is a special joy to acknowledge the continuing love, support and nourishment of Elysa (who also provided an added incentive for finishing by promising to kill me if I didn't receive a Ph.D. by June, 1988).

Has the gain been worth the countless victims? Has our present structure ofthe life of the mind been sufficiently developed, and is it likely to endure long enough, to justify as worthwhile sacrifices all the sufferings, convulsions, and abnormalities: the trials of heretics, the burnings at stake, the many "geniuses" who ended in madness or suicide? For us it is not permissible to ask these questions.

- Herman Hesse, Magister Ludi.

Treading once upon the tiger's tail, he is a fool.
Treading twice upon the tiger's tail, he is a master.
Treading thrice upon the tiger's tail, he is devoured.

ABSTRACT

The generation of integrated behaviors and motivational states is commonly hypothesized to involve neuropeptides acting at diverse sites throughout the central nervous system. This dissertation describes research into some of the basic functions of neuropeptides, performed with electrophysiological and immunohistochemical techniques in the experimentally convenient abdominal ganglion of the marine mollusk *Aplysia*.

Three experimental studies focused on the bag cells, model neuroendocrine cells which control egg-laying behavior. The neuropeptide egg-laying hormone was previously demonstrated to function as a bag cell neurotransmitter. Chapter 2 reports the identification and characterization of alpha-bag cell peptide(α-BCP) as a putative cotransmitter mediating central inhibitory actions of the bag cells. This was the first demonstration that two distinct neuropeptides derived from a common precursor protein function as cotransmitters. Chapter 3 reports that α-BCP, as well as the structurally related β- and γ-BCPs, have autoexcitatory effects on the bag cells, mediated by a second receptor subtype, which provide positive feedback and contribute to the episodic, all-or-none activity pattern of the bag cells. Chapter 4 demonstrates that the bag cells are activated by a small cluster of neurons in the right pleural ganglion which also contain the BCPs. These pleural

neurons are hypothesized to constitute part of the descending pathway that triggers bag cell activity *in vivo*.

Two studies investigated the possible neurotransmitter role of the molluscan neuropeptide FMRFamide. Chapter 5 reports the wide distribution of immunoreactive FMRFamide in the abdominal ganglion, including its localization to many identified neurons. Chapter 6 reports the widespread pharmacological activities of FMRFamide and its analogs on abdominal ganglion neurons, provides evidence for multiple FMRFamide receptor subtypes, and describes the identification by bioassay of native FMRFamide in abdominal ganglion extracts.

Chapter 7 describes phasic and tonic central actions of neuron R_{15}, a peptidergic neurosecretory cell which contains an antidiuretic hormone and regulates water balance.

These diverse peptidergic neurons in *Aplysia* share many of the general characteristics of vertebrate neuropeptide systems, but allow for more detailed analysis of their properties at the cellular level. The general functional significance of these studies, and their relation to vertebrate neuropeptide systems, are discussed in Chapter 8.

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Publication of the Material in this Thesis

In all instances, Earl Mayeri directed and supervised the research, and was a coauthor of all publications.

Chapter 2 contains material which has mostly been published in Rothman et al. 1983 and Sigvardt et al. 1986. It includes only the electrophysiological results Richard Brown obtained, although some of these overlapped with Karen Sigvardt's work. The other authors were not directly involved in the electrophysiological experiments reported here. Some additional work mentioned in chapter 2 was published in Rothman et al., 1985 and 1986. The results on the biphasic responses in RUQ cells have not previously been published.

Chapter 3 is in press (Brown and Mayeri 1989).

Chapter 4 has been submitted for publication (Brown, Pulst, and Mayeri 1989). The contribution of Stefan Pulst to this work consisted of the generation and affinity purification of the α-BCP antiserum used for the immunocytochemistry, and which has been documented in detail in 4 previous publications by Pulst et al.

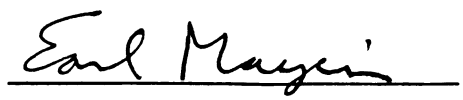
Chapter 5 was published in Brown, Gusman, Basbaum and Mayeri 1985. Dennis Gusman conducted the pilot experiments with FMRFamide immunocytochemistry which served as the

rationale for this project, but Richard Brown generated all the data and results presented in the paper.

Chapter 6 has not been published. Some of these results may be combined with vintage data from Stone and Mayeri (1981) and prepared for publication (Brown, Stone, and Mayeri). The isolation of native FMRFamide was reported in Rothman et al. 1985.

Chapter 7 was published in Brown and Mayeri 1987.

I certify that this is an accurate representation of the contributions of Richard Brown and coauthors to the work presented in this dissertation.



Earl Mayeri

Research Advisor

Publications

Brown, R.O., D.I. Gusman, A.I. Basbaum and E. Mayeri (1985). Identification of *Aplysia* neurons containing immunoreactive FMRFamide. Neuropeptides 6: 517-526.

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Rothman, B.S., E. Mayeri, R.O. Brown, P.-M. Yuan, and J.E. Shively (1983). Primary structure and neuronal effects of alpha-bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia*. Proc. Natl. Acad. Sci. (USA) 80, 5753-5757.

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Sigvardt, K.A., B.S. Rothman, R.O. Brown, R.O., and E. Mayeri (1986). The bag cells of *Aplysia* as a multi-transmitter system: identification of alpha bag cell peptide as a second neurotransmitter. J. Neurosci. 6, 803-813.

Chapter 1

INTRODUCTION

The purpose of neurobiology is to explore the biological basis of behavior and perception. Neurobiology has had great success in discovering many of the fundamental mechanisms underlying nervous activity and cellular communication. The question of how these local, physical events throughout the nervous system are integrated to produce unified perceptions and coherent behaviors remains baffling. At the deepest level, this question echoes the venerable issue of the relation between mind and matter, and many neurobiologists (including myself) are ultimately driven by the goal of gaining insight into this mystery (see Lorenz 1963 and Delbruck 1986). A more mundane, but more experimentally approachable facet of this question is how the many diverse components of complex behaviors and motivational states are centrally generated and coordinated. An emerging theme that has been vigorously pursued over the last decade is that these functions involve the diffuse central actions of neuropeptides.

Many mammalian neuropeptides produce very specific behavioral effects. Early studies by de Weid indicated effects of vasopressin and adrenocorticotropic hormone (ACTH) on memory and learning (see de Weid 1969). Centrally administered angiotensin was found to initiate specific, motivated drinking behavior (Epstein, Fitzsimmons and Rolls 1970; Severs et al. 1970). Other neuropeptides that are generally associated with particular behaviors when centrally administered include luteinizing hormone-releasing

hormone (LHRH) with sexual receptivity (McCann and Moss 1975; Sakuma and Pfaff 1980), enkephalin with analgesia (Pert et al. 1976), cholecystokinin (CCK) with satiety (Della-Ferra and Baile 1979), and oxytocin with maternal behavior (Pedersen and Prange 1979). In addition, some of the behaviorally active drugs which are not themselves peptidic have been found to interact with receptors for endogenous neuropeptides, notably morphine and phencyclidine (PCP) with opiate receptors (Hughes et al. 1975; Zukin and Zukin 1981) and benzodiazepines with receptors for either CCK (Bradwejn and de Montminy 1984) or a novel endogenous peptide (Guidotti et al. 1983). The profound and specific behavioral effects of these peptides, coupled with their concentration in regions of the diencephalon which were known from earlier lesion and stimulation studies to play a key role in generating motivation and behaviors, led to the development of the hypothesis that neuropeptides may be key mediators of drives, motivations or behaviors (see Bloom 1972; Nicoll 1975; Barker 1976; Olds 1977; Moss 1977).

Although it is possible that some of the behavioral actions of neuropeptides are secondary to peripheral actions (see Iversen 1981), it is generally thought that neuropeptides act directly on central neurons to affect behavior. The specific hypothesis is that they function as central neurotransmitters (see Branton et al. 1978; Hokfelt 1980a; Snyder 1980), although some of their properties may differ from those of classical transmitters. The combined

power of immunocytochemistry, peptide biochemistry and molecular genetics has generated a prodigious amount of data about the identity and distribution of neuropeptides throughout the nervous system, and many of these neuropeptides have pharmacological actions on central neurons. However, it has not been possible to unequivocally demonstrate a transmitter role for any neuropeptide within the vertebrate central nervous system (CNS), and the experimental difficulties of vertebrate CNS preparations make it very problematic to satisfy the classical strict criteria for transmitter identification (see Werman 1966). In particular, it has not been possible to record from both the presynaptic and postsynaptic elements of any peptidergic interaction in the vertebrate CNS (Zieglgansberger 1980; Bloom 1987). In the relatively accessible spinal cord, there is considerable experimental support for transmitter roles for the neuropeptides substance P (see Otsuka and Yanagisawa 1988) and enkephlin (see Yaksh 1987).

There are a number of important functional questions about neuropeptides in the CNS, in addition to that of possible transmitter roles. For instance, neuropeptides are generally produced by cleavage of a large polyprotein precursor. The multiple peptides on the precursor may be covalently linked, as with insulin (Steiner et al. 1974), or tightly associated, as with the neurophysins (Russell, Brownstein and Gainer 1980) to form a single functional peptide. Alternatively, the precursor may yield several

independently bioactive neuropeptides, as with proopiomelanocortin (POMC) (Mains, Eipper and Ling 1977; see Douglass, Civelli and Herbert 1984). The appealing idea that these multiple neuropeptides may function coordinately as cotransmitters remains to be demonstrated experimentally in the vertebrate CNS. In addition to this coexistence of multiple peptide products of precursor polyproteins, peptides also frequently coexist with independently derived peptides or classical transmitter substances (Hokfelt et al. 1980b), raising further questions about possible functional interactions between cotransmitters (see O'Donohue et al. 1985; Hokfelt 1987). A second common feature of neuropeptide systems is a diversity of related peptides and their receptors. The best example of this is that of the endogenous opiate systems, in which three distinct genes yield at least 11 structurally related opiate peptides, which act on at least 4 receptor subtypes (reviewed in Akil et al. 1984). The implications of this diversity for the functions of neuropeptides in the vertebrate CNS are not clear. A third property of many neuropeptide systems is a wide distribution throughout the nervous system, in addition to the concentrations in classical neurosecretory regions. Even the cerebellum, which is often fancied to be a computer-like logical device, has been shown to contain immunoreactivity for a wide variety of neuropeptides, from cerebellin to corticotropin-releasing factor (CRF). It is an important question whether some of these distributed

peptide systems represent functionally cohesive groups, perhaps representing the integration of a single behavioral function, as opposed to independent neural centers which happen to use the same neuropeptide for unrelated functions (see Iversen, Nicoll and Vale 1978; Bloom 1984). A fourth property found in a subset of neuropeptide systems is episodic, pulsatile release (see Lincoln et al. 1985; Leng 1988). In the case of oxytocin, these episodic bursts of release are associated with the all-or-none behavioral events of parturition (Summerlee 1981; Fuchs et al. 1982), milk letdown (reviewed in Robinson 1986), and orgasm (Fox and Knaggs 1969; Murphy et al. 1987). The mechanism by which graded, continuous stimuli are transduced into pulsatile neurohormone release events is generally unknown.

The gap between the rapidly growing knowledge of the anatomical and biochemical properties of neuropeptides on the one hand, and the limited understanding of their functional significance in the CNS on the other, suggests the use of invertebrate model preparations (see Newmark 1983). Invertebrate preparations provide the experimental advantages of accessibility, hardiness, and apparent simplicity. The fundamental roles played by peptides appear to be strongly conserved (Niall 1982), indicating that the basic principles to be gleaned from studying these systems may have general applicability. Many classical studies on the physiological roles of hormones were carried out in invertebrates (see O'Shea and Schaffer 1985), especially

involving hormones from the corpora-cardiaca complex of insects, which has analogous functions to the vertebrate hypothalamus/neurohypophysis (reviewed in Truman and Riddiford 1974). The central nervous system of Aplysia, with its large, identified and often well-characterized neurons (see Kandel 1976), is particularly well suited for studies at the cellular level on the functions of neuropeptides. The abdominal ganglion of Aplysia contains many neurosecretory cells (Coggeshall 1967; Frazier et al. 1967), and these were used as model systems in a number of studies of peptide synthesis, processing and transport (reviewed in Gainer, Loh and Sarne 1977).

The neuroendocrine bag cells in the abdominal ganglion are the best characterized neuropeptide system in Aplysia, and the one with which much of this thesis deals. The bag cells were shown to contain a hormone that induces egg-laying behavior when injected into the hemocoel (Kupfermann 1967). This egg-laying hormone (ELH) was identified as a polypeptide (Arch, Early and Smock 1976) and its primary structure determined by Chiu et al. (1979).

The bag cells fire in episodic, all-or-none burst discharges, usually lasting 15-30 minutes (Kupfermann and Kandel 1970). These burst discharges precede all episodes of spontaneous egg-laying, and stimulation of the bag cells to discharge causes egg-laying (Pinsker and Dudek 1977). The bag cell burst discharge causes release of eggs from the ovotestis due to the hormonal actions of ELH, and initiates

the complex pattern of egg-laying behavior which lasts for hours.

Bag cell activation also has widespread effects on many target neurons in the abdominal ganglion, as shown in fig 1A (Mayeri et al. 1979a, 1979b). These central actions of the bag cells are thought to underly components of the egg-laying behavior. The neuronal responses were categorized into four types: slow inhibition, prolonged excitation, burst augmentation, and transient excitation, as illustrated in fig 1B (from Mayeri and Rothman 1982). ELH is the putative bag cell transmitter for the two effects of prolonged excitation and burst augmentation (Branton et al. 1978; Mayeri et al. 1985), but cannot account for the other effects, suggesting the bag cells use more than one transmitter. In addition, analogous with many vertebrate systems, ELH is expressed in other regions of the Aplysia CNS (Chiu and Strumwasser 1981), and a family of ELH genes encodes structurally related peptides which are expressed in other tissues (Scheller et al. 1982; Mahon et al. 1985).

The work reported here was undertaken to address several of the basic questions of the functions of neuropeptides in the central nervous system, using the bag cells and other model peptidergic systems in Aplysia. The general approach has been to study the electrophysiological properties of the presynaptic peptidergic neurons and the postsynaptic responses of target neurons, and to link these with each other and with their possible behavioral

functions. The motif is the exploration of the integrative roles of neuropeptides in the generation and modulation of behavior.

The beginning of modern science can be dated from the time when such general questions as "How was the universe created? What is matter made of? What is the essence of life?" were replaced by such limited questions as "How does a stone fall? How does water flow in a tube? How does blood circulate in vessels?" - Francois Jacob.

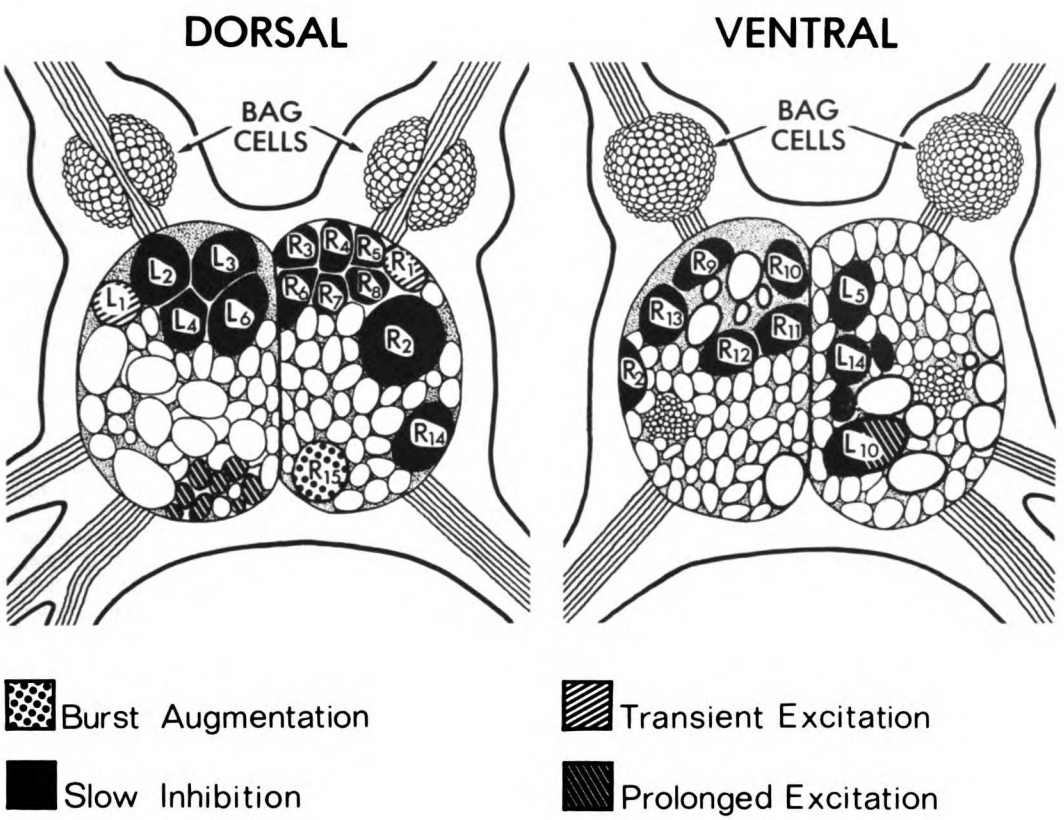
I am not interested in this or that phenomenon, in the spectrum of this or that element. I want to know His thoughts, the rest are details. - Albert Einstein.

Figure 1. Neuronal responses to bag cell burst discharges.

A. Schematic map of the abdominal ganglion shows the distribution of identified neuronal responses to bag cell burst discharges. (from Mayeri et al. 1979b)

B. Bag cell activity and the four major respones to bag cell activity in identified abdominal ganglion neurons. Records are aligned so that bag cell burst discharges begin at the arrow. Burst augmentation is shown in R15, prolonged excitation in an LC cell, prolonged inhibition in L6, and transient excitation in R1. (from Mayeri and Rothman 1982)

A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Burst Augmentation
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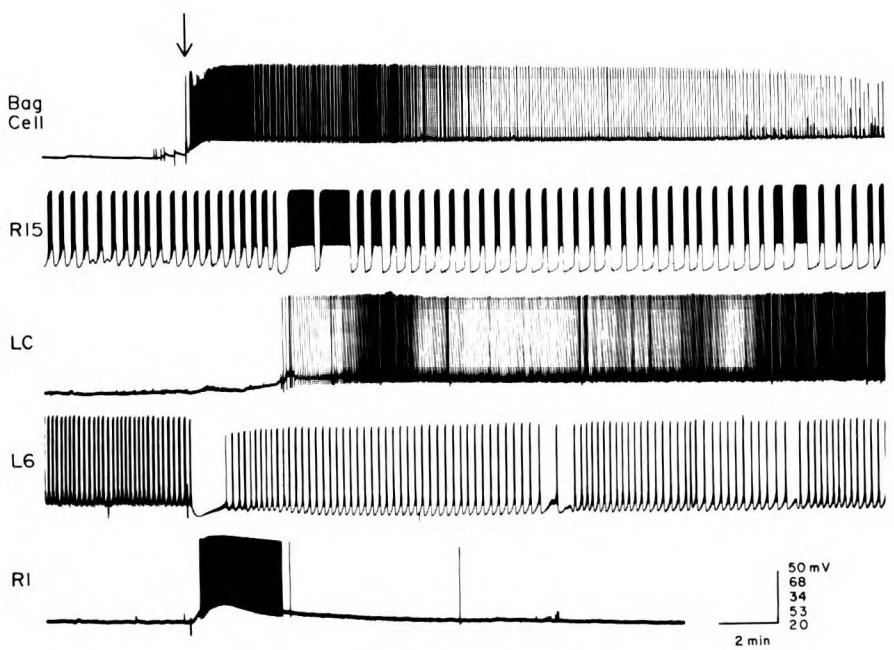
Transient Excitation
Prolonged Excitation

B


Bag Cell
R15
LC
L6
RI
50 mV
68
34
53
20
2 min

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Chapter 2

ALPHA-BAG CELL PEPTIDE AS A COTRANSMITTER IN THE BAG CELL NEURONS OF *APLYSIA*

SUMMARY

The neuroendocrine bag cells in the abdominal ganglion of Aplysia control the complex, hours-long behavior pattern of egg-laying. The bag cells fire in episodic, all-or-none burst discharges which cause egg release from the ovotestis as well as long lasting central actions on numerous target neurons in the abdominal ganglion. Egg-laying hormone (ELH) is an identified bag cell neuropeptide which acts as a hormone to produce egg release, and is also the putative transmitter for some of the bag cells' excitatory actions onto target neurons. However, many of the other neuronal responses seen following extracellular stimulation of the bag cells, including the inhibition of the left upper quadrant (LUQ) neurons L_{2-6}, are not produced by ELH.

The inhibition of LUQ neurons was produced by direct intracellular stimulation of individual bag cells. The LUQ neurons were also inhibited by the releasate collected in the presence of protease inhibitors during an elicited bag cell burst discharge, implicating a second bag cell peptide as the cotransmitter. Samples of HPLC fractionated bag cell extracts were assayed on LUQ neurons to identify candidate cotransmitters, and one peptide, named alpha-bag cell peptide (α-BCP), was identified with inhibitory actions. The inhibition was dose-dependent, and three fragments of the peptide, α-BCP(1-7), (1-8), and (1-9), had relative potencies of 10:30:1, respectively. Cross-desensitization

of the bag cell-induced inhibition with the α-BCP-induced inhibition was demonstrated, suggesting a common mechanism. Many other abdominal ganglion neurons were also inhibited by α-BCP, including the right upper quadrant neurons R_{3-13}, which have biphasic inhibitory-excitatory responses to the bag cells but only excitatory responses to ELH. The bag cells themselves were depolarized and activated by α-BCP, suggesting the peptide also has autoexcitatory functions. These results, in combination with other data, indicate that α-BCP and ELH act as cotransmitters to mediate the central actions of the bag cells.

INTRODUCTION

Until recently, one of the working hypotheses of neurobiologists was the parsimonious idea that each neuron secretes only one neurotransmitter. This notion, often (mistakenly) called 'Dale's Principle', has been largely discredited in recent years by a wealth of anatomical and biochemical evidence that many neurons contain multiple biologically active substances that are putative neurotransmitters (Hokfelt et al. 1980, O'Donohue et al. 1985). This is particularly true for peptidergic neurons, which typically synthesize large polyprotein precursors that are subsequently processed to yield several neuropeptide products. For instance, adrenocorticotropic hormone (ACTH), β-endorphin, and β-lipotropic hormone (β-LPH) are biologically active products of the proopiomelanocortin (POMC) precursor which are colocalized to individual hypothalamic neurons (Watson et al. 1978).

In contrast to the anatomical and biochemical evidence, the physiological evidence for cotransmission has been difficult to come by. Probably the strongest cases for cotransmission have been made for acetylcholine and vasoactive intestinal polypeptide (VIP) in the cat submandibular gland (Lundberg et al. 1981a,b) and for acetylcholine and a luteinizing hormone-releasing hormone-(LHRH-) like peptide in the frog sympathetic ganglion (Jan and Jan 1983). It has not been possible to establish similar physiological evidence for cotransmission in the

central nervous systems of vertebrates. And while it is widely believed that the different neuropeptide products derived from common precursors may have coordinate actions on targets (Jacquet 1979; Weid 1982; Sirinathsinghji et al. 1983;), in no case has it been established that they actually function as cotransmitters.

The central nervous system of *Aplysia californica*, with its large, accessible identified cells, is experimentally convenient for physiological, biochemical, and anatomical studies at the cellular level (Frazier et al. 1967). Studies in *Aplysia* have helped elucidate some of the basic principles of central nervous function (Kandel 1976). The neuroendocrine bag cells in the abdominal ganglion of *Aplysia*, which control egg-laying, are a model system for studying central peptidergic neurotransmission (reviewed in Mayeri and Rothman 1985a). Bag cells fire episodic burst discharges which produce multiple actions on identified target neurons in the abdominal ganglion. These actions include prolonged excitation of LB and LC cells, prolonged burst augmentation in R_{15}, transient excitation of R_1 and L_1, and inhibition of a large number of neurons, including the left upper quadrant (LUQ) neurons L_{2-6} (Mayeri et al. 1979a,b). Egg-laying hormone (ELH), a bag cell neuropeptide which acts hormonally to causes egg release from the ovotestis (Chiu et al. 1979; Rothman et al. 1983b), is also the putative transmitter mediating the first two long-lasting excitatory actions of the bag cells (Branton et al.

1978b, Mayeri et al. 1985b). However, ELH does not produce the other bag cell effects, suggesting that the bag cells use a cotransmitter in addition to ELH to mediate its central actions. This work identifies alpha-bag cell peptide (α-BCP) as a candidate for this cotransmitter, and provides additional physiological evidence for such a function.

MATERIALS AND METHODS

Preparations

Abdominal ganglia were dissected from large (400+ g) sexually mature *Aplysia californica* that were collected by our laboratory from Elkhorn Slough in Monterey Bay, CA or obtained from Sea Life Supply (Sand City, CA) or Pacific Biomarine (Venice, CA). In most cases animals were anesthetized by injection of isotonic $MgCl_2$ (1/3 of body weight) 30 min prior to dissection. Ganglia were pinned onto a Sylgard-lined 1 ml capacity chamber under 1/3 $MgCl_2$, 2/3 bathing medium (consisting of seawater buffered to pH 7.6 with HEPES plus 250 mg/L dextrose). After pinning, the ganglion was continuously superfused with bathing medium for the duration of the experiment at a rate of 30 ml/hr.

Electrophysiology

Standard elelctrophysiological techniques were used as previously described (Mayeri et al. 1979; Mayeri et al. 1985). Intracellular recordings were made from up to four neurons simultaneously using glass microelectrodes filled with 1.5 M potassium acetate. In most experiments the electrodes were inserted through the connective tissue sheath overlying the ganglion by tapping. An extracellular electrode was placed over one or both bag cell clusters and used for recording and stimulating bag cell activity. Experiments were performed at room temperature, which was in

the range of 19-24°C, and usually did not vary nore than 1°C over the course of an indiviual experiment.

Application of peptides

The caudal artery of the abdominal ganglion was cannulated with a short piece (10 cm) of drawn polyethylene tubing (PE 260) held steadily in place by a clamp attached to the recording table. A 100-200 cm length of polyethylene tubing (PE 20) with a drawn tip that made a tight friction fit into the cannula was attached at the other end to a Gilmont micrometer syringe. The microsyringe and the PE 20 tubing were filled with distilled H_2O. Peptide solutions (usually 10 µl) were drawn into the smaller tubing through the tip, separated from the distilled H_2O by a small (1 µl) air bubble. The tip was inserted into the cannula, and the peptide solution was perfused into the ganglion by turning the syringe at a rate of 3 µl/min.

Serial perfusion

In serial perfusion experiments, two ganglia were used as described in Mayeri et al. (1985b) to study the neuronal effects of bag cell releasate. One ganglion, the source ganglion, was placed in a small sealed chamber (approximate volume 200 µl). The caudal artery of the ganglion was cannulated as described above with a piece of drawn PE-20 tubing attached to the chamber, and two stainless steel electrodes inside the chamber were placed over the bag cell

clusters for recording and stimulating bag cell activity. This ganglion was continuously perfused and superfused (7 µl/min each) with bathing medium containing protease inhibitors. The second ganglion, the assay ganglion, was pinned in the standard recording chamber as in the other experiments, except that the cannula was continuously perfused with the outflow from the assay ganglion chamber (total flow 14 µl/min). A bag cell burst discharge was elicited in the source ganglion, and intracellular recordings in the assay ganglion demonstrated the responses to materials released from the source ganglion during the bag cell burst discharge.

Peptides and protease inhibitors

The native bag cell peptides were purified from abdominal ganglion extracts by Barry Rothman and coworkers in our laboratory as described (Rothman et al. 1983; Sigvardt et al. 1986). The synthetic peptides were synthesized by Peninsula laboratories. Peptides were made up in solutions of filtered (0.22 µm pore size) bathing medium plus protease inhibitors. The standard cocktail of protease inhibitors used included 250 µg/ml each of bacitracin (Sigma), hen egg-white trypsin inhibitor (Sigma), lima bean trypsin inhibitor (Sigma), and ovomucoid (Worthington); 100 µg/ml each of antipain (Peninsula), leupeptin (Peninsula), and phenylalanylalanine (Vega); and 25 µg/ml of angiotensin converting enzyme inhibitor

(Peninsula). Some of the earlier experiments did not include the last four of these protease inhibitors.

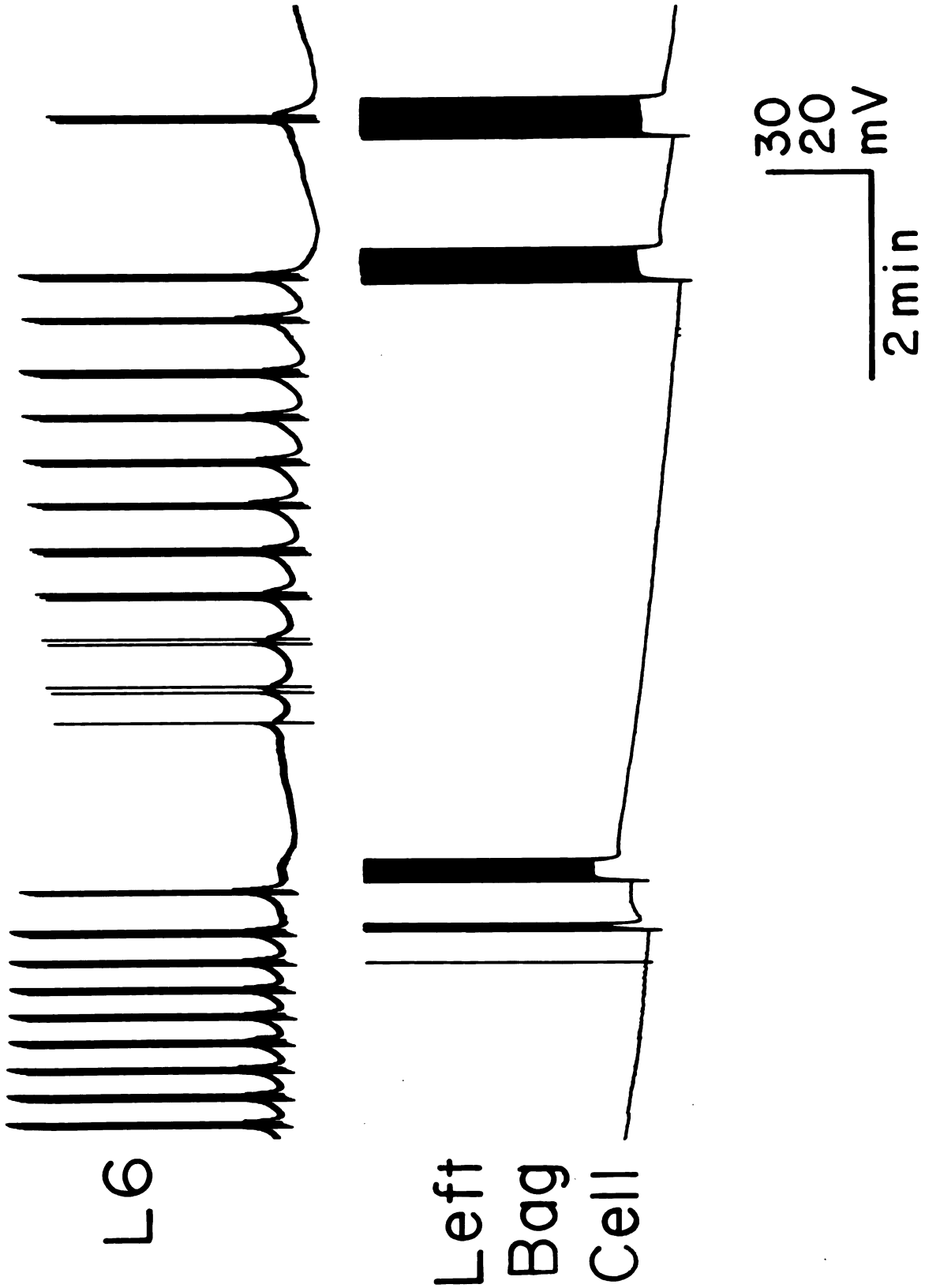
RESULTS

Inhibition of LUQ neurons by direct bag cell stimulation

The LUQ neurons are strongly inhibited when bag cell burst discharges are elicited by brief focal extracellular stimulation of a bag cell cluster or of regions containing concentrations of bag cell processes (Mayeri et al. 1979a). While the stimulation parameters in these experiments were carefully adjusted to minimize the possible stimulation of neuronal processes other than bag cells, this possibility still allowed for uncertainty that the bag cell burst discharges were causing the inhibitory responses, and this uncertainty was heightened by the finding that the known bag cell peptide ELH did not mimic this inhibition. To directly demonstrate that the bag cells were causing inhibition of the LUQ neurons, simultaneous intracellular recordings were made from a left bag cell and an LUQ cell. As shown in Fig 1, the direct stimulation of bag cell spikes by intracellularly injected depolarizing current pulses (50 msec pulses at 5/sec) produced hyperpolarization and a cessation of bursting in the LUQ cell. In the experiment shown in Fig 1, the bag cell stimulation did not lead to a prolonged bag cell afterdischarge, and the inhibition of the LUQ neuron was relatively brief. The stimulation of this bag cell probably caused spiking in only a subpopulation of the bag cell cluster (perhaps only in the stimulated cell), and only bag cells located near the target neuron seemed effective in producing inhibition.

Figure 1 Inhibition of LUQ neuron by direct stimulation of a bag cell.

Simultaneous intracellular recordings were made from L_6 and a left bag cell located near L_6. Activation of spikes in the bag cell by injection of trains of depolarizing current pulses (bars) produced inhibition of L_6.


L6
Left
Bag
Cell
30
20
mV
2 min

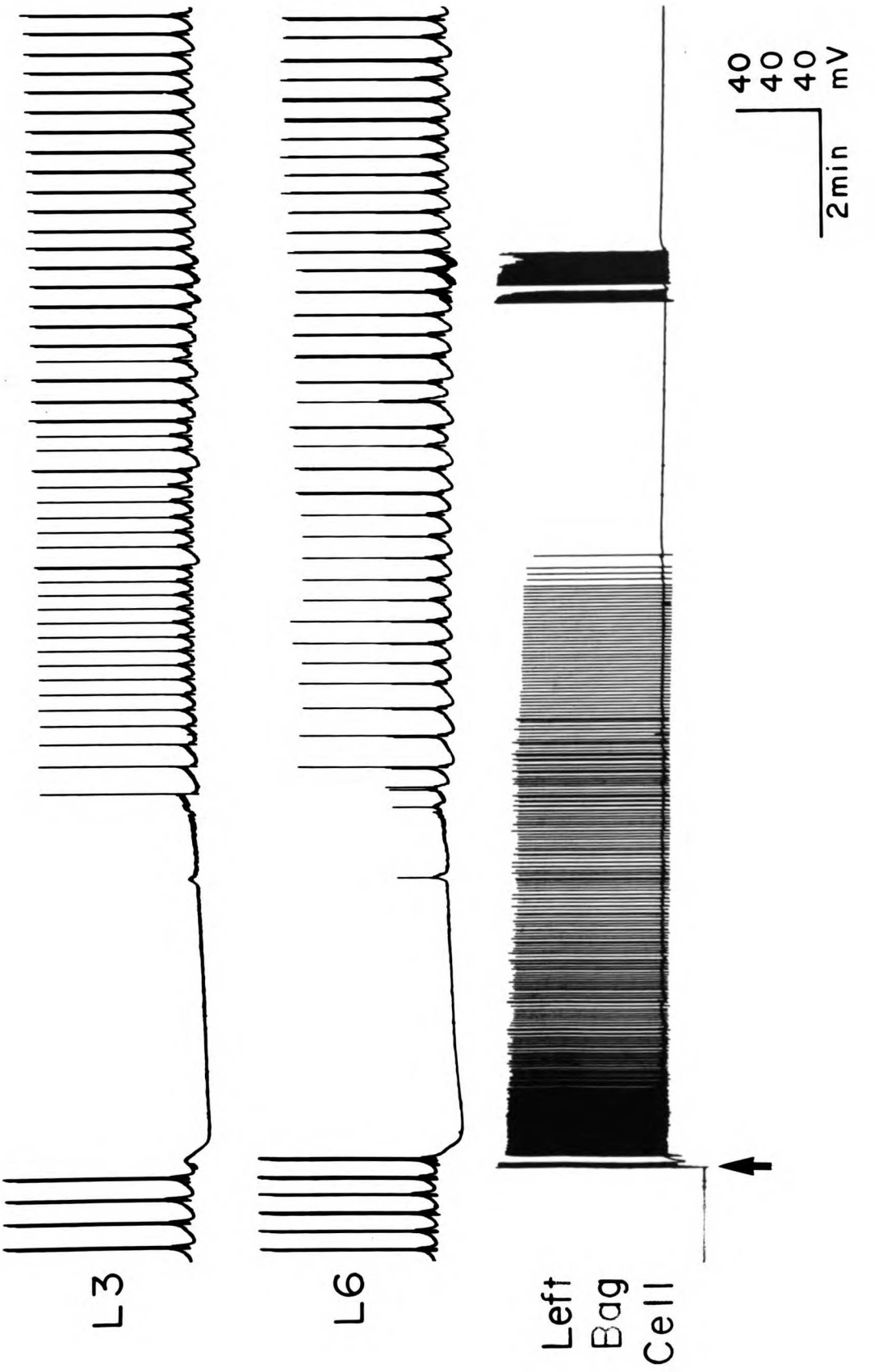
In other experiments, direct intracellular stimulation of bag cells did initiate long-lasting, synchronous afterdischarges in the entire population of bag cells. One of these directly elicited afterdischarges is shown in Fig 2. The bag cell activity in this case produced a more powerful, longer-lasting inhibition of the LUQ neuron, as was previously described for extracellularly stimulated bag cell burst discharges. Other responses described for extracellularly stimulated bag cell burst discharges, including burst augmentation in R15, long-lasting excitation of LLQ cells, and transient excitation of L1 and R1, were also verified with intracellularly stimulated bag cell burst discharges. These experiments demonstrate that the responses were caused by activity in the bag cells, rather than by nonspecific activation of other neuronal processes. These experiments by themselves do not indicate whether these were direct effects of bag cell transmitters on the target cells, or indirect, polysynaptic effects.

Release of inhibitory activity during bag cell burst

The central actions of the bag cells are presumed to be due to neuropeptides released during burst discharges. The bag cells are thought to act on the target neurons through nonsynaptic (or parasynaptic) transmission, in which transmitters diffuse relatively long distances to their targets from diffuse release sites in the ganglion (Branton et al. 1978a; Mayeri et al. 1985b). Thus releasate

Figure 2 Inhibition of LUQ neuron by bag cell burst discharge.

Simultaneous recordings were made from L_6 and a left bag cell. A brief train of depolarizing current pulses injected into the bag cell (arrow) initiated a bag cell burst discharge, with synchronous spike activity throughout the bag cell cluster. This directly elicited discharge produced a strong inhibition in L_6. Minutes after the discharge ended, further stimulation of the bag cell had no effect on L_6.

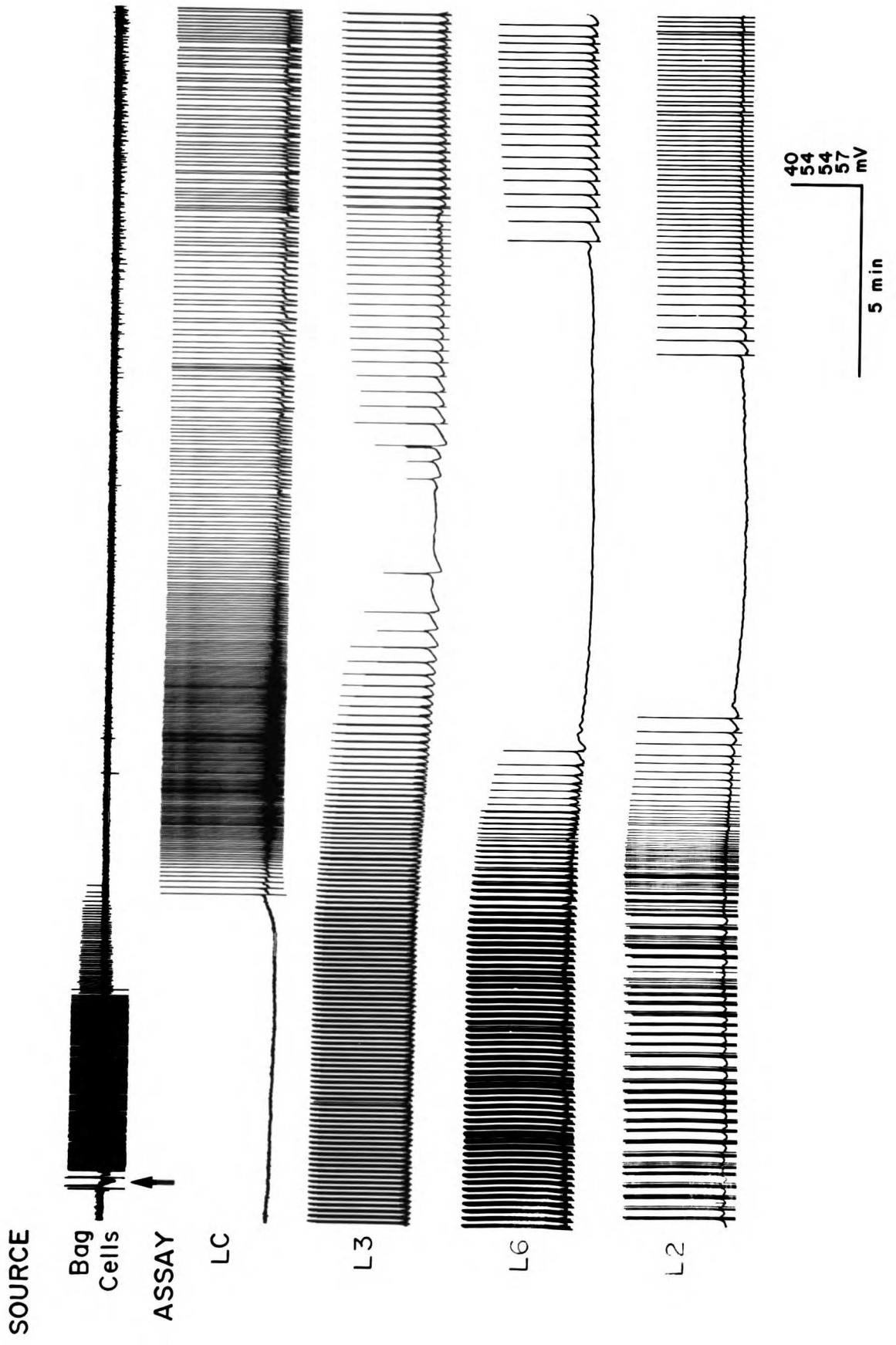

L3
L6
Left
Bag
Cell
40
40
40
mV
2min

collected during bag cell activity might be expected to contain these neuropeptides, and to have neuronal effects mimicking those produced by the bag cells. In an earlier study (Mayeri et al. 1985b), it was demonstrated that bag cell releasate produced the excitatory responses mediated by ELH, but did not cause the transient excitation of L_1 and R_1, nor the inhibitory responses in LUQ and other neurons. However, no protease inhibitors were used in that study, and it was possible that ELH, a blood-borne hormone, was more resistant to proteolysis after release than the other bag cell peptides.

These experiments were repeated using a cocktail of protease inhibitors to protect released peptides against degradation. The serial perfusion apparatus described in Mayeri et al. (1985b) and in METHODS was used, in which the outflow from one (source) ganglion in a sealed chamber is perfused directly into a second (assay) ganglion. Intracellular recordings from four neurons in the assay ganglion displayed steady activity during a 60 min control period. A bag cell burst discharge was elicited by extracellular stimulation of the bag cells in the assay ganglion. After a 6 min delay (due largely to the transit time in the plumbing between the ganglia), the 3 LUQ neurons in the assay ganglion were inhibited and the LC cell was excited (Fig 3). In conjunction with the earlier study, this suggests that the bag cell inhibition of the LUQ neurons is mediated by a second bag cell peptide that is

Figure 3 Inhibition of LUQ neurons by bag cell releasate

The source abdominal ganglion was placed in a small sealed chamber with extracellular electrodes on the bag cell clusters. The assay abdominal ganglion was placed in an open chamber, and intracellular recordings were made from 3 LUQ neurons (L_3, L_6, and L_2) and 1 LC neuron. The source ganglion was perfused and superfused with seawater containing protease inhibitors, and the outflow was directly perfused into the assay ganglion. Brief electrical stimulation of the bag cells in the source ganglion (arrow) initiated a bag cell burst discharge (top trace). After a delay, this caused an inhibition of the LUQ neurons, and excitation of the LC neuron, in the assay ganglion.


SOURCE
Bag Cells
ASSAY
LC
L3
L6
L2
40
54
54
57
mV
5 min

more susceptible to proteolytic inactivation than ELH. The releasate also contained excitatory activity on L_1 and R_1 (data not shown).

Identification of alpha-bag cell peptide

To identify candidate cotransmitters for the mediation of LUQ inhibition, peptides fractionated by Barry Rothman and coworkers in our laboratory were perfused into the abdominal ganglion while intracellular recordings were made from 1-4 LUQ neurons, assaying for inhibitory effects similar to those produced by bag cell burst discharges. Initially, bag cell proteins were separated by size into three fractions: low (0-2 kDa), medium (2-12 kDa), and high (>12 kDa) molecular weight. The medium molecular weight fraction contained ELH, and had strong excitatory effects on R_{15} and LB and LC cells, but little or no inhibitory activity on LUQ neurons. The inhibitory activity was almost entirely within the low molecular weight fraction, which was then separated by reverse phase HPLC into 14 resolveable peaks. Two of these peaks had potent inhibitory activity, mimicking the early, minutes-long inhibitory response of all five LUQ neurons to bag cell burst discharges. They did not mimic the second, hours-long inhibitory phase which occurs only in L_3 and L_6, and which had previously been shown to involve a distinct conductance mechanism from the first phase (Brownell and Mayeri 1979), nor did they mimic the transient excitation of L_1 and R_1. The larger of these 2

peaks was purified to homogeneity, and sequenced by Pau-Miau Yuan and John E. Shively at City of Hope. The sequenced peptide was a seven residue peptide which was named alpha-bag cell peptide (α-BCP(1-7)). This peptide was synthesized, and the synthetic peptide had identical inhibitory activity with the fraction containing the native peptide, as shown in Fig 4. Fig 4 also shows that the second active fraction, which was subsequently identified as α-BCP(1-8), was somewhat more potent than the native and synthetic α-BCP(1-7). The responses to α-BCP were dose-dependent, as shown in Fig 5, and thresholds (for detectable inhibitory responses) were generally around 100 nM for α-BCP(1-7). Native and synthetic α-BCP(1-7) had virtually identical dose-response relationships, while α-BCP(1-8) was approximately 3 times as potent. α-BCP also inhibited LUQ neurons when it was pressure ejected locally onto individual LUQ somata in a desheathed preparation, indicating it acts directly on receptors located on these target cells. Thus α-BCP was identified as a candidate bag cell cotransmitter.

Subsequent to these findings, the gene encoding ELH was cloned and sequenced, and the precursor protein was found to contain a nine residue predicted cleavage product with the sequence corresponding to carboxyl-terminal extended α-BCP (Scheller et al. 1983). The nonapeptide α-BCP(1-9) was also synthesized, and found to be about one-thirtieth as potent as α-BCP(1-8) in inhibiting LUQ neurons. In more recent

Figure 4 Inhibitory actions of α-BCP on LUQ neuron.

Intracellular recording was made from L_3 while 3.2 μM solutions of α-BCP with protease inhibitors were perfused into the abdominal ganglion. The three traces show responses of the same cell to successive applications of purified native α-BCP(1-7), synthetic α-BCP(1-7), and purified native α-BCP(1-8). The native and synthetic forms of α-BCP(1-7) had identical effects, while α-BCP(1-8) was more potent.

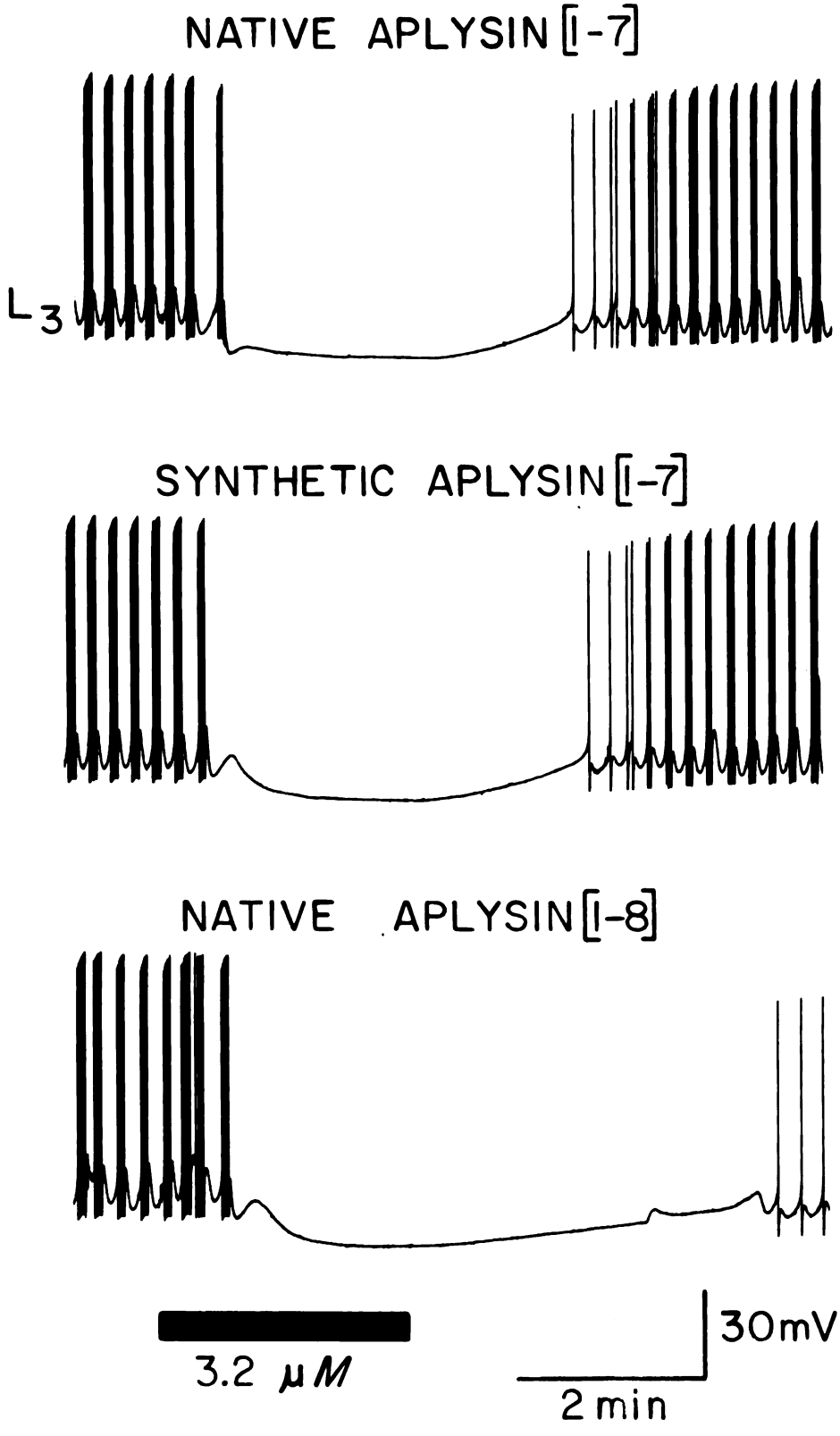
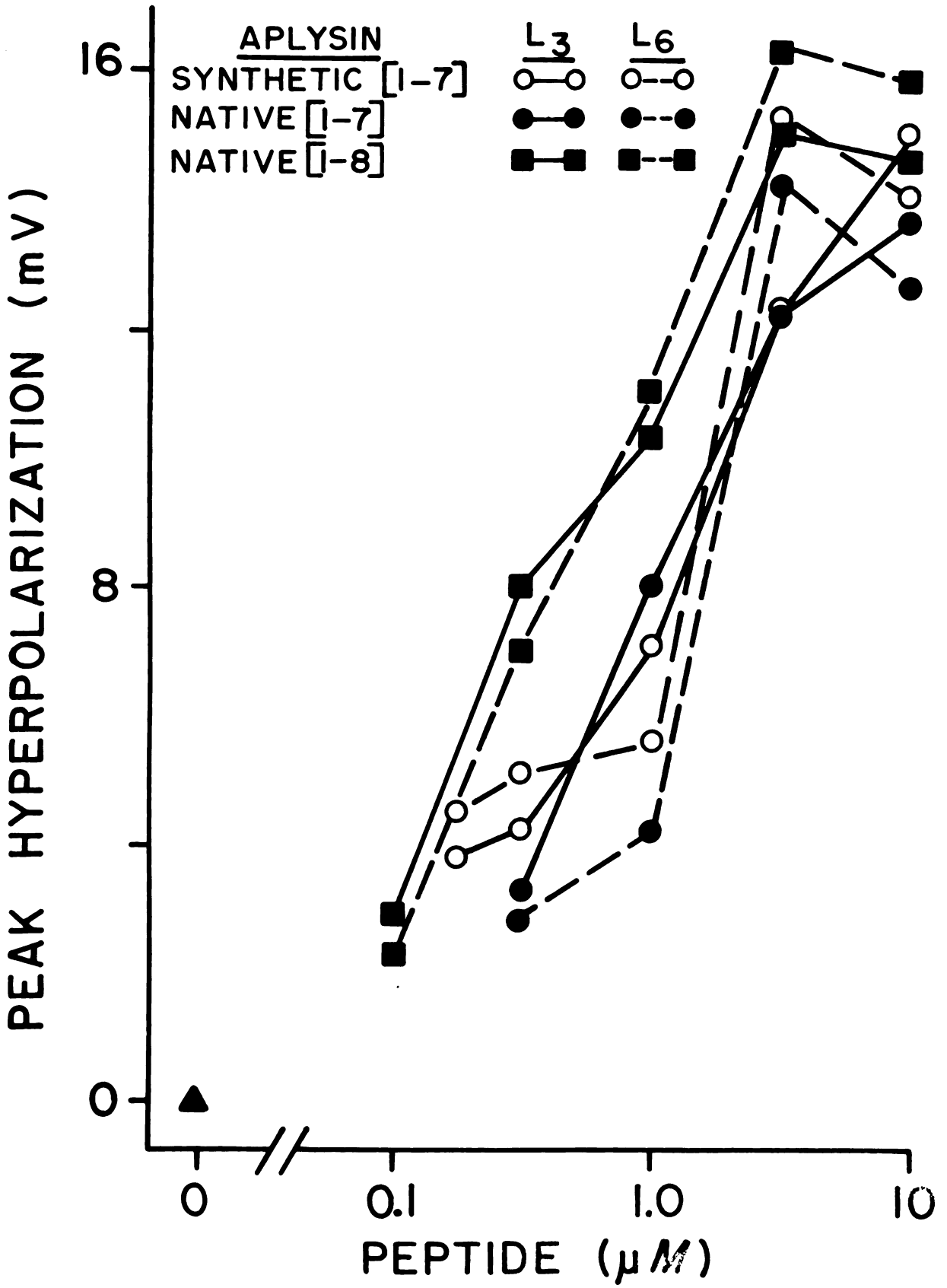

NATIVE APLYSIN [1-7]
L3
SYNTHETIC APLYSIN [1-7]
NATIVE APLYSIN [1-8]
3.2 μM
30mV
2 min

Figure 5 Dose-Response relationships for native and synthetic α-BCP.

The inhibition of LUQ neurons L_3 and L_6, measured by the peak hyperpolarization during interburst intervals, is compared for purified native α-BCP(1-7) [filled circles], synthetic α-BCP(1-7)[open circles], and purified native α-BCP(1-8)[filled squares], all in the presence of protease inhibitors. The dose-response curves for native and synthetic α-BCP(1-7) were nearly superimposed, while the curve for α-BCP(1-8) was shifted to the left, representing about 3 times greater potency. Data are from the same preparation as Fig 4. The triangle represents the average response to control application of protease inhibitors. Every other point represents a single trial on either L_3 (connected by solid lines) or L_6 (connected by dashed lines).


APLYSIN
SYNTHETIC [1-7]
NATIVE [1-7]
NATIVE [1-8]
L3
L6
16
8
0
PEAK HYPERPOLARIZATION (mV)
0
0.1
1.0
10
PEPTIDE (μM)

purifications of bag cell extracts in which proteolysis was more thoroughly controlled, only α-BCP(1-8) and α-BCP(1-9) are found, suggesting that these are the naturally occuring forms, and that α-BCP(1-7) was a fragment caused by proteolysis during the purification procedure. α-BCP(1-8) seems to be a naturally occuring form, suggesting a novel carboxypeptidase processing step that converts a fraction of the α-BCP(1-9) into this more potent fragment (Pulst et al. 1987) However, many of the physiological studies were done with α-BCP(1-7) since that was the form initially available. The results obtained with α-BCP(1-7) have been confirmed for α-BCP(1-8) and α-BCP(1-9), with differences in the potencies as discussed but no qualitative differences.

Cross-desensitization

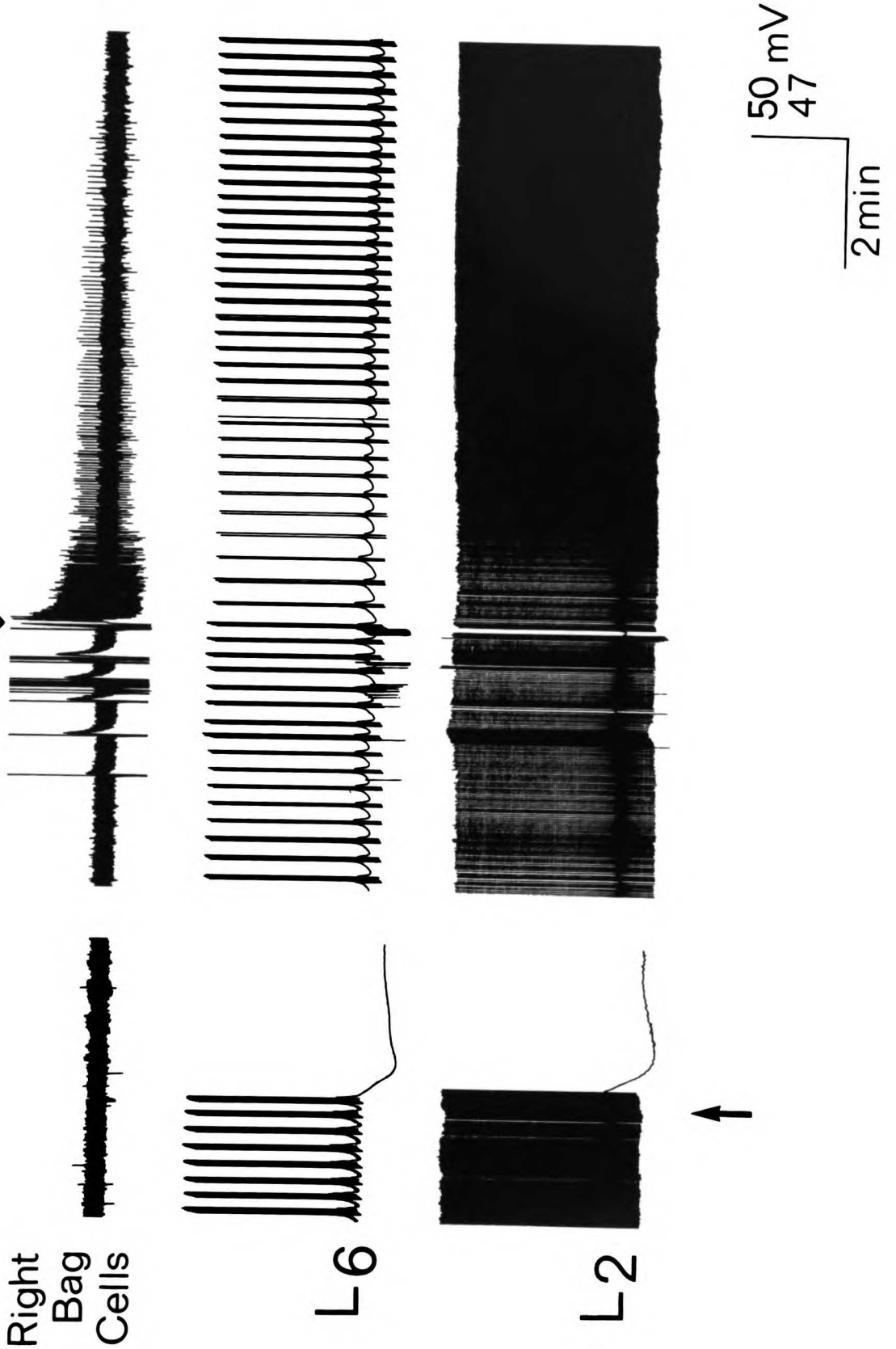
The response of the LUQ neurons to α-BCP could be desensitized by prolonged perfusions of high (>10^{-4} M) concentrations. If the bag cell inhibition of the LUQ neurons is mediated by α-BCP, then this inhibition should be absent when the response to α-BCP is desensitized. This was tested as shown in Fig 6. Perfusion of 1 mM α-BCP(1-7) (beginning at arrow) initially caused a very strong hyperpolarization of two LUQ cells (left side of Fig 6). After 25 min of continued perfusion, the response had almost

Figure 6 Cross desensitization of bag cell-induced inhibition with α-BCP.

Simultaneous intracellular recordings were made from the LUQ neurons L_6 and L_2, with an extracellular recording from the right bag cells.

Left: Continuos perfusion of the ganglion with 1 μM a-BCP began at the arrow, producing an initial strong hyperpolarizing response which soon began desensitizing.

Right: After 25 min of continuous perfusion, the firing patterns in the LUQ neurons were nearly back to baseline, indicating desensitization to α-BCP. With the perfusion continuing, an electrically stimulated bag cell burst discharge (upper arrow) was elicited. In contrast to the usual strong inhibition, this burst discharge produced only a very weak inhibition of L_6, and a slight excitation of L_2.


Right
Bag
Cells
L6
L2
50 mV
47
2 min

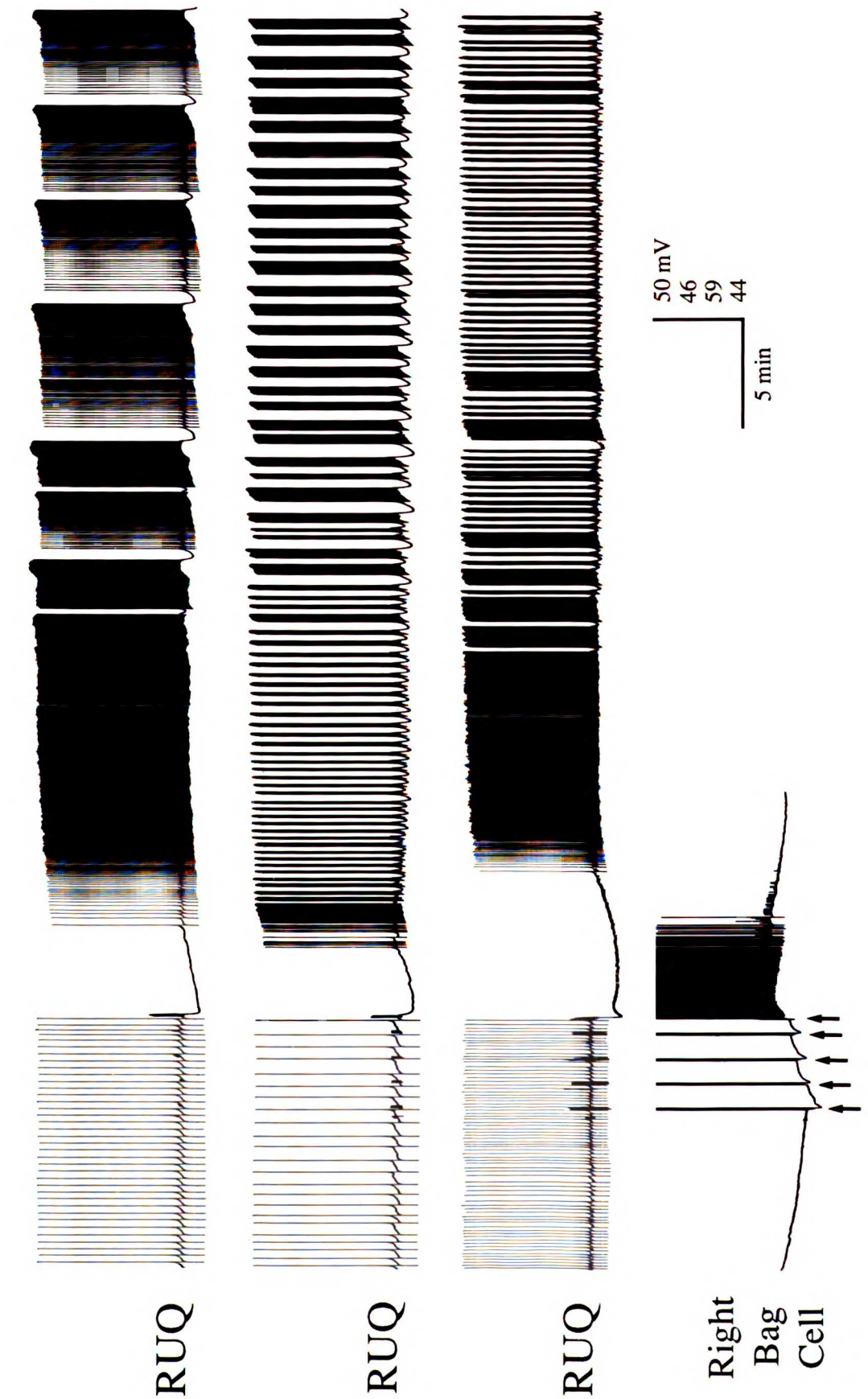
completely desensitized, and the LUQ neurons were near their original levels of activity (right side of Fig 6). A bag cell burst discharge was initiated by extacellular stimulation (top arrow). This produced little or no inhibition of the LUQ cells, suggesting that the bag cell transmitter that normally inhibits LUQ neurons cross-desensitized with α-BCP, and thus may act at the same receptors.

Biphasic inhibition-excitation in RUQ neurons

In contrast to the bag cell-induced inhibition of the LUQ neurons L_{2-6}, R_2, L_{14}, and other target neurons, and the excitation of L_1, R_1, R_{15}, and the LB and LC neurons, a biphasic inhibitory-excitatory response usually occurs in the cardiac pacemaker neuron L_{10} and in the right upper quadrant (RUQ) neurons R_{3-13} (Mayeri et al. 1979b). The biphasic response, consisting of an early transient inhibition followed by a prolonged excitation (and often a transition to bursting activity) is shown in three simultaneously recorded RUQ neurons in Fig 7. This was the most common response seen in RUQ neurons, but there was more variability in this response than in most of the other reported responses; this may in part reflect heterogeneity in the RUQ neurons. Because the RUQ neurons send very many processes to the sheath around the bag cells, it was especially important to determine that this was a response to the bag cells, and not due to nonspecific extracellular

Figure 7 Biphasic inhibitory-excitatory response of right upper quadrant (RUQ) neurons to bag cells.

Simultaneous recordings were made from 3 RUQ neurons and 1 right bag cell. A bag cell burst discharge was elicited by extracellular stimulation of the left bag cell cluster (arrow). The RUQ response consisted of an initial inhibition lasting several minutes, followed by prolonged excitation and a transition to a bursting firing pattern that lasted for over 1 hour.

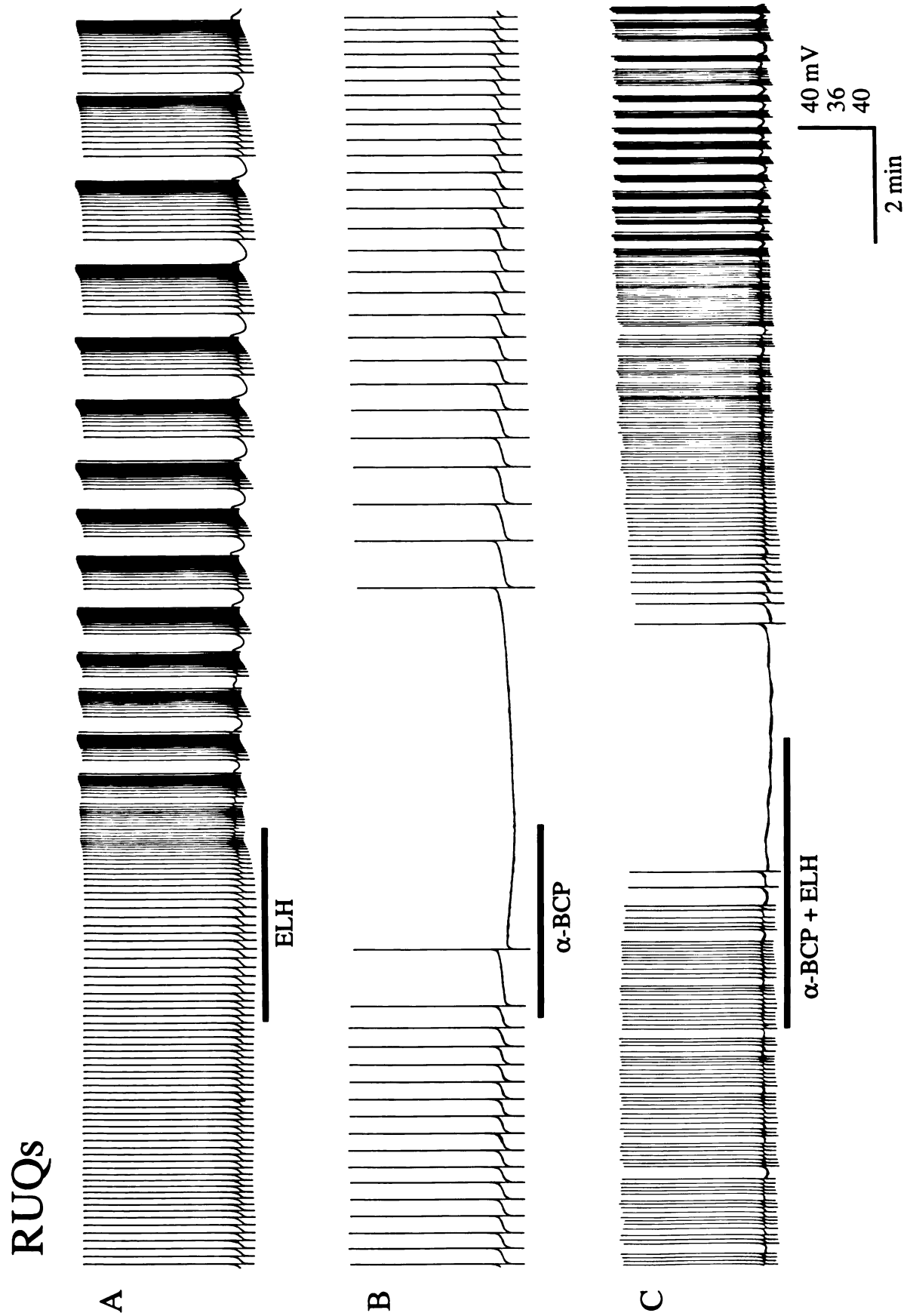

RUQ
RUQ
RUQ
Right Bag Cell
50 mV
46
59
44
5 min

stimulation. The biphasic response was also seen when the bag cells were stimulated directly with intracellular pulses. α-BCP produces only a transient inhibition of RUQcells (Fig 8A), while ELH produces only a prolonged excitation (Fig 8B). Mixtures of α-BCP and ELH produced biphasic responses which qualitatively mimicked the response to the bag cells (Fig 8C). The concentrations of peptides needed to mimic bag cell effects were higher on the RUQ neurons than on other target neurons, with the threshold for ELH being approximately 100 times that needed on the LLQs. However, the density of RUQ processes in the vicinity of the bag cell clusters (Frazier et al. 1967) makes it plausible that they are exposed to such high concentrations under physiological conditions during bag cell burst discharges. These results suggest that the biphasic response in the RUQ cells is mediated by both ELH and α-BCP functioning as cotransmitters onto common target neurons. This may also be the case for the biphasic response in L_{10}. L_{10} is inhibited by α-BCP, but the possible role of ELH in mediating the excitatory phase was difficult to address, in part because of the normal variability and slow excitatory trend in L_{10}'s spike activity (Mayeri et al. 1979b), and also because L_{10} is particularly sensitive to the pressure artifacts caused by arterial perfusion (Furgal and Brownell 1987).

Figure 8 α-BCP and ELH mimic separate components of the biphasic response in RUQ neurons.

A. Transient inhibitory response of an RUQ neuron to arterial perfusion of 1 μM α-BCP(1-8). There was no excitation following the inhibitory response.
B. Long-lasting excitatory response produced by arterial perfusion of 20 μM ELH. There was no initial inhibitory response.
C. Biphasic inhibitory-excitatory response to simultaneous arterial perfusion of 1 μM α-BCP (1-8) and 10 μM ELH.

The three responses shown are taken from three different preparations. Voltage calibration scale is for the three different cells; all traces have the same time scale.


RUQs
A
ELH
B
α-BCP
C
α-BCP + ELH
40 mV
36
40
2 min

Activity of related peptides

The inhibitory activity of α-BCP was compared with that of several related peptides (Table 1). The three forms of α-BCP had relative potencies of 10:30:1 for the (1-7), (1-8), and (1-9) forms, respectively. After release, α-BCP(1-9) and (1-8) are apparently degraded by both carboxypeptidases and post-proline diaminopeptidases, yielding the fragments α-BCP(1-8), (3-9), (1-7), and (3-8) (Rothman et al. 1987). The cleavage of the amino terminal residues alanylprolyine from α-BCP did not significantly alter the inhibitory activity when assayed in the presence of protease inhibitors, although it conceivably may affect the stability of the molecules in their natural milieu.

The two pentapeptides β-BCP and γ-BCP are also derived from the bag cell precursor protein and contain α-BCP(3-6) in their sequences at positions (1-4) (Scheller et al. 1983). Of these, β-BCP had very weak inhibitory effects, and γ-BCP had no apparent inhibitory activity. Peptides closely related to α-BCP are also encoded by other members of the ELH/BCP gene family, which are expressed in the atrial gland (Mahon et al. 1985). Atrial gland peptides A and B are biologically active and can initiate bag cell burst discharges when injected into *Aplysia* or applied to the head ganglia (Heller et al. 1980), but they had no inhibitory activity on LUQ neurons nor direct excitatory activity on the bag cells. (In contrast, the atrial gland ELH-like peptides, called califins, mimicked the excitatory

Table 1. Relative potencies of α-BCP and related peptides

PEPTIDE	SEQUENCE*	POTENCY
α-BCP(1-9)	APRLRFYSL	1
α-BCP(1-8)	APRLRFYS	30
α-BCP(1-7)	APRLRFY	10
α-BCP(3-9)	RLRFYSL	1
α-BCP(3-8)	RLRFYS	20
β-BCP	RLRFH	0.05
γ-BCP	RLRFD	<0.01
Peptide A	AVKLSSDGNYPFDLSKEDGAQPYFMTPRLRFYPIa	<0.01
Peptide B	AVKSSSYEKYPFDLSKEDGAQPYFMTPRLRFYPIa	<0.01
Somatostatin-25	SNPAMAPRERKAGCKNFFWKTFTSC	<0.01
PO_4 Acceptor	RGYSLG	<0.01

*Standard one-letter notation for amino acid sequences of IUPAC/IUB Commission on Biochemical Nomenclature, 1968

effects of ELH on LC neurons (Rothman et al. 1986). Two commercially available peptides, the synthetic phosphate acceptor peptide RGYSLG and somatostatin 25, each include sequences with 4 out of 5 amino acids in common with α-BCP, but neither had inhibitory activity on the LUQ neurons. No simple necessary and sufficient sequence emerged from these studies. Neither residues (1-2) nor (8-9) were necessary for activity, suggesting that α-BCP(3-7) contains the biologically active core of the peptide. However, when embedded in the atrial gland peptides A and B, α-BCP(2-7) is inactive, suggesting some dependence on context (or accessibility).

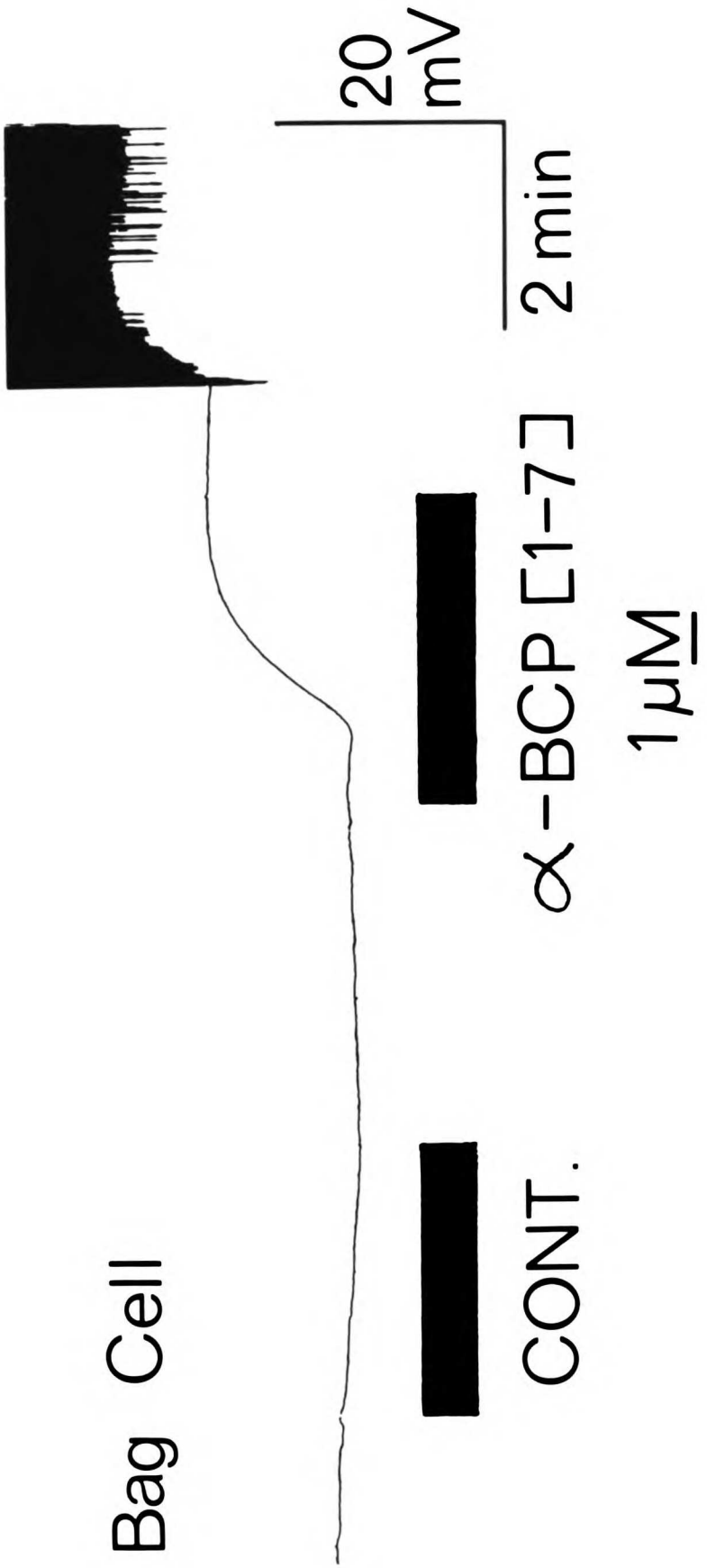
Bag cell excitation by a-BCP

While α-BCP had inhibitory effects on LUQ, RUQ, and many other neurons in the abdominal ganglion, it also had excitatory effects on the bag cells. In Fig 9, perfusion of 1 μM α-BCP(1-7) depolarized a bag cell and initiated a bag cell burst discharge. In 10 experiments in which intracellular recordings were made from bag cells, perfusion of 1 μM α-BCP depolarized the bag cells by 6.2 ± 1.1 mV (mean ± S.E.M.). Application of varying concentrations of α-BCP (0.5 μM to 1 mM) initiated bag cell burst discharges within 5 min of application in 10 of 32 preparations. In 3 of 6 serial perfusion experiments in which protease inhibitors were used, a bag cell burst discharge occurred in the assay ganglion within 3 minutes of the time released bag

cell peptides reached the assay chamber (as timed from the onset of inhibition in LUQ neurons in the assay ganglion). Thus α-BCP apparently has autoexcitatory effects on the bag cells which may provide positive feedback that would contribute to the regenerative burst discharge. The variability in the responses of bag cells to α-BCP may reflect the observed variability in the excitability of the bag cells. Further investigations of the autoexcitatory actions of α-BCP and their function in the burst discharge are taken up in Chapter 3.

Figure 9 Excitation of a bag cell by α-BCP

Intracellular recording was made from a left bag cell. 1 μM α-BCP(1-7) was arterially perfused into the abdominal ganglion. α-BCP produced a slow depolarization of the bag cell. This depolarization eventually reached threshold for a regenerative burst discharge, accompanied by a further depolarization in the bag cell. This suggests that α-BCP may function as an excitatory autotransmitter.


20 mV
2 min
Bag Cell
CONT.
α-BCP [1-7]
1 μM

DISCUSSION

Evidence that α-BCP is a bag cell neurotransmitter

These results, in combination with other data, provide strong evidence that α-BCP is the bag cell neurotransmitter mediating inhibition of the LUQ neurons. α-BCP meets most of the criteria needed to establish it as a neurotransmitter: **(1) Synthesis** α-BCP was the most potent inhibitory fraction purified from bag cell extracts. α-BCP incorporated radiolabel when isolated bag cell clusters were incubated in tritiated amino acids, indicating it was synthesized by bag cell somata (Rothman et al. 1983). α-BCP is encoded on the ELH/BCP gene (Scheller et al. 1983) which is specifically expressed in the bag cells (McAllister et al. 1984). **(2) Presence at release sites** α-BCP-like immunoreactivity is found in bag cell processes and beaded varicosities (representing presumed release sites), including those in close proximity to the LUQ neurons (Pulst et al. 1986). α-BCP immunoreactivity is primarily localized to dense core vesicles in the bag cells (Kreiner et al. 1986). **(3) Release** In a set of release experiments in collaboration with Barry Rothman, Karen Sigvardt, Susan Kansky, and others in the laboratory, material collected from source ganglia during bag cell bursts was fractionated on HPLC, yielding peaks of α-BCP(1-8) and (1-9) (Sigvardt et al. 1986). The amounts recovered in the releasate per bag cell burst were approximately 50 and 7 times as much (respectively) as required to mimic the neural inhibition

when arterially perfused. This fulfills the important, but often overlooked, requirement that the quantity of transmitter released is sufficient to account for the biological effect. **(4) Mimicry** α-BCP inhibits all the identified targets for bag cell inhibition. This is presumably a direct effect on receptors located on target cells, including somatic receptors, since inhibitory responses were seen to local pressure ejection of α-BCP directly onto LUQ somata (Rothman et al. 1983a; Sigvardt et al. 1986; Jansen and Mayeri 1986). α-BCP and the bag cell-induced inhibition of LUQ neurons have apparently identical effects on the conductance (Sigvardt et al. 1986) and specific ionic currents (Jansen and Mayeri 1986) of LUQ cells. **(5) Pharmacology** No antagonists have yet been identified for α-BCP. However, evidence that α-BCP and the endogenous bag cell transmitter may act on the same receptors was obtained from the cross-desensitization of the bag cell-induced inhibition with α-BCP. **(6) Inactivation** α-BCP is quickly inactivated after release by proteases in the abdominal ganglion (Sigvardt et al. 1986; Rothman et al. 1987).

Significance of peptidergic cotransmisison

Previous data established ELH as a putative bag cell neurotransmitter mediating the excitation of R_{15} and LB and LC neurons in the abdominal ganglion (Mayeri et al. 1985b). The finding that α-BCP acts as a second bag cell

neurotransmitter, mediating inhibition of the LUQ and other abdominal ganglion neurons, indicates that the bag cells use two neuropeptides derived from a common precursor as cotransmitters. While most of the responses studied are apparently mediated by either α-BCP or ELH, the biphasic inhibitory/excitatory response of the RUQ neurons may involve the conjoint actions of both peptides.

The demonstration that different neuropeptide products derived from a common precursor act in parallel to mediate different components of the central actions of neuroendocrine cells provides one possible explanation for the widespread occurrence of polyprotein precursors. In this case, the peptides not only have opposite "signs" to their actions (inhibitory and excitatory), they also have different resistances to proteolysis, resulting in different spreads of the peptides through time and space. α-BCP has relatively transient actions on neuronal targets within a short distance from release sites, while ELH diffuses widely throughout the animal, with long-lasting actions on peripheral as well as central targets (see Mayeri et al. 1985b).

It is noteworthy that although 90% of the α-BCP recovered from either bag cell extracts or bag cell releasate is in the (1-9) form (Sigvardt et al. 1986; Pulst et al. 1987), the remaining 10% which is in the (1-8) form would account for approximately 75% of the total inhibitory activity, since it is 30 times as potent. This sensitivity

of the total inhibitory activity to the relatively small fraction of the α-BCP pool in the (1-8) form suggests that this ratio may be subject to regulation, either by posttranslational processing in the vesicles or by extracellular peptidases acting on released α-BCP(1-9). The bag cell system is presumably constrained to produce α-BCP and ELH in equimolar stoichiometry, since are synthesized on a common precursor polyprotein, unlike other cotransmitter systems in which the transmitters are independently synthesized. The regulation of the relative activities of α-BCP and ELH by the alternate processing of α-BCP into fragments with different potencies may serve to introduce this type of flexibilty.

There remain two identified bag cell responses which are not produced by either α-BCP or ELH, such as the transient excitation of L_1 and R_1, and the prolonged phase of inhibition in L_3 and L_6, suggesting that additional bag cell transmitters are also involved. These may be some of the other peptides encoded on the ELH/BCP precursor. The complex pattern of egg-laying behavior may require the coordinate actions of most or all of the multiple neuropeptides on the ELH/BCP precursor, each acting to mediate some components of the physiology and behavior. The encoding of all these peptides on a single gene has been suggested to serve to ensure the coordinate evolution and regulation of these many components of a single instinctive behavior (Scheller et al. 1983).

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Chapter 3

POSITIVE FEEDBACK BY AUTOEXCITATORY NEUROPEPTIDES IN NEUROENDOCRINE BAG CELLS OF *APLYSIA*

SUMMARY

Neurohormones are often secreted in large amounts from neuroendocrine cells during episodes of synchronous, repetitive spike activity. We report evidence that this pattern of activity in the neuroendocrine bag cells of *Aplysia* involves positive feedback by autoexcitatory transmitters. Intracellular stimulation of individual bag cells caused slow depolarizing afterpotentials and synchronous afterdischarges in the entire population of bag cells. Application of the bathing medium collected during bag cell activity mimicked these responses. Application of α-, β-, or γ- bag cell peptides, three structurally related neuropeptides which are released from bag cells, also mimicked these responses. These autoexcitatory bag cell peptides fulfill most of the strict criteria necessary for classification as neurotransmitters in this system. This is the first biological activity reported for β- and γ- bag cell peptides, and brings to 4 the number of bag cell neuropeptides derived from the ELH/BCP precursor which are putative co-transmitters. Positive feedback by autoexcitatory transmission may provide a general mechanism for the generation of episodic activity in neuroendocrine systems.

INTRODUCTION

Neuroendocrine systems regulate physiology and behavior by releasing neurohormones which enter the circulation to act on distant tissues. To meet this functional requirement, neuroendocrine cells must secrete large amounts of chemical messengers, and this secretion often occurs in discrete episodes. In a variety of invertebrate and vertebrate systems such pulsatile neurohormone release has been associated with coordinated bursts of action potentials in neuroendocrine cells (Kupfermann and Kandel 1970; Vlieger et al. 1980; Copenhaver and Truman 1986; Lincoln et al. 1985). However, the neuronal mechanisms underlying this pattern of activity are not well understood.

The bag cells are neuroendocrine cells in the marine mollusk Aplysia which release multiple neuropeptides to control egg-laying behavior (reviewed by Mayeri and Rothman 1985). The bag cells are an apparently homogeneous group of neurons which comprise 2 clusters of approximately 400 neurons each at the rostral end of the abdominal ganglion. They are normally electrically silent, but episodically fire synchronous, repetitive burst discharges (also termed afterdischarges) which typically last 15-30 minutes. In intact animals, each bag cell burst discharge is associated with egg release from the ovotestis and the accompanying stereotyped egg-laying behavior. During burst discharges, the bag cells release large amounts of neuropeptides derived from a common precursor, including egg-laying hormone (ELH),

and α-, β-, and γ- bag cell peptides (BCPs). ELH functions both as a neurohormone to cause egg release from the ovotestis (Rothman, Weir, and Dudek 1983) and as an excitatory transmitter onto various target neurons (Mayeri et al. 1985). α-BCP is an inhibitory transmitter onto various other target neurons (Rothman et al. 1983; Sigvardt et al. 1986). β- and γ- BCPs are structurally related to α-BCP, but do not share its inhibitory activity (Sigvardt et al. 1986).

In the isolated nervous system, the bag cells can be activated by a variety of electrical or chemical stimuli (Kupfermann and Kandel 1970; Dudek and Blankenship 1977; Haskins and Blankenship 1979; Heller et al. 1980; Strumwasser, Kaczmarek, and Jennings 1982; Rothman et al. 1983). The characteristics of the resulting burst discharges seem independent of the type and intensity of the stimulus, and are essentially the same as those of the spontaneous burst discharges recorded from bag cells <u>in vivo</u> (Pinsker and Dudek 1977). This suggests that the stereotyped burst discharge is an intrinsic property of the bag cells.

Many of the bag cell processes wrap around the pleuroabdominal connectives to form dense neuritic cuffs just rostral to each bag cell cluster (Frazier et al. 1967). This neuritic cuff is the site at which bag cell activity is normally initiated (Haskins and Blankenship 1979), and is capable of supporting normal burst discharges even when

dissected away from the bag cell somata (Kaczmarek, Jennings and Strumwasser 1978). Electrotonic connections between the bag cell processes in this cuff are thought to synchronize the firing of the bag cells (Kupfermann and Kandel 1970; Dudek and Blankenship 1977). Bag cell processes in the cuff label very heavily for immunoreactive ELH and α-BCP (Chiu and Strumwasser 1981; Pulst et al. 1986; Painter et al. 1986), suggesting that very high local concentrations of bag cell peptides may be reached here after release during burst discharges. Thus the critical interactions underlying the generation of bag cell burst discharges are likely to occur in this neuritic cuff.

The intrinsic all-or-none pattern of bag cell activity, and the explosive acceleration of firing rate in the initial stage of burst discharges, suggest the involvement of positive feedback. α-BCP was previously shown to have excitatory effects on the bag cells (Rothman et al. 1983). We now report evidence that α-, β-, and γ- BCP's all function as autoexcitatory transmitters to provide positive feedback in this system. It is suggested that the BCPs released during the initial spikes of bag cell activity act on bag cell autoreceptors to produce depolarizing potentials and further spike activity, contributing to the explosive, all-or-none bag cell burst discharge.

Some of this work was previously presented in abstract form (Brown and Mayeri 1986).

MATERIALS AND METHODS

Electrophysiology

Abdominal ganglia were dissected from large (400-1200 g), sexually mature specimens of Aplysia californica, and pinned out in a 1 ml capacity chamber lined with Sylgard resin (Dow-Corning). The preparation was continuously superfused (30 ml/hr) with seawater containing 10 mM HEPES buffer (pH 7.6) and 250 mg/L dextrose. All experiments were performed at room temperature (19-23°C). Animals were anesthetized by injection 30 min prior to dissection with isotonic $MgCl_2$ (118 gm $MgCl_2 \cdot 6H_2O$ added to 1L H_2O; amount injected equal to 1/3 of body weight). Ganglia were pinned under 2/3 seawater, 1/3 isotonic $MgCl_2$, then washed with normal seawater for at least 1 hour prior to the experiments. The ganglionic sheath was left intact for all experiments except when otherwise specified. Intracellular recordings were made from up to four neurons at a time through potassium acetate (1.5 M) filled glass microelectrodes. The details of the electrophysiology and arterial perfusion of peptides have been previously published (Mayeri et al. 1985). Peptides were applied with protease inhibitors by perfusion through the caudal artery at a flow rate of 3 μL/min.

In tests for direct action of the peptides, isolated and desheathed bag cell clusters were bathed in high divalent cation artificial seawater containing (in mM):

NaCl, 317; KCl, 10; $CaCl_2$, 33; $MgCl_2$, 165; with 250 mg/L dextrose and buffered at pH 7.6 with 10 mM HEPES. These ionic concentrations were identical to those used to eliminate interneuronal input in the abdominal ganglion by Simmons and Koester (1986).

Serial Perfusion

The serial perfusion experiments were conducted as described previously (Mayeri et al. 1985; Sigvardt et al. 1986). Briefly, an abdominal ganglion was placed in a small sealed source chamber with extracellular electrodes placed for stimulating and recording bag cell activity. The source abdominal ganglion was continuously superfused and perfused with seawater containing a battery of protease inhibitors. A second ganglion was placed in the assay chamber for intracellular and extracellular recordings. The assay ganglion was continuously perfused with the outflow from the source ganglion. The methods of the serial perfusion experiments are further shown in Fig 3.

Peptides and Protease Inhibitors

All the synthetic bag cell peptides used in these experiments were synthesized by Peninsula Laboratories. The protease inhibitors were 250 µg/ml each of bacitracin (Sigma), hen egg-white trypsin inhibitor (Sigma), lima bean trypsin inhibitor (Sigma), ovomucoid (Worthington), and 100 µg/ml each of phenylalanylalanine (Vega), leupeptin

RESULTS

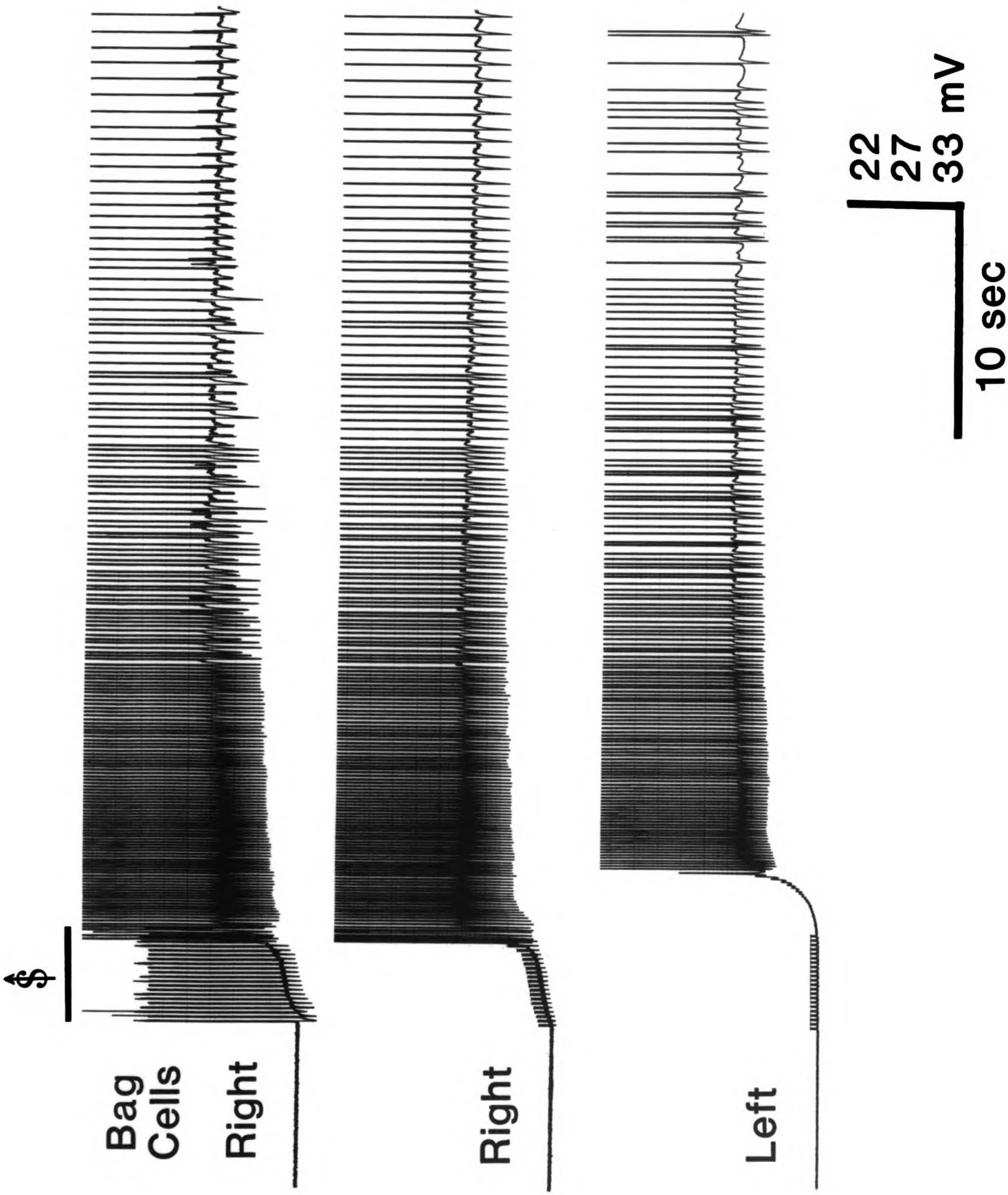
Positive feedback in the bag cells

Intracellular recordings were made from bag cells in isolated abdominal ganglia from large, sexually mature specimens of Aplysia californica. Figure 1 shows a typical bag cell burst discharge initiated by the injection of a short (4 sec) train of depolarizing current pulses into a single bag cell (top trace). The stimulated spike activity in this cell produced a slow depolarization of the ipsilateral bag cells (top and middle traces), leading to a synchronous ipsilateral burst discharge. This led after a delay to depolarization and activation of the contralateral bag cells (bottom trace). The duration of this bilateral discharge was 18 minutes. Such intracellular stimulation produced full burst discharges in 16 of 35 preparations, though several trains of pulses were often necessary. The bag cells within each cluster fired highly synchronous action potentials (upper 2 traces), and the two clusters were usually nearly synchronous during the early part of the burst discharge. This synchronization of action potentials during bursts is very likely mediated by electrotonic connections in the bag cell cuff neurites (Kupfermann and Kandel 1970; Dudek and Blankenship 1977).

The slow depolarizations produced by intracellular stimulation of a bag cell are seen more clearly in Fig 2. Here intracellular stimulation of a single bag cell produced slow depolarizing afterpotentials in the ipsilateral bag

Figure 1 Positive feedback in bag cells.

Bag cell discharge produced by intracellular electrical stimulation of an individual bag cell. Simulataneous intracellular recordings were made from two right and one left bag cells using standard electrophysiological techniques as previously described (Mayeri et al. 1985). The right bag cell in the top trace was stimulated with a 4 second train of depolarizing pulses (bar; 10^{-7} A, 50 msec pulses, 5 Hz) to cause spiking. This caused a depolarization and discharge of the right bag cell cluster and, subsequently, of the left bag cell cluster. The figure shows the first 40 sec of the discharge, which lasted 18 min.


Bag Cells
Right
Right
Left
22
27
33 mV
10 sec

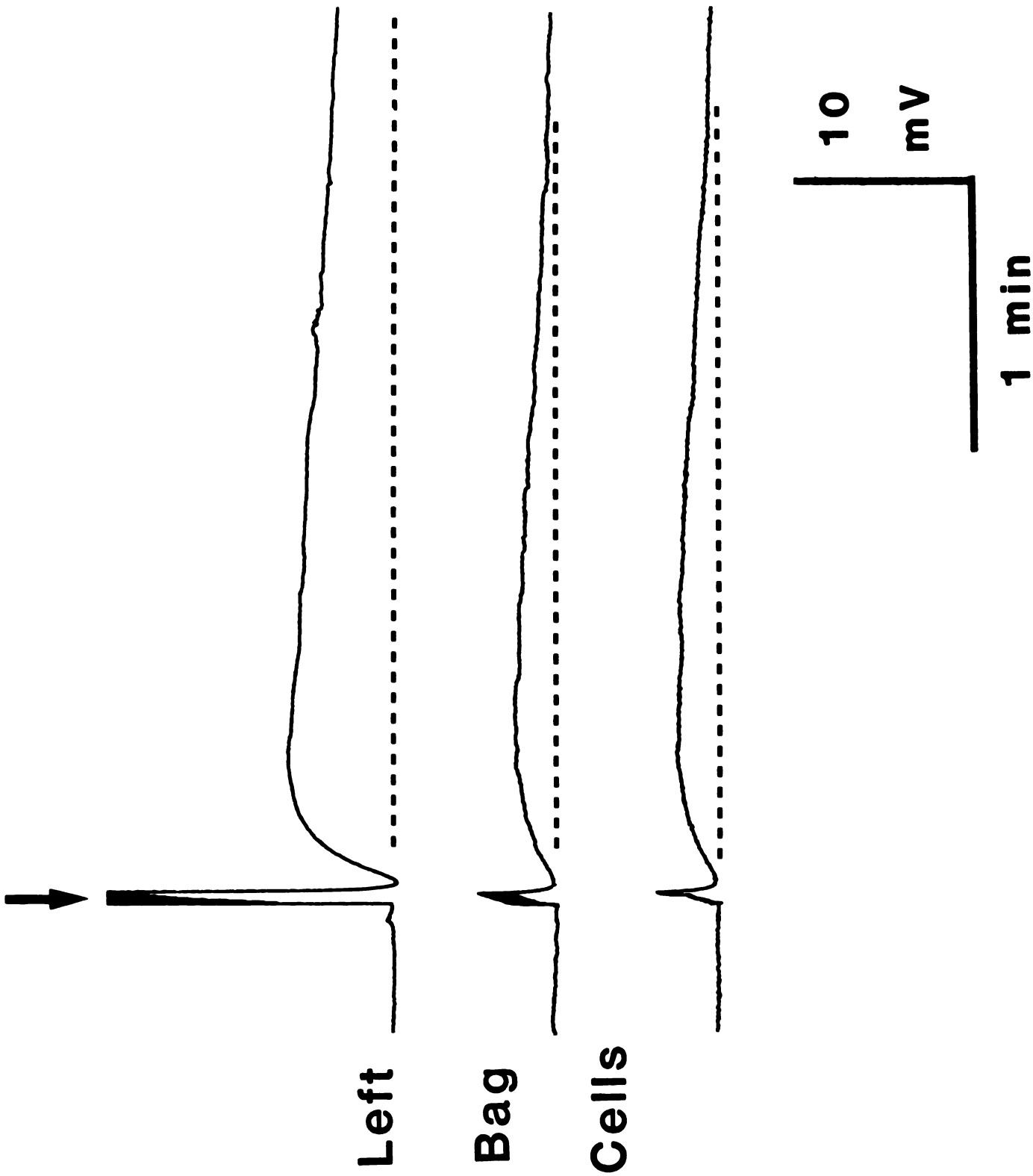
cells which was subthreshold to elicit a regenerative afterdischarge. These slow depolarizations typically rose to a peak in 20-60 sec, and lasted for up to several minutes. The slow depolarizations and prolonged burst discharges caused by brief intracellular stimulation of individual bag cells indicate a positive feedback mechanism underlies bag cell activation.

Release of bag cell excitatory factors during bag cell burst discharges

One possible positive feedback mechanism is autoexcitatory transmission by bag cell transmitters acting on bag cell autoreceptors (initially proposed by Kupfermann and Kandel 1970). Previous experiments designed to test this hypothetical mechanism have yielded negative results (Kupfermann and Kandel 1970; Strumwasser, Kaczmarek, and Jennings 1982). We used the serial perfusion apparatus shown in Fig 3A to investigate whether factors with excitatory actions on bag cells are released during bag cell burst discharges. An assay ganglion was continuously perfused with the outflow from a small, sealed chamber containing a source ganglion. A bag cell discharge was initiated in the source ganglion by a brief extracellular stimulation, and the substances released from the source ganglion during the discharge entered the flow into the assay ganglion. After a delay due to the transit time of

Figure 2 Depolarizing afterpotentials caused by bag cell stimulation.

Depolarizing afterpotentials were produced by intracellular electrical stimulation of individual bag cells. Intracellular recordings were made from three left bag cells. The bag cell in the top trace was stimulated as in Fig 1 to cause spiking (arrow), but the stimulation was not sufficient to initiate an afterdischarge. Following the stimulation, slow depolarizing afterpotentials developed which reached peaks of 6 mV in the stimulated cell and 2.5 mV in the other bag cells.


Left
Bag
Cells
10
mV
1 min

fluid in the plumbing between the ganglia, the bag cells in the assay ganglion began depolarizing, and eventually reached threshold for a regenerative discharge (Fig 3B). Bag cell depolarizations of 3-12 mV were seen in 5 of 6 such serial perfusion experiments, and led to burst discharges in 3 of them. In a previous set of serial perfusion experiments with only extracellular bag cell recordings, afterdischarges also occurred in 3 of 6 experiments (Sigvardt et al. 1986). These results provide evidence that chemical factors released during bag cell burst discharges have autoexcitatory effects on bag cells.

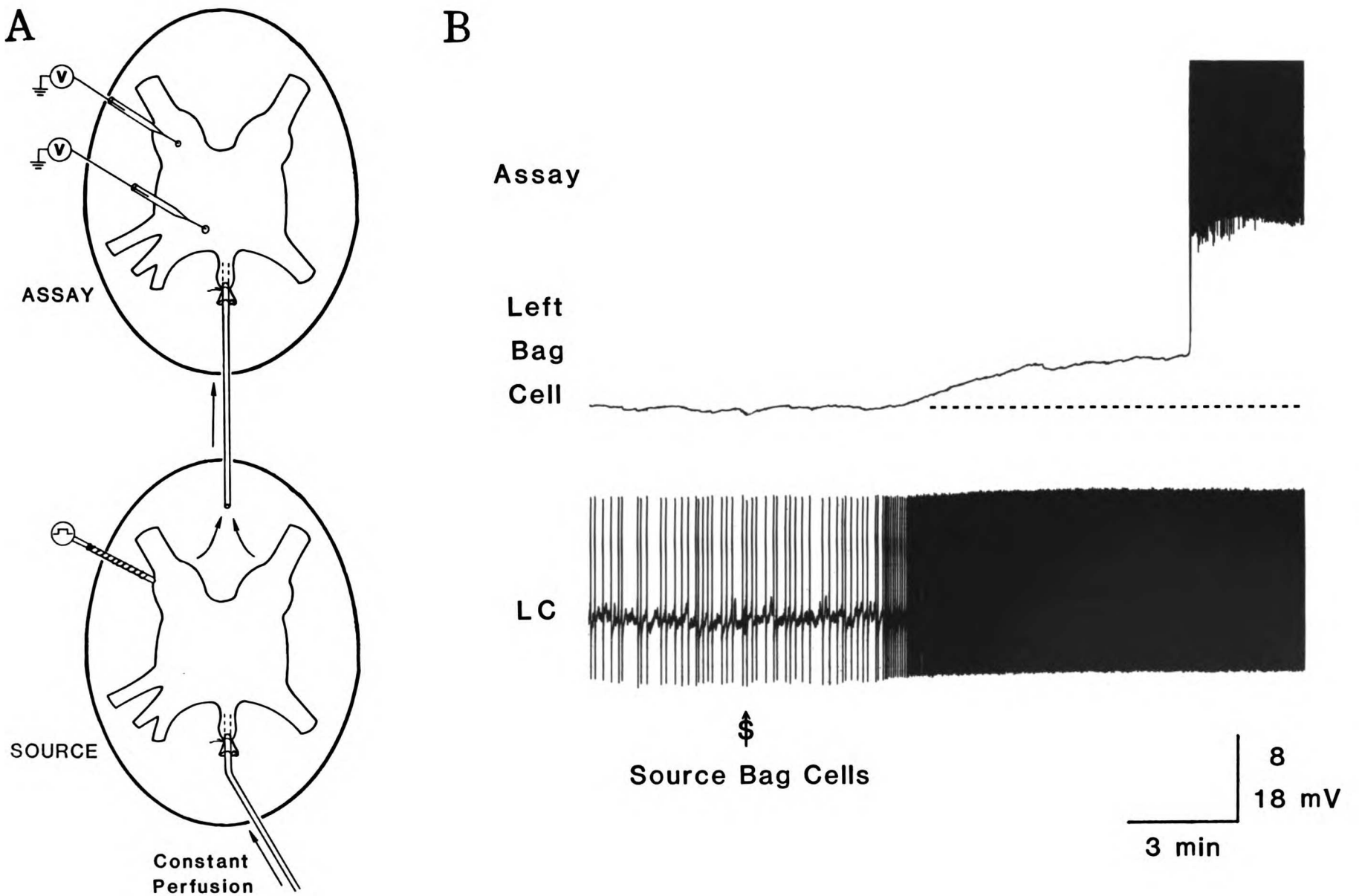
Autoexcitatory effects of α-, β-, and γ-bag cell peptides

We found that the structurally related neuropeptides α-, β-, and γ-BCP all have excitatory actions on bag cells. Fig 4A-1 shows the slow depolarization of a bag cell produced by arterial perfusion of α-BCP(1-9), the predominant released form of α-BCP (Sigvardt et al. 1986). All 3 neuroactive forms of α-BCP, (1-7), (1-8), and (1-9), were approximately equipotent on the bag cells. Arterial perfusion of 8 μL of 10^{-6} M α-BCP produced bag cell depolarizations of 2-16 mV in 28 of 32 preparations (combined data for the three forms of α-BCP). β-BCP had similar effects (Fig 4A-2) and was approximately equipotent with α-BCP. γ-BCP also depolarized bag cells (Fig 4A-3), but was less potent than α- and β-BCP, and was used in fewer experiments. The time course of these depolarizations

Figure 3 Chemical factors that excite bag cells are released during bag cell activity.

A. Diagram of the apparatus used in the serial perfusion experiment. The source abdominal ganglion was placed in a small, sealed chamber (bottom of diagram), and continuously arterially perfused and superfused (7 µL/min each) with buffered seawater containing a mixture of protease inhibitors. Two extracellular electrodes inside the chamber were placed on the bag cell clusters and used for stimulation and recording. The assay abdominal ganglion was placed in an open recording chamber (top of diagram), and arterially perfused with the outflow from the source ganglion (total flow of 14 µL/min into the assay ganglion).

B. Simultaneous intracellular recordings from a bag cell and an identified target neuron (of the LC cluster) in the assay ganglion. At the arrow, the bag cells in the source ganglion were stimulated, initiating a bag cell discharge which continued beyond the end of the record. The substances released into the flow by this discharge reached the assay ganglion about 3 minutes later. The response in the LC cell, an identified target neuron for ELH, is typical of that produced by arterial perfusion of ELH or by normal bag cell activity (Mayeri et al. 1985). Simultaneous with the onset of the LC response, the assay bag cells began depolarizing, leading to an afterdischarge. Bag cell depolarizations were seen in the assay ganglion in 5 out of 6 preparations, with afterdischarges in 3 of these.


A
V
V
ASSAY
SOURCE
Constant
Perfusion
B
Assay
Left
Bag
Cell
LC
Source Bag Cells
8
18 mV
3 min

was similiar to that of the depolarizing afterpotentials produced by intracellular bag cell stimulation.

There was day-to-day variability in the sensitivity of the bag cells to the BCP's, but the responses in individual experiments were dose-dependent (Fig 4B). The typical thresholds for depolarizing responses were between 10^{-8} and 10^{-7} M for α-BCP and β-BCP, and between 10^{-7} and 10^{-6} M for γ-BCP. Higher concentrations of α-BCP or β-BCP can initiate regenerative burst discharges (see also Rothman et al. 1983; Rock, Shope, and Blankenship 1986).

The responses to the BCPs quickly desensitized at concentrations above 10^{-6} M, or at even lower concentrations with repeated applications. This desensitization process, together with the slow time course of the responses and the difficulty in holding bag cell recordings through many arterial perfusions, made it difficult to compare precisely the potencies of the different BCPs. However, when the BCPs were compared, α- and β- BCPs had similar potencies, while γ-BCP was approximately one-tenth as potent. In contrast to the BCPs, the atrial gland peptides A and B, which are homologous to α-BCP and can trigger bag cell activity when applied to the head ganglia (Heller et al. 1980; Painter et al. 1986), did not have depolarizing actions on bag cells at concentrations of 20-125 μM (10 bag cells in 6 preparations). This indicates that they are not active on the same receptors as the BCPs. While the present study and that of Painter et al. (1986) found no effects of these

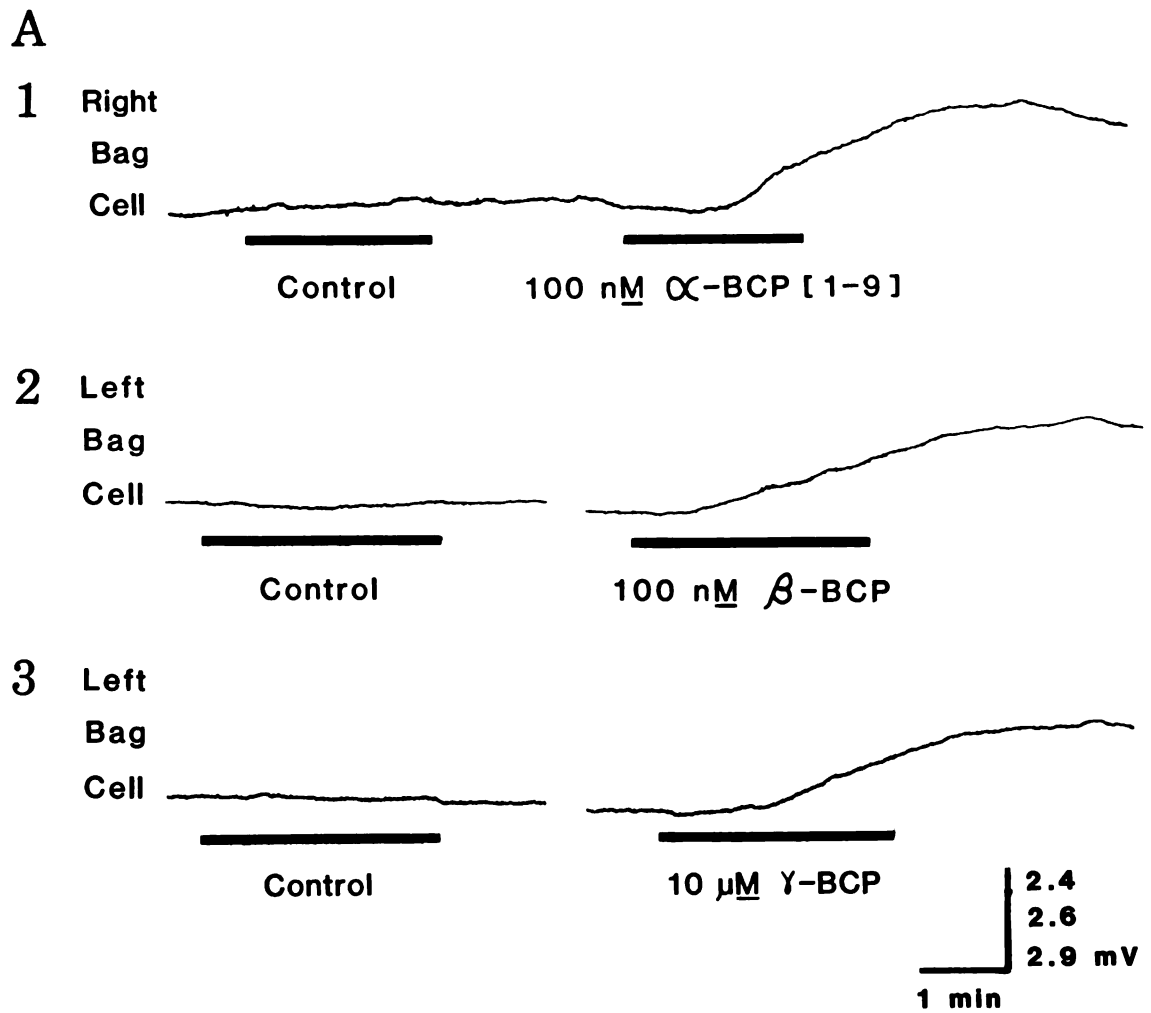
Figure 4 Excitatory actions of bag cell peptides on bag cells.

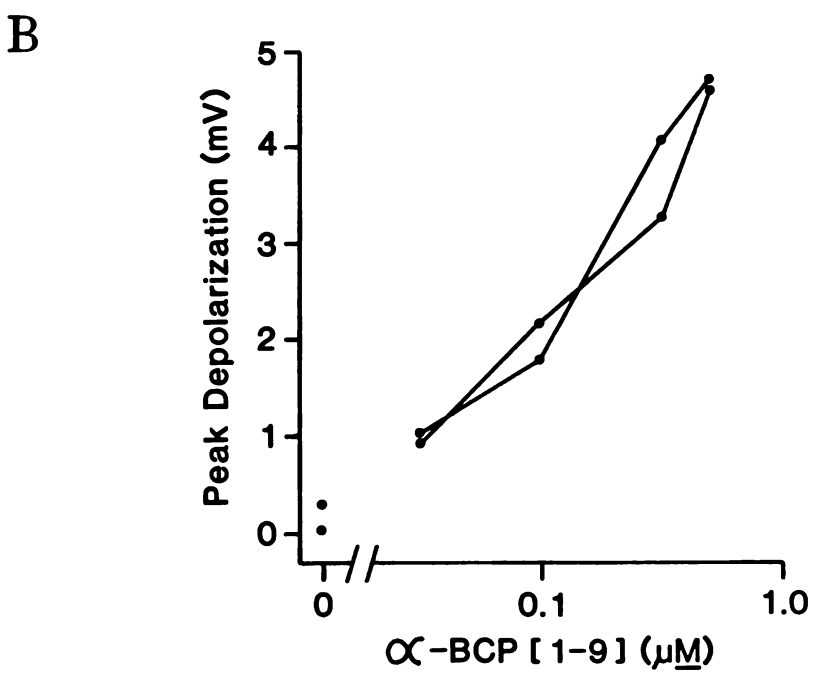
A-1. Arterial perfusion of 6 μL of 100 nM α-BCP(1-9) produced a slow depolarization in a bag cell (right). Control application of carrier solution containing the protease inhibitors had no effect (left).

A-2. Depolarization of bag cell by 8 μL of 100 nM β-BCP.

A-3. Depolarization of bag cell by 8 μL of 10 uM γ-BCP.

B. Dose dependency of the excitatory response to α-BCP. 6 μL of varying concentrations of α-BCP(1-9) were arterially perfused, and the peak depolarization measured. The dose-dependent responses shown were simultaneously recorded from 2 bag cells, including the one shown in A-1.


A
1
Right
Bag
Cell
Control
100 nM α-BCP [1-9]
2
Left
Bag
Cell
Control
100 nM β-BCP
3
Left
Bag
Cell
Control
10 μM γ-BCP
2.4
2.6
2.9 mV
1 min


B
Peak Depolarization (mV)
5
4
3
2
1
0
0
0.1
1.0
α-BCP [1-9] (μM)

atrial gland peptides in the absence of attached head ganglia, Heller et al. (1980) reported that peptides A and B initiated bag cell burst discharges, with mean latencies of 16 minutes, when applied to bag cells with the pleuroabdominal connective intact. They were uncertain whether these were postsynaptic effects on the bag cells or presynaptic effects on their inputs, and Strumwasser et al. (1980) favored the latter interpretation.

This is the first biological activity demonstrated for β-BCP and γ-BCP. In an earlier study, a fraction of bag cell extract with excitatory effects on neurons L1 and R1 contained a pentapeptide with the same amino acid composition as β-BCP, and β-BCP was thus considered a candidate transmitter for the bag cell excitation of these cells (Mayeri et al. 1985). However, applications of synthetic β-BCP have failed to mimic this activity except when they also activated the bag cells.

Tests for direct action of BCPs on bag cells

Arterially perfused peptides are distributed throughout the abdominal ganglion, so the cellular sites of their actions are undetermined. To address whether the BCPs act directly on bag cell autoreceptors, we dissected bag cell clusters away from the abdominal ganglion. In this reduced preparation, all the neuronal somata are bag cells. To minimize the possible involvement of severed processes which arose from other cells, the bag cell clusters were bathed in

high divalent cation solution. Peptides were pressure ejected over the isolated cluster and neurites through a wide-mouthed (approximately 100 μm) pipette. Fig 5A shows an excitatory response to β-BCP seen under these conditions, and Fig 5B shows the dose-dependency of the response. Excitatory responses to α-BCP or β-BCP were seen in 6 of 8 such preparations, suggesting that the peptides act directly on bag cell autoreceptors. In a previous test for direct action, BCPs were applied locally by pressure ejection from broken micropipettes (diameter approximately 10 μm) onto individual bag cell somata. However, no responses were observed in these experiments unless such a large amount of peptide was applied that it diffused beyond the somata (data not shown). We interpret this to indicate that bag cell BCP receptors are not on the somata, but may be localized to the neurites. Further evidence of bag cell autoreceptors comes from the finding that BCPs have direct excitatory effects on isolated bag cells in primary cell culture (K.J. Loechner and L.K. Kaczmarek, personal communication; see Discussion).

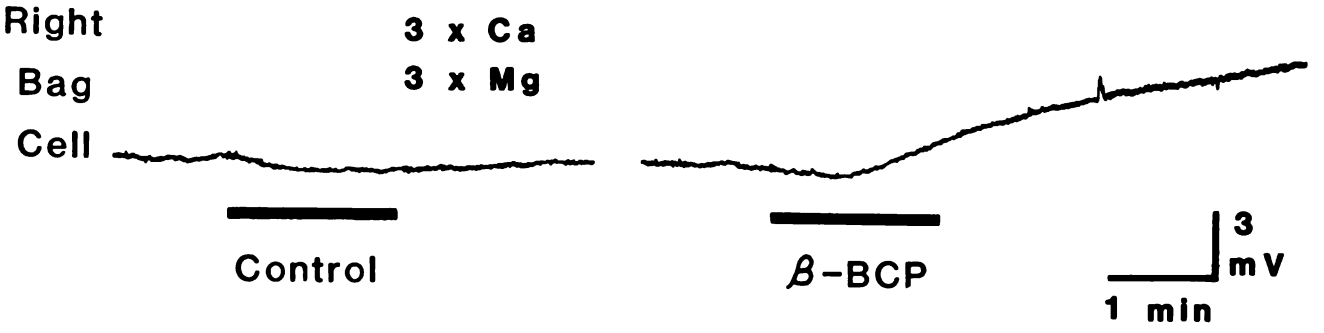
The responses to applied BCPs desensitized at concentrations above 10^{-6} M. If high local concentrations of BCPs occur during bag cell burst discharges, these would then be expected to cause desensitization. Fig 6 shows that the excitatory response to β-BCP was desensitized following bag cell discharges. Arterial perfusion of 10^{-5} M β-BCP, which normally causes a large consistent depolarization of about 10 mV (Fig 6A, upper trace), was ineffective when

Figure 5 Excitatory response to β-BCP in isolated bag cell clusters in high Ca++, high Mg++ medium suggests direct action.

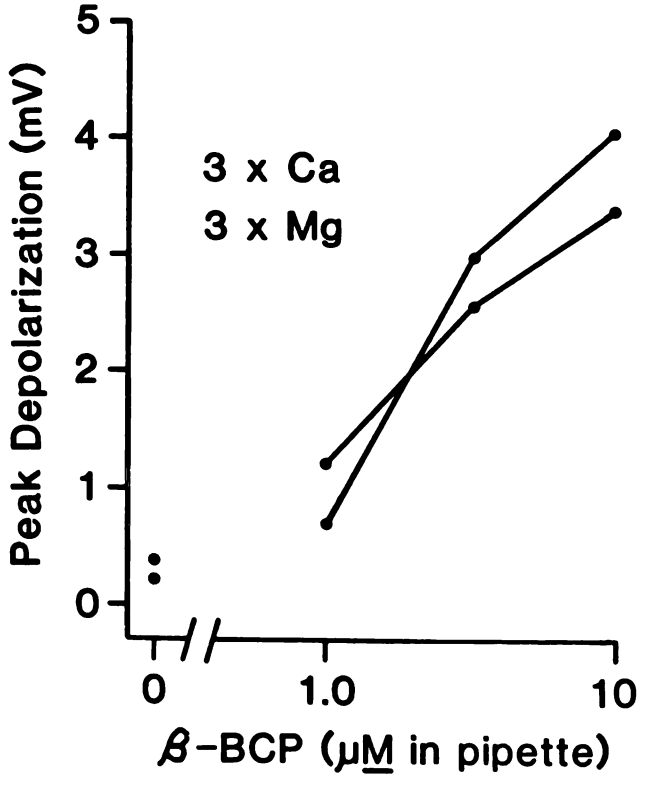
A. A bag cell cluster, with 2 cm of connective, was dissected away from the abdominal ganglion, partially desheated, and bathed in high divalent cation solution for at least 2 hr. before the experiment began. 100 μL of carrier solution alone or 10 μM β-BCP were pressure ejected from a pipette over the cluster.

B. Dose-dependency of the excitatory response to β-BCP in isolated bag cell clusters in high divalent cation solution. The plot shows the dose dependent responses of two bag cells, from the same preparation as in A, to varying concentrations of β-BCP. Concentrations shown are those in the pipette; the actual concentrations at the target cell were diluted by an unknown factor.

A


Right
Bag
Cell
3 x Ca
3 x Mg
Control
β-BCP
3
mV
1 min

B

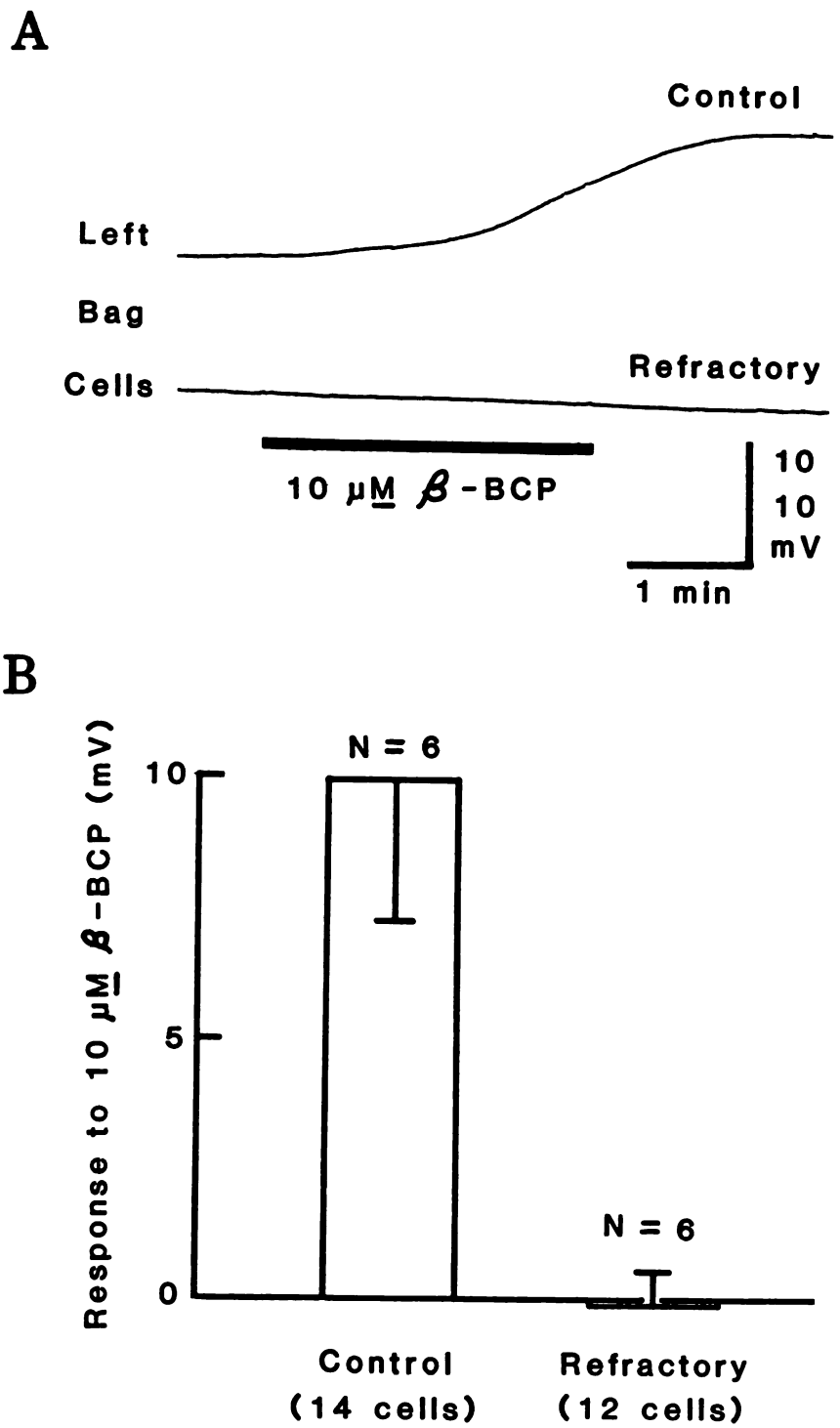

Peak Depolarization (mV)
5
4
3
2
1
0
3 x Ca
3 x Mg
0
1.0
10
β-BCP (μM in pipette)

applied 10 minutes after the end of an electrically stimulated bag cell discharge (Fig 6A, lower trace). The Control and Refractory experiments were done in separate preparations to ensure that all of the desensitization seen was due to the bag cell discharge and not to the desensitizing effects of a previous application of β-BCP. The response to α-BCP was also desensitized during the refractory period (not shown). Desensitization to the BCPs during a discharge may provide a necessary mechanism to turn off the positive feedback.

Figure 6 Desensitization of the excitatory response to β-BCP during the refractory period that follows bag cell discharges.

A. Intracellular recordings from bag cells in separate preparations. 10 μM β-BCP was arterially perfused into the abdominal ganglion with no bag cell discharge (Control) or 10 minutes after the termination of a bag cell discharge (Refractory).

B. Results from application of 10 μM β-BCP to 6 Control and 6 Refractory preparations. For each preparation, the response was taken as the mean response of 1 to 4 bag cells. Control: 9.9 ± 2.8 mV. Refractory: -0.04 ± 0.63 mV. This difference is significant at the $p < 0.001$ level by Cochran's method for the comparison of independent samples with unequal variances (Snedecor and Cochran 1967).


A
Control
Left
Bag
Cells
Refractory
10 μM β-BCP
10
10
mV
1 min
B
Response to 10 μM β-BCP (mV)
10
5
0
N = 6
N = 6
Control
(14 cells)
Refractory
(12 cells)

DISCUSSION

α-, β-, and γ-BCPs as autoexcitatory transmitters

The present findings indicate α-, β-, and γ-BCPs have autoexcitatory effects which provide positive feedback in the bag cells. These results, taken with previous data, fulfill most of the strict criteria needed to establish the three BCPs as neurotransmitters in this system. The key data are as follows: (1) The BCPs act on bag cell autoreceptors to mimic depolarizations caused by bag cell activity; (2) The BCPs are synthesized by the bag cells and cleaved from a common precursor encoded by a bag cell-specific messenger RNA, and are present in bag cell extracts in approximately equimolar amounts (Rothman et al. 1983; Scheller et al. 1983; Rothman, Sigvardt, and Mayeri 1985; Rothman et al., in preparation); (3) Immunoreactive α-BCP is localized to bag cell somata and processes, and in particular, to the neuritic cuff region which is thought to be the critical site for the generation of bag cell activity (Pulst et al. 1986; Painter et al. 1986a); (4) All three BCPs are co-released during bag cell discharges (Sigvardt et al. 1986; Rothman, Sigvardt, and Mayeri 1985;), and in quantities which are apparently sufficient to account for the neurally evoked autoexcitatory responses (see below); (5) The BCPs are rapidly inactivated by proteolysis after release (Sigvardt et al. 1986; Rothman, Phares, and Groves 1987; Rothman, Sigvardt, and Mayeri, in preparation). This

is in contrast to ELH, which functions as a neurohormone and is much more stable (Mayeri et al. 1985).

It is possible to directly compare the neuroactive doses of the BCPs with the quantities released during bag cell activity. The 8 μL of 1 μM α-BCP doses which consistently depolarized bag cells each contained 8 x 10^{-12} mol of peptide. By comparison, approximately 10^{-10} mol of each of the three BCPs could be recovered from the material released during a single bag cell discharge in the presence of protease inhibitors (Rothman, Sigvardt, and Mayeri 1985). To accurately compare these estimates, several additional factors would have to be considered, such as the normal degradation of BCPs after release, and the losses of BCPs when collecting released material. But as a first estimate, the quantity of BCPs released by bag cells seems more than sufficient to account for the autoexcitatory activity seen in the serial perfusion experiments. Since it is probable that the local BCP concentrations in the critical neuritic cuff during a discharge are much higher than the concentrations reached in the serial perfusion flow, strong autoexcitatory feedback by the BCPs would then be expected to occur during bag cell activity.

These data all indicate autotransmitter roles for the BCPs. An additional critical test, blockade of the neurally evoked response by pharmaclogical antagonists, cannot be performed until specific antagonists to the BCPs are developed. An alternative approach would be to desensitize

the bag cell autoreceptors with high concentrations of BCPs, as was done with the inhibitory α-BCP receptors on target neurons (Sigvardt et al. 1986). However, preliminary experiments using this approach have not demonstrated dramatic effects on bag cell activity in the presence of 1 mM α-, β-, and γ-BCPs (unpublished experiments). While this may indicate that the autoexcitatory actions of the BCPs are not necessary for the generation of bag cell burst discharges, an alternative interpretation is that there was poor penetration of the undegraded BCPs into the extracellular spaces surrounding the cuff neurites, so that only some of the autoreceptors were actually desensitized. The determination of the precise causal role of autoexcitatory transmission in this system requires further study.

Multiple neuroactive bag cell peptides and receptors

Previous data has shown α-BCP (Rothman et al. 1983; Sigvardt et al. 1986) and egg-laying hormone (Mayeri et al. 1985) are bag cell neurotransmitters onto target neurons in the abdominal ganglion. The present data indicating that α-, β-, and γ- BCPs are autoexcitatory transmitters brings to four the number of bag cell neuropeptides derived from the common ELH/BCP precursor which are thought to function as neurotransmitters. The demonstration that these neuropeptides act on the releasing neurons as well as on other central neurons and on peripheral tissues provides a

further rationale for the frequent coexistence of multiple putative transmitters in neurons.

α-BCP(1-7), α-BCP(1-8), α-BCP(1-9), and β-BCP were approximately equipotent in depolarizing bag cells, while γ-BCP was about one-tenth as potent. In contrast, for inhibition of the left upper quadrant neurons L2 - L6, α-BCP(1-8) was 3 times as potent as α-BCP(1-7), and 30 times as potent as α-BCP(1-9), while β-BCP and γ-BCP had little or no activity (Rothman et al. 1983; Sigvardt et al. 1986). These pharmacological distinctions provide evidence for two subtypes of BCP receptor. One subtype mediates the inhibitory response to α-BCP, and occurs in a large proportion of abdominal ganglion neurons. The other subtype mediates the excitatory response to α-, β-, and γ-BCPs, and was seen only in the bag cells, suggesting that it may be specific for bag cell autoreceptors. The atrial gland peptides A and B, which are homologous to α-BCP and can trigger bag cell burst discharges when applied to head ganglia (Heller et al. 1980; Painter et al. 1986b), but did not appear to have direct excitatory effects on the bag cells, may be acting on yet another related receptor subtype.

Kauer, Fisher, and Kaczmarek (1987) reported that α-BCP hyperpolarized bag cells in primary cultures that were up to 2 days old, and lacked extensive neuritic processes. However, hyperpolarizing responses to BCPs were never observed in bag cells *in situ*, and in subsequent work by

this group on bag cells in longer term cultures, all three BCPs were seen to depolarize bag cells (K.J. Loechner and L.K. Kaczmarek, personal communication). If the autoexcitatory receptors are mainly restricted to bag cell neurites, it may be necessary for cultured bag cells to regenerate extensive functional neurites before the autoexcitatory responses return. It is interesting to note that the bag cells in short term cultures which lacked autoexcitatory responses also did not generate burst discharges following electrical stimulation. This is consistent with the hypothesis that autoexcitatory transmission is necessary for the generation of bag cell burst discharges, although there are other possible explanations. Kauer, Fisher, and Kaczmarek (1987) also found that α-BCP had only occasional weak depolarizing effects on bag cells *in situ*, in apparent contrast to our findings, and suggested that the bag cells' autoexcitatory response to α-BCP may depend on the use of $MgCl_2$ anesthesia during the dissection (which we routinely used, as described in Methods, but they did not). However, we have seen autoexcitatory responses as well in preparations which were not anesthetized for dissection.

Functions of positive feedback

Positive feedback by autoexcitatory neuropeptides in the bag cells is postulated to have two conceptually distinct consequences. First, the positive feedback in

individual cells produces a temporal spread of activity, in which a brief stimulation of a bag cell leads to a prolonged afterdischarge (Fig 7A). This is most apparent in the first minute of bag cell burst discharges, when the firing rate of bag cells accelerates rapidly to as much as 10/sec. Second, the positive feedback within a population of cells produces a spatial spread of activity, in which the activation of one or a few cells leads to the coordinated activation of the entire population (Fig 7B). This includes the spread of activity among cells in a cluster, between the bilateral clusters, and possibly between different ganglia. In this last regard, we have recently found that intracellular stimulation of pleural ganglion cells which contain α-BCP-like immunoreactivity causes depolarizations and afterdischarges in the bag cells (Brown and Mayeri, 1987, 1988).

Both aspects of the positive feedback contribute to all-or-none, synchronous activity in the bag cells. The resulting prolonged, stereotyped discharge in the bag cells is thought to be important for ensuring that the bag cells, once activated, release a large, fixed quantity of neuropeptides appropriate to generate a complete episode of egg-laying behavior (Kupfermann and Kandel 1970).

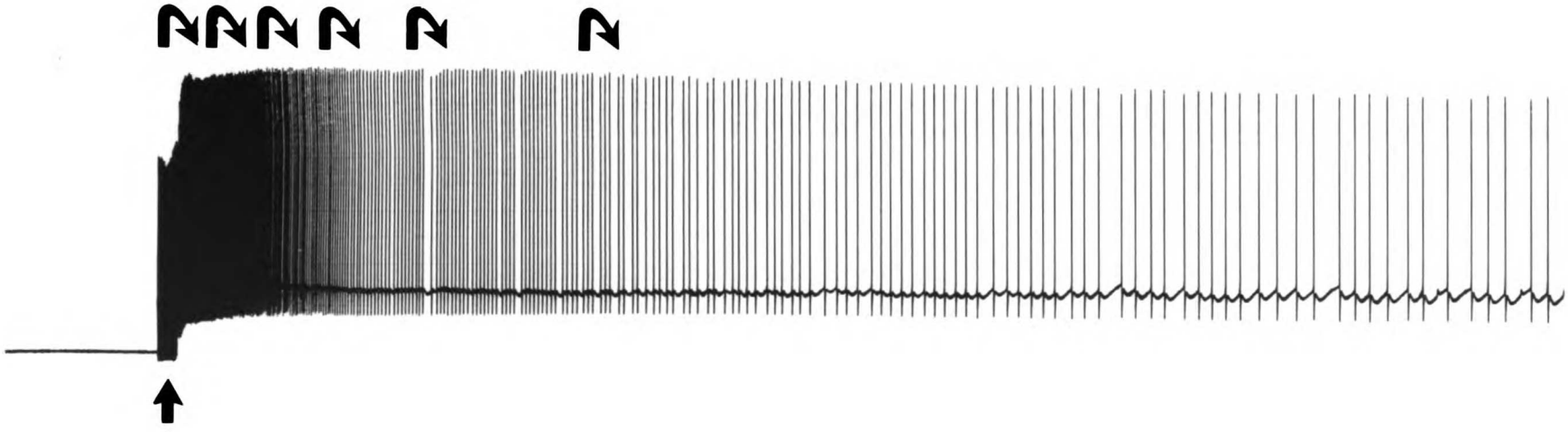
The positive feedback may be turned off by depletion of the autoexcitatory transmitters, desensitization of the autoexcitatory responses (Fig 6), and/or biochemical modifications of bag cell proteins during the discharge

Figure 7 Postulated functions of positive feedback by autoexcitatory transmitters

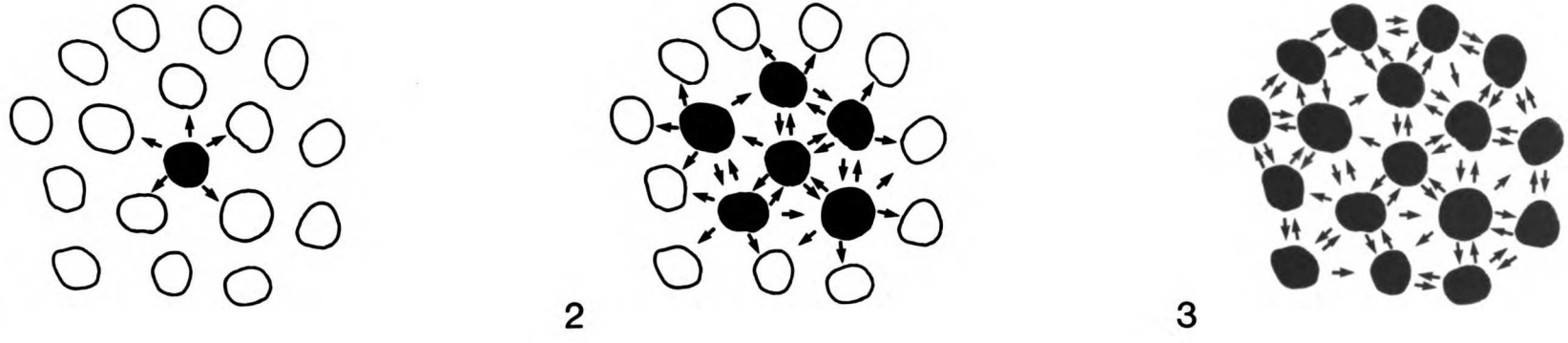
A. Temporal spread of activation in individual neurons. Transient stimulation of an individual cell (arrow at bottom) initiates regenerative autoexcitatory process (represented by arrows above), leading to a prolonged afterdischarge.

B. Spatial spread of activation in a population of neurons. Positive feedback within a functional group of autoexcitatory cells spreads activation from a single cell to the entire cluster. Three successive stages of activity are represented. Filled cells represent active neurons; arrows represent the spread of excitation to neighboring neurons.

A. TEMPORAL SPREAD OF EXCITATION



B. SPATIAL SPREAD OF EXCITATION



(Kaczmarek, Strong, and Kauer 1986). This could contribute both to the termination of the discharge and to the subsequent refractory period, during which the bag cells are relatively inexcitable and lack slow depolarizing afterpotentials when stimulated (Kupfermann and Kandel 1970; Kaczmarek and Kauer 1983).

The positive feedback by the autoexcitatory BCPs is in addition to other presumed positive feedback mechanisms, such as electrotonic coupling (Kupfermann and Kandel 1970; Kaczmarek et al 1979; Blankenship and Haskins 1979) and voltage- and calcium-dependent currents (Dudek and Blankenship 1977; De Reimer et al. 1985). Cyclic AMP and polyphosphatidylinositol turnover are also thought to play important roles in the generation of bag cell activity (Kaczmarek, Jennings, and Strumwasser 1978; Strumwasser, Kaczmarek, and Jennings 1982; Kaczmarek, Strong, and Kauer 1986). The possible relationships among these different mechanisms, and their relative contributions to patterns of bag cell activity, remain to be determined.

While there is growing evidence that autoinhibitory transmission is a common mechanism used to stabilize neuronal activity (Chesselet 1984), the role of autoexcitatory transmission has not been well established. Pharamcological studies of transmitter release have suggested conjoint regulation by both inhibitory and excitatory autoreceptors on mammalian noradrenergic (Adler-Graschinsky and Langer 1975) and cholinergic (Wessler et al.

1986) nerves, and at a central cholinergic synapse in Aplysia (Fossier et al. 1988). Autoexcitatory transmission was reported in the turtle olfactory bulb, where it may function in signal amplification, but is thought to be under the control of inhibitory feedback (Nicoll and Jahr 1982). A shift toward net positive feedback in such systems has been suggested as an epileptogenic mechanism (Ayala et al. 1973; Nicoll and Jahr 1982). In the abdominal ganglion of Aplysia, the electrically coupled L25 neurons, which produce the stereotyped behavior of respiratory pumping, have also been suggested to use autoexcitatory transmitters (Byrne 1982).

Under normal conditions, the type of intermittent, explosive activity autoexcitatory transmission tends to produce is most often reported in neuroendocrine systems. Besides the bag cells, pulsatile neurohormone secretion has been associated with episodic, coordinated electrical activity in the caudodorsal cells (CDC) and the "Light-Yellow Cells" (LYC) in the pond snail Lymnaea stagnalis (Vlieger et al. 1980; Swigchem 1981), the eclosion hormone cells in the moth Manduca sexta (Copenhaver and Truman 1986), and some hypothalamic nuclei in mammals (reviewed in Lincoln et al. 1985). Evidence for the involvement of autoexcitatory transmission has been reported for the Lymnaea CD cells, although the autoexcitatory transmitter has not yet been identifed (Maat et al. 1988), and for the hypothalamic oxytocin cells (Freund-Mercier and Richard

1984; Belin and Moos 1986; Yamashita et al. 1987). An emerging hypothesis is that autoexcitatory transmission provides a general mechanism for the generation of episodic activity in neuroendocrine systems.

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Chapter 4

THE NEUROENDOCRINE BAG CELLS OF *APLYSIA* ARE ACTIVATED BY BAG CELL PEPTIDE-CONTAINING NEURONS IN THE PLEURAL GANGLION

SUMMARY AND CONCLUSIONS

1) The generation of egg-laying behavior in the marine mollusk *Aplysia* involves a prolonged burst discharge in the neuroendocrine bag cells, which secrete neuropeptides derived from the ELH/BCP precursor protein.

(2) Besides the bag cells, which are located in the abdominal ganglion, small clusters of neurons in the cerebral and pleural ganglia also express the ELH/BCP neuropeptides. We made intracellular recordings from 32 of these ELH/BCP cells in right pleural ganglia, in 18 preparations, to characterize their physiological properties and their functional relationship to the bag cells.

3) The identification of these ELH/BCP cells was confirmed by pressure injection of Lucifer Yellow and subsequent immunocytochemical processing for α-BCP immunoreactivity.

4) The basic electrophysiological properties of the pleural ELH/BCP cells were similar to those of the bag cells. These pleural cells were directly demonstrated to be electrically coupled, and direct intracellular stimulation of individual pleural ELH/BCP cells initiates prolonged, synchronous burst discharges in the entire cluster through a positive feedback mechanism.

5) Burst discharges elicited in the pleural ELH/BCP cells consistently initiated burst discharges in the bag cells. Bag cell burst discharges were less effective in initiating burst discharges in the pleural ELH/BCP cells, indicating that there were reciprocal but asymmetrical connections.

6) The results show that the pleural ELH/BCP cells are functionally coupled to the bag cells. They support the hypothesis that the pleural ELH/BCP cells are part of the descending pathway that initiates bag cell activity, and egg-laying behavior, *in vivo*.

INTRODUCTION

The bag cells are a population of 800 homogeneous neuroendocrine cells that are located in bilateral clusters in the abdominal ganglion of *Aplysia* and involved in the control of egg-laying behavior. The bag cells are usually electrically silent, but are activated episodically to fire synchronous, all-or-none burst discharges lasting about 15-30 minutes. Bag cell burst discharges precede, and cause, egg-laying in intact animals (37). During burst discharges the bag cells release large amounts of several neuropeptides which act on both central and peripheral targets. Their central actions are thought to coordinate egg-laying behavior (reviewed in 33). One of the peptides, egg-laying hormone (ELH), functions as a neurohormone to cause egg release from the ovotestis (2,14,43) as well as producing excitatory responses on many identified target neurons in the abdominal ganglion (9,32) and on neuron B16 in the buccal ganglion (52).

Alpha, beta, and gamma-bag cell peptides (α, β-, and γ-BCPs) are three structurally related neuropeptides encoded on the same precursor protein as ELH (47) and coreleased during bag cell burst discharges (44,50). α-BCP functions as an inhibitory transmitter onto many target neurons in the abdominal ganglion (42,50). α-, β-, and γ-BCPs depolarize bag cells and are thought to function as autoexcitatory transmitters to provide positive feedback in the bag cells

and contribute to the all-or-none firing pattern of the bag cells (11,13).

Sexually mature *Aplysia* lay eggs approximately once a week. Egg-laying occurs in physically isolated *Aplysia*, indicating it can be triggered endogenously, though under natural conditions it also depends on environmental and possibly pheromonal cues (3,38,45). Several lines of evidence suggest that the physiological pathway for initiation of bag cell burst discharges (and thus, of egg-laying) comes from the cerebral and pleural ganglia, via the cerebropleural and pleuroabdominal connective nerves. Bag cell burst discharges can be initiated by electrical stimulation of these connectives (22,26,29), or by focal stimulation of specific points in the cerebral ganglion (20,52). Application of the atrial gland peptides A and B to the cerebral or pleural ganglia also activates the bag cells (25,36). These studies indicate that a neural pathway from the cerebral and pleural ganglia is sufficient to activate the bag cells. That this pathway is also necessary was suggested by lesion studies, which demonstrated that bilateral transections of either the cerebropleural or the pleuroabdominal connectives eliminated spontaneous bag cell burst discharges and egg-laying *in vivo* (20,37). However, the neuronal components of this putative descending pathway have not been identified at the cellular level.

The cerebral and pleural ganglia contain small clusters of neurons which also express ELH genes, and are

immunoreactive for both ELH and α-BCP (16,34,36,40). These neurons presumably express the same ELH/BCP precursor protein as the bag cells, since other members of the ELH gene family would not be expected to yield peptides cross-reacting with the α-BCP antisera used (40), and mRNA corresponding to the bag cell gene has been isolated from these ganglia (49). This is similar to many vertebrate neuroendocrine systems, in which the neuropeptides released from neuroendocrine cells are often present in neurons in other parts of the central nervous system. The functions of these other neurons, and their possible relationship to the neuroendocrine cells, are in general poorly understood. The expression of the bag cell ELH/BCP gene in the cerebral and pleural ganglion cells raises the possibility that they may be functionally related to the bag cells. Because these cells are also in the approximate locations of the descending neural pathway for activating the bag cells, and presumably release transmitters with known excitatory actions on bag cells (α-, β-, and γ-BCPs), they are likely candidates to comprise this descending pathway.

This paper reports studies of the ELH/BCP cells in the pleural ganglia, and their functional relationship to the bag cells. A preliminary report of this work was presented as an abstract (12).

METHODS

Electrophysiology

Ganglia were dissected from large (700+ g), sexually mature *Aplysia californica* that had been anaesthetized by injection of isotonic $MgCl_2$ (1/3 of body weight) 30 min prior to dissection. Three types of preparations were used: isolated right pleural ganglion; right pleural and abdominal ganglia with intact right pleuroabdominal connective; or cerebral, right pleural and abdominal ganglia with intact right cerebropleural and pleuroabdominal connectives. The connective tissue sheaths overlying ganglia were generally left intact, though when the sheath over the pleural ganglion was unusually thick it was partly trimmed away to permit easier access to the neurons.

Conventional intracellular recording and stimulating techniques were used as previously described (30,32). Ganglia were pinned out in a dish lined with clear silicone resin (Sylgard, Dow-Corning), and continually superfused with seawater containing 10 mM HEPES (adjusted to pH 7.6 with NaOH and HCl) and 250 mg/L dextrose. Pleural ELH/BCP cells were visualized under bright epi-illumination, and the pinning of the sheath to the Sylgard was adjusted to increase their accessibility and the tension of the sheath lying directly over them. Intracellular recordings were made through the sheath with glass microelectrodes filled with 1.5 M potassium acetate, except in two experiments in which electrodes contained 5% Lucifer Yellow in 0.15%

lithium chloride. Double-barreled microelectrodes were used for the data shown in Fig 4.

Histochemistry

The basic immunocytochemical procedures used have been previously published (10,39,40). Briefly, ganglia were rinsed thoroughly in filtered artificial sea water, and fixed overnight in 4% paraformaldehyde/ 0.1 M phosphate buffered saline (1% NaCl, pH 7.6) at 4°C. Ganglia were cryoprotected 24 hr in 30% sucrose (also 4°C), then cut in 30 micron sections on a cryostat. Floating sections were blocked with 3% normal goat serum (at room temperature), then incubated 24 hr at 4°C with 10 µg/ml αφφινιτψ-πυριφιεδ α-BCP IgG from rabbit. This antiserum was previously characterized (39,40). Sections were rinsed, then incubated 1 hr at room temperature with secondary antiserum (goat-anti-rabbit conjugated with either fluorescein or rhodamine, Cappel), rinsed again, floated onto gelatin coated slides, and cover-slipped. Usually only every second or third section was processed in order to conserve antisera.

For retrograde tracing studies, ganglia were dissected and loosely pinned down in sterile Sylgard-lined covered dishes. Broken glass micropipettes (tip diameters approximately 10 µm) were used to pressure inject tracers under the sheath in the region of the cuff neurites at either the abdominal or pleural ganglion. Retrograde tracers used in these studies were fluorescent latex

microspheres (Lumafluor Inc.) and three kinds of labelled wheat germ agglutinin (WGA): fluorescein-WGA (Sigma), phycoerythrin-WGA (Biomeda), and WGA-apoHorseradish Peroxidase Gold (kindly provided by Dr. Allan Basbaum). After injection, ganglia were kept at room teperature on a slow shaker table, with L-15 medium (made up in ASW containing only 8 m$\underline{M}$ Ca^{++} to minimize precipitation), which was changed daily. Ganglia remained apparently healthy for up to a week under these conditions, with normal electrophysiological behavior of identified neurons, including normal responses of target neurons to bag cell burst discharges.

RESULTS

Histochemistry of ELH/BCP cells in the pleural ganglia

Right and left pleural ganglia were fixed, sectioned, and processed for immunocytochemistry using highly specific antisera to α-BCP(1-9). This α-BCP antiserum labels the same set of neuronal somata as ELH antiserum (40), but has little or no cross-reactivity with the homologous atrial gland peptides A and B or the ELH/BCP precursor protein (39,40). Thus, labelled neurons are presumed to process peptides from the ELH/BCP precursor as in the bag cells.

As previously described (16,36,40), positive staining somata had the morphological characteristics of bag cells in the abdominal ganglion, and immunoreactive fibers formed a neuritic cuff around the proximal pleuroabdominal connective similar to (though not as dense as) the bag cell neuritic cuff at the abdominal end of the pleuroabdominal connective nerve. Some of the fibers in the neuritic cuffs appeared to arise from somata in the pleural ganglia.

In left pleural ganglia, ELH/BCP cells were seen in only 2 of 5 ganglia sectioned and processed for immunocytochemistry, and in both of these cases only a single ELH/BCP cell was seen. An α-BCP-immunoreactive cell in a left pleural ganglion, with an immunoreactive neuritic cuff around the left pleuroabdominal connective, is shown in Fig 1A. Processes from the immunoreactive cell body appear to comprise much of the immunoreactive cuff in this section. However, immunoreactive cuffs were seen around the left

pleuro-abdominal connective in all left pleural ganglia examined, even when no immunoreactive somata were found.

In contrast to the variability in the presence of immunoreactive somata in the left pleural ganglia, clusters of 3-13 ELH/BCP cells were seen in 6 of 6 right pleural ganglia that had been arbitrarily selected for immunocytochemical processing, and in 2 of 2 in which they had first been recorded from and stained (see below). An example of an ELH/BCP-immunoreactive cell in a right pleural ganglion is shown in Fig 1B. An axon from this cell can be seen heading out to the pleuroabdominal connective, though it is not clear whether this process projects out the connective and/or contributes to the neuritic cuff. The immunoreactive right pleural cuffs were generally much more extensive than the left pleural cuffs.

In an attempt to follow possible α-BCP-immunoreactive projections between the pleural and abdominal ganglia, several experiments were done using a variety of retrograde tracers (see Methods) injected into the cuff neurites at either the pleural or abdominal end of the connective. In none of these experiments was there any sign of retrograde transport of the tracers between these ganglia (which were approximately 10 cm apart), even after 7 days in culture at room temperature. However, the WGA-apoHRP Gold tracer (4) was apparently transported from the pleural to the cerebral ganglion (approximately 1 cm), and seemed the most promising technique for future studies. Haskins, Price, and

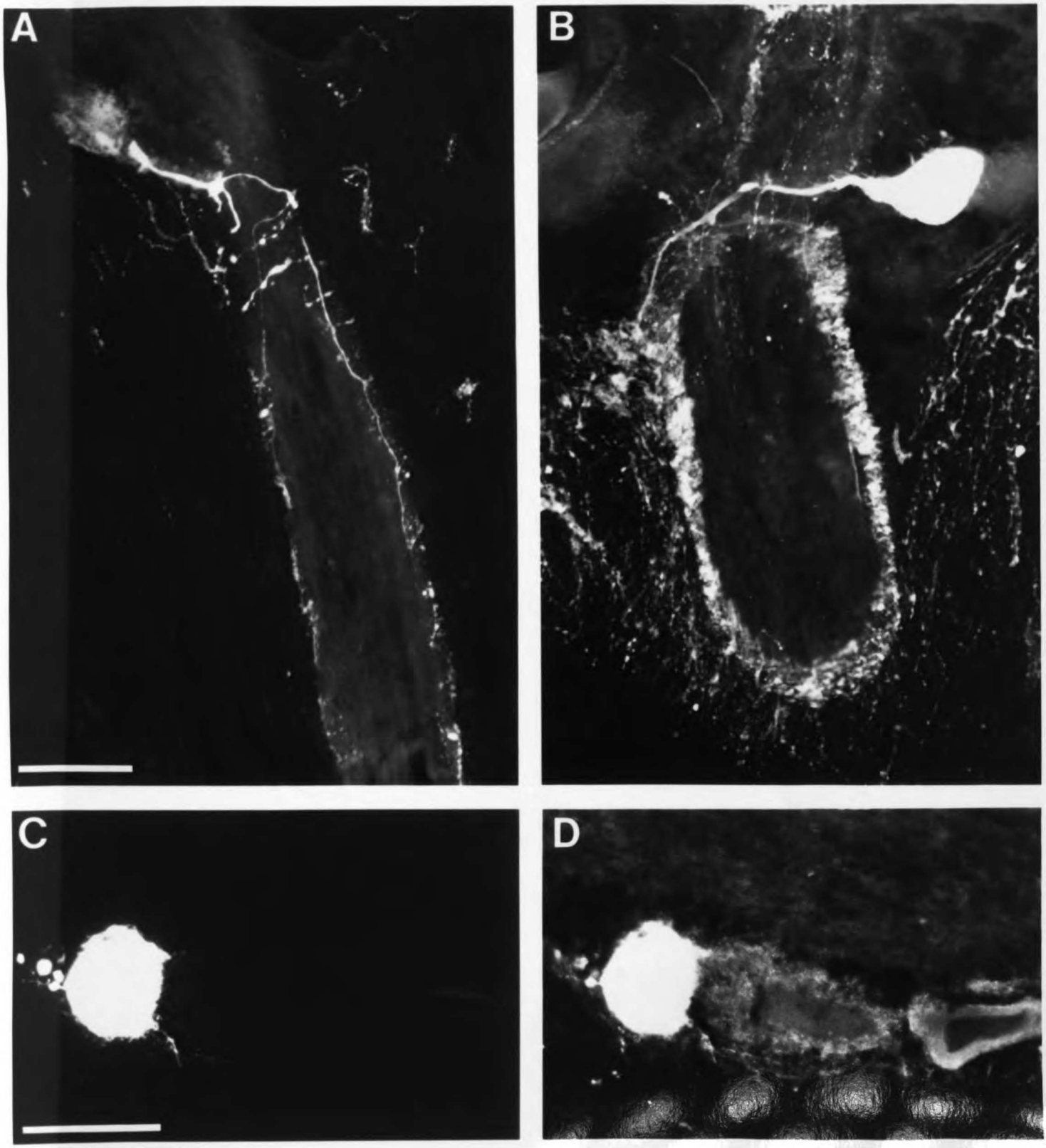
Figure 1 α-BCP-immunoreactive cells in the pleural ganglia

A. Left pleural ganglion. An immunoreactive cell body is seen, with processes going to the pleuroabdominal connective and contributing to the sparse immunoreactive neuritic cuff around the left pleuroabdominal connective connective. This section just grazed the immunoreactive soma, which was more clearly defined in neighboring sections. However this section showed more of the details of the neuritic cuff, and is similar to the section shown in part B for comparison. Scale bar: 100 μm.

B. Right pleural ganglion. An immunoreactive cell body is seen, with its axon heading out to the right pleuroabdominal connective nerve. The extensive neuritic cuff can be seen wrapping around the connective, with additional immunoreactive fibers lying in the sheath. Same scale as A.

C. Right pleural ELH/BCP cell stained with Lucifer Yellow. After electrophysiological characterization, this cell was pressure injected with 5% Lucifer Yellow in 0.15% LiCl. Scale bar: 100 μm.

D. Same section as in C, labelled for α-BCP immunoreactivty with a Rhodamine-conjugated second antibody. The same cell seen under Lucifer Yellow optics in C contained immunoreactive α-BCP, confirming that we have properly identified the pleural ELH/BCP cells.


A
B
C
D

Blankenship previously tried to trace bag cell processes between the abdominal and pleural ganglia, using tritiated amino acids, but could not find label more than 1 cm from the bag cell somata (23). To our knowledge, the long-range (>1 cm) labelling of axonal projections with retrograde or anterograde tracers has not yet been accomplished in any molluscan system. Other studies have used much smaller, juvenile animals (1-2 g) to study projections between the pleural and abdominal ganglia (1,24, and R.D. Haekins, personal communication), but this approach is probably inappropriate for studying this circuit, since the bag cells and their processes are immature in such small animals (21).

Electrophysiological properties of pleural ELH/BCP cells

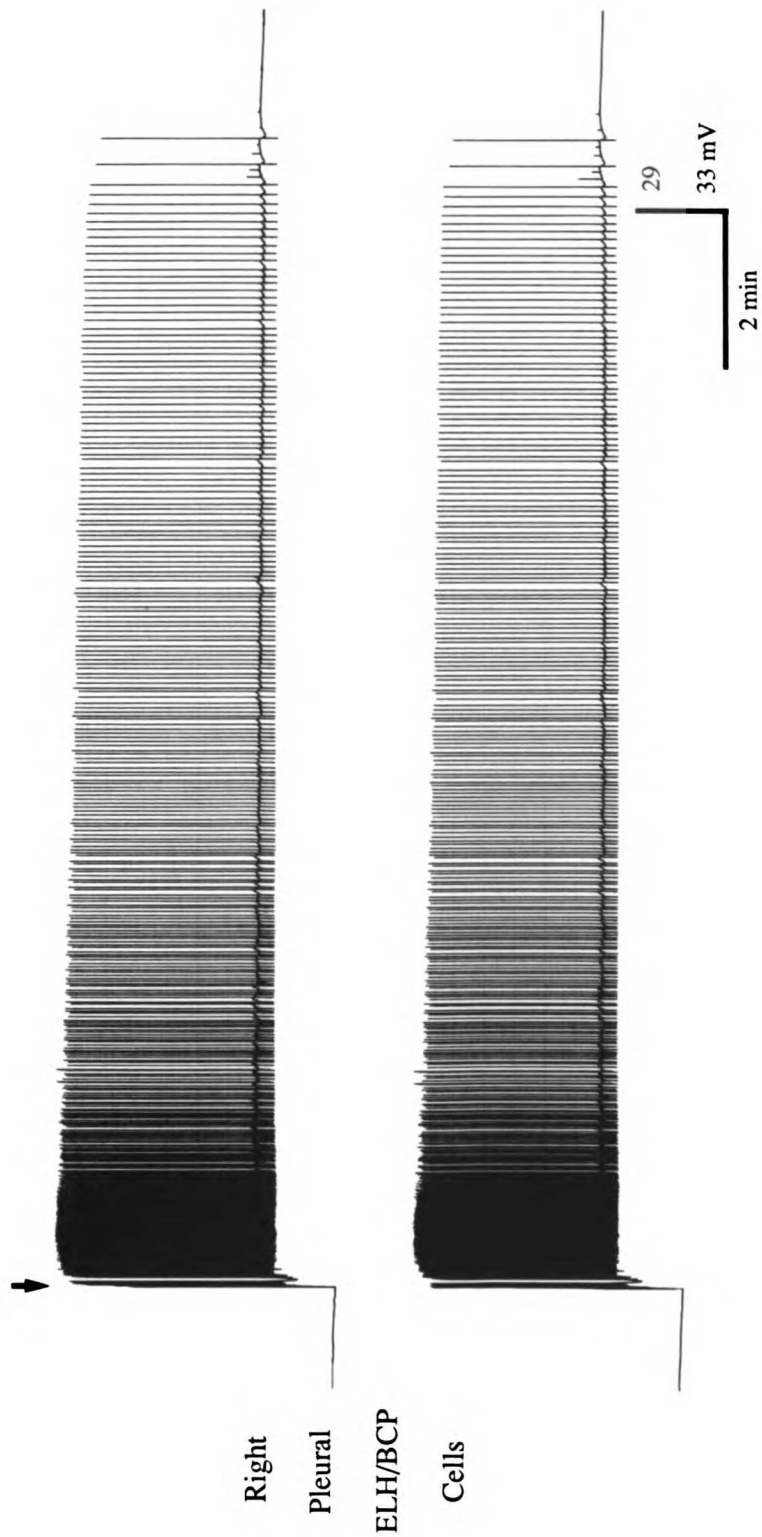
ELH/BCP neurons with the characteristic size and whitish appearance of bag cells could be directly visualized in approximately half of intact right pleural ganglia examined. (This does not imply that such cells were present in only half of right pleural ganglia, since many of the cells found after immunocytochemical processing had not been seen in the intact ganglia.) When succesfully impaled with intracellular eléctrodes, these cells also possessed the basic electrophysiological properties of bag cells (see below). A total of 32 of these cells were recorded from in 18 right pleural ganglia. In 2 of these preparations, cells were pressure injected with Lucifer Yellow after electrophysiological characterization, and processed for α-BCP

immunocytochemistry. The Lucifer Yellow-stained cells (Fig 1C) also labelled for immunoreactive α-BCP (Fig 1D) in both preparations, confirming that we had correctly identified the pleural ELH/BCP cells.

When impaled, pleural ELH/BCP cells were always silent, and received no apparent spontaneous synaptic PSPs. The resting potentials were typically about -60 mV. Following brief intracellular electrical stimulation, they depolarized 20-30 mV and fired prolonged synchronous discharges lasting 2-15 minutes. Fig 2 shows such a synchronous burst discharge in 2 ELH/BCP cells in an isolated right pleural ganglion. The burst discharge in this preparation lasted 14 minutes, and contained 442 action potentials, of which 119 occured during the first 30 seconds of the burst discharge. The action potentials were of long duration (20-40 msec at half-maximal potential), and spike activity was highly synchronous among pleural ELH/BCP cells throughout the burst discharge. In these parameters they strongly resembled the bag cells (29).

The activation of pleural ELH/BCP cells in another preparation is shown in greater detail in Fig 3. Intracellular stimulation of a single pleural ELH/BCP cell (upper trace) with depolarizing current pulses (first arrow) produced slow depolarizing afterpotentials in the stimulated cell as well as in another ELH/BCP cell (lower trace) in the isolated right pleural ganglion. These slow depolarizing afterpotentials indicate an intrinsic positive feedback

Figure 2 Synchronous burst discharge in pleural ELH/BCP cells. Simultaneous intracellular recordings were made from 2 ELH/BCP cells in an isolated right pleural ganglion. This characteristic burst discharge, accompanied by a 30 mV depolarization and lasting 15 min, was initiated by intracellular stimulation of the cell in the top trace with several 50 msec depolarizing pulses (arrow).


29
33 mV
2 min
Right
Pleural
ELH/BCP
Cells

mechanism like that in the bag cells (11,13). Further stimulation (second arrow) raised this depolarizing afterpotential past threshold, and an all-or-none burst discharge began. Burst discharges were produced by intracellular stimulation of pleural ELH/BCP cells in 6 of 6 isolated right pleural ganglion preparations, and in 8 of 8 preparations containing right pleural ganglia with attached abdominal ganglion. This was more reliable than the initiation of bag cell burst discharges by intracellular stimulation of individual bag cells, which was succesful in 16 of 35 cases under similar conditions in a previous study (13).

The strong synchrony of firing among the pleural ELH/BCP cells suggests they are electrically coupled, as are the bag cells (6,27,29). The electrical coupling between pleural ELH/BCP cells was directly demonstrated as shown in Fig 4a. Simultaneous intracellular recordings were made from three cells, with a double barreled electrode used in the cell in the bottom trace to both record voltage and pass current. Injection of negative or positive current steps (bars) into the cell shown in the bottom trace produced hyperpolarizing or depolarizing responses in both the injected cell and the other ELH/BCP cells (top 2 traces). The voltage step responses in the coupled cells were proportional to the voltage response in the injected cell (Fig 4B), corresponding to coupling coefficients of approximately 0.11 and 0.14 (r=0.995 and 0.997,

Figure 3 Activation of pleural ELH/BCP cells involves positive feedback. Simultaneous intracellular recordings were made from 2 ELH/BCP cells in an isolated right pleural ganglion. A brief train of intracellular depolarizing pulses given to the cell in the top trace (first arrow) produced slow depolarizing potentials in both cells. A subsequent, identical stimulation (second arrow) brought the cells past the threshold for a burst discharge.

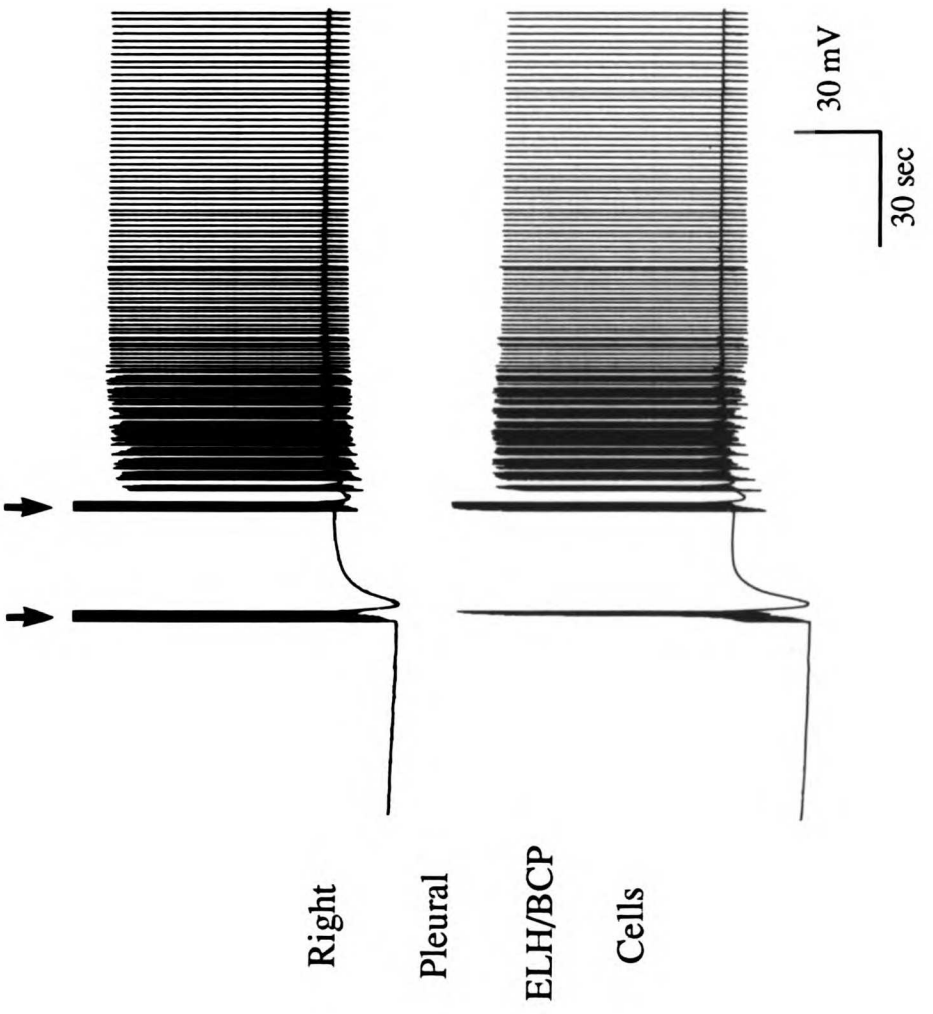
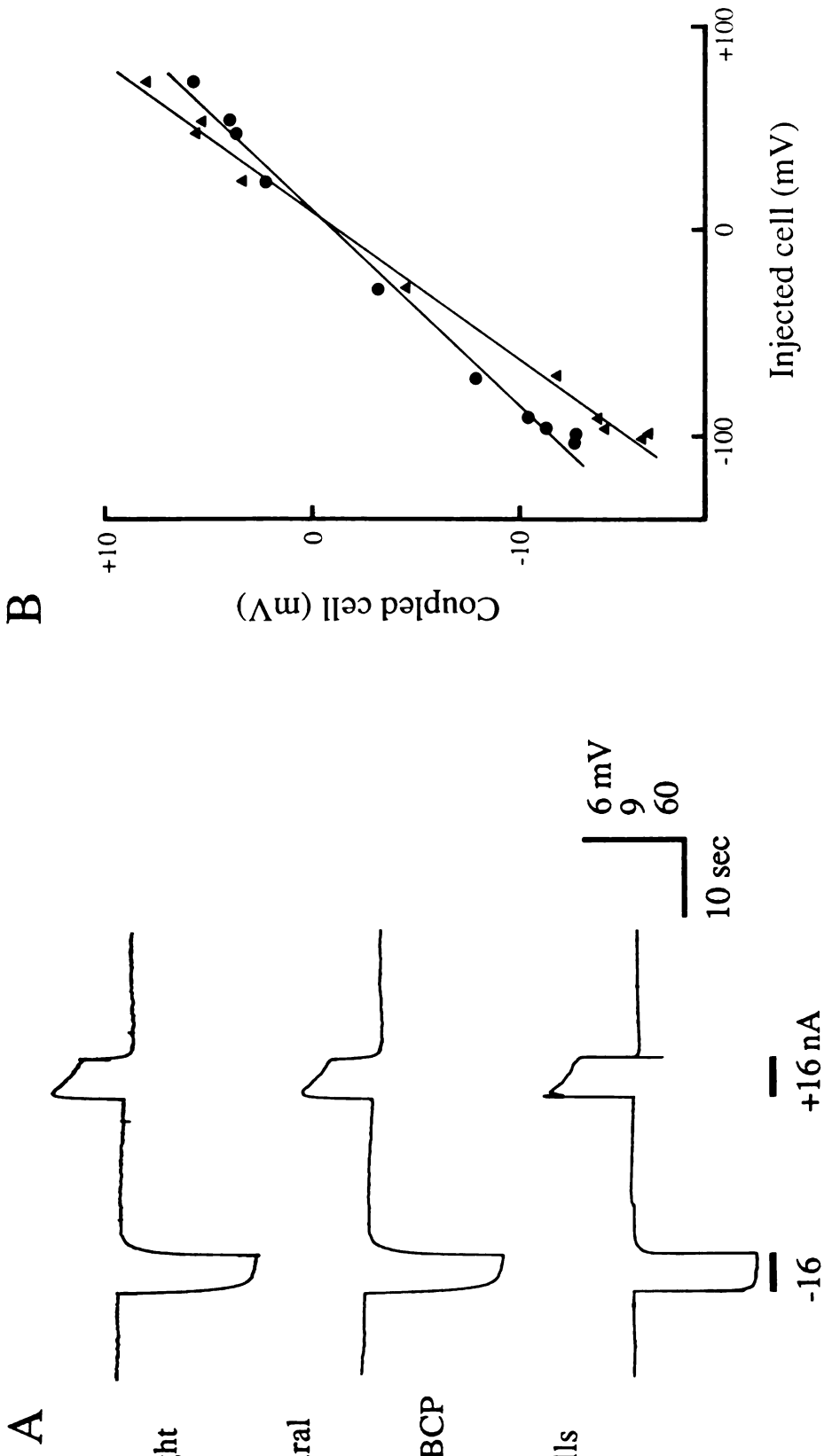

30 mV
30 sec
Right
Pleural
ELH/BCP
Cells

Figure 4 Electrical coupling between pleural ELH/BCP cells. Simultaneous intracellular recordings were made from 3 ELH/BCP cells in an isolated right pleural ganglion.

A. Hyperpolarizing or depolarizing current (bars) was injected through a double-barreled electrode into the cell shown in the bottom trace. The voltage responses in the cell being injected and in two other pleural ELH/BCP cells are shown in the traces. (When the double electrode was pulled out of the cell, passing current did not cause significant voltage shifts in any of the three electrodes)

B. Plot of the voltage responses to current injections in the injected cell (abscissa) vs. the responses in the two other cells (ordinate). The responses were approximately linear. The solid lines drawn represent coupling coefficients of 0.11 and 0.14, and are the least-squares best linear fits for the data from each cell.


A
Right
Pleural
ELH/BCP
Cells
-16
+16 nA
10 sec
9
60
6 mV
B
Coupled cell (mV)
+10
0
-10
-100
0
+100
Injected cell (mV)

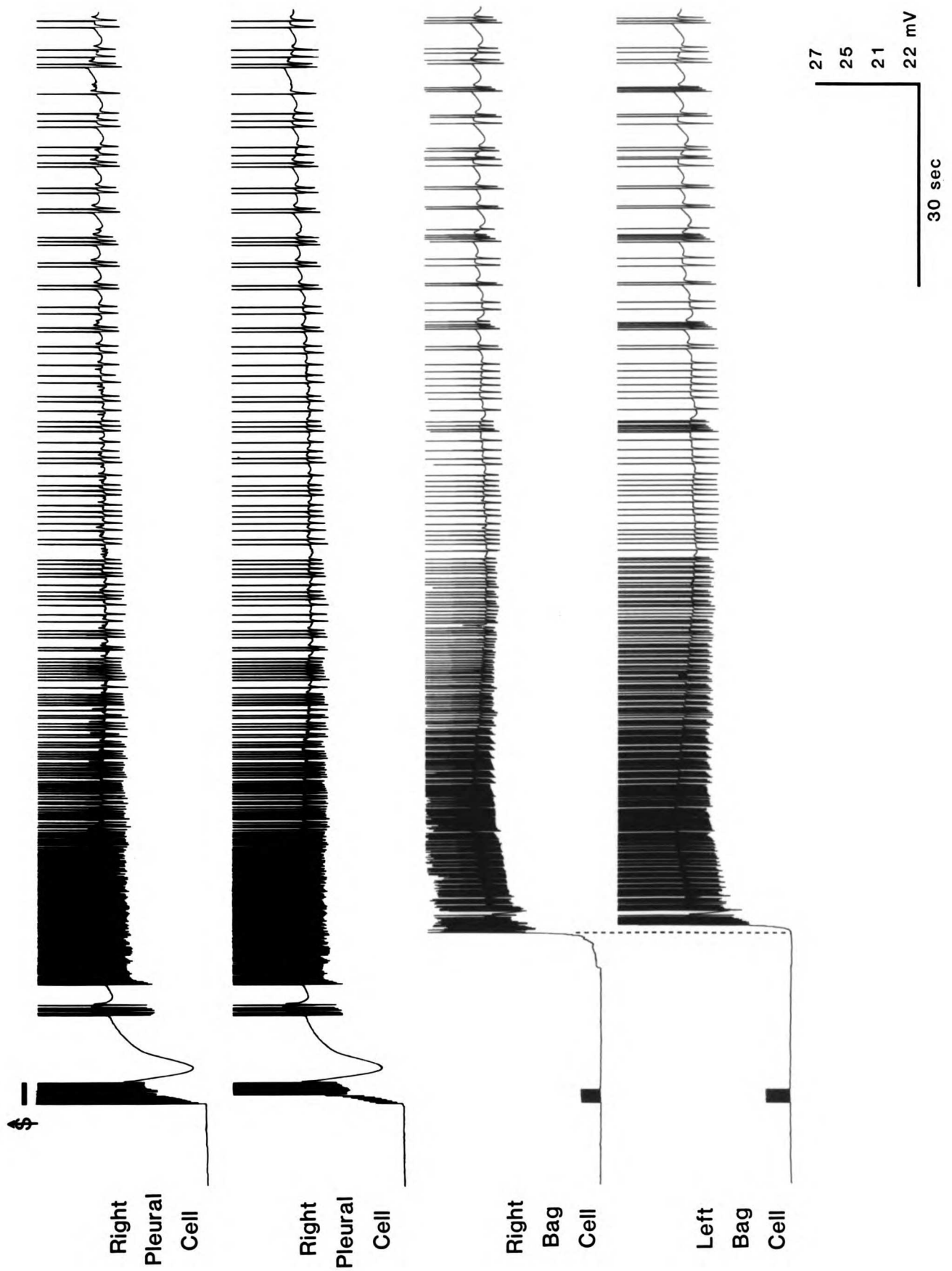
respectively). Similar coupling coefficients were observed in two other experiments, although precise measurements could not be made because single barreled electrodes were used to inject current.

Functional relationship between pleural ELH/BCP cells and the bag cells

To determine whether the pleural ELH/BCP cells activate the bag cells, simultaneous recordings were made from right pleural ELH/BCP cells and bag cells in preparations containing a right pleural ganglion and abdominal ganglion, with intact right connectives. Intracellular stimulation of a single pleural ELH/BCP cell (Fig 5, top trace) produced a synchronous burst discharge in the pleural ELH/BCP cells (top 2 traces). After a delay, this produced a depolarization and burst discharge in the ipsilateral bag cells (third trace), which in turn caused a depolarization and burst discharge in the contralateral bag cells (bottom trace). The spikes in the bag cells did not appear to be synchronized with spikes in the pleural cells, even with a transmission delay between the ganglia taken into consideration. Bag cell burst discharges were initiated by intracellular stimulation of pleural ELH/BCP cell burst discharges in 7 of 7 preparations.

The converse experiment was also performed, to see whether bag cell activity causes pleural ELH/BCP cell activity. Intracellular recordings were made from right

Figure 5 Activation of bag cells by pleural ELH/BCP cells. Simultaneous intracellular recordings were made from 2 right pleural ELH/BCP cells, 1 right bag cell, and 1 left bag cell, with the right pleuroabdominal connective intact. The pleural cell in the top trace was intracellularly stimulated with depolarizing pulses (bar) to initiate a burst discharge. This caused depolarization and activation of the right bag cells. The left bag cells also depolarized and began discharging after the right bag cell burst discharge began (marked by the dotted line). (Spikes are clipped).


Right
Pleural
Cell
Right
Pleural
Cell
Right
Bag
Cell
Left
Bag
Cell
27
25
21
22 mV
30 sec

pleural ELH/BCP cells and bag cells, and bilateral bag cell burst discharges were initiated by focal extracellular stimulation of the left bag cell cluster. The stimulation procedure was previously shown to activate bag cells quite selectively (30). In 2 of 5 experiments, bag cell burst discharges did trigger burst discharges in the pleural ELH/BCP cells. Fig 6 shows one of these experiments in which activation of the bag cells caused depolarization and initiation of a burst discharge in the pleural ELH/BCP cells.

In another 2 of these 5 experiments, bag cell burst discharges led to slow depolarizations, but no spike activity, in the pleural ELH/BCP cells. One of these cases is shown in Fig 7 (left side). Extracellular stimulation of the left bag cells (arrow) initiated a burst discharge in the left bag cells, which then triggered a right bag cell burst discharge. This was followed by a slow depolarization of 3.5 mV in the right pleural ELH/BCP cell, which did not reach threshold for a burst discharge. In the same experiment (Fig 7, right side), the pleural ELH/BCP cell was subsequently stimulated (arrow) to produce a burst discharge shortly after the bag cell burst discharge ended, during the bag cells' refractory period. This demonstrated that the pleural ELH/BCP cell was excitable and capable of a burst discharge. In this instance, however, the burst discharge in the pleural ELH/BCP cells had no effect on the bag cells, presumably due to the refractory period in the bag cells,

Figure 6 Activation of pleural ELH/BCP cell by the bag cells. Simultaneous intracellular recordings were made from a right bag cell and a right pleural ELH/BCP cell, with the right pleuroabdominal connective intact. A contralateral bag cell burst discharge was initiated by brief extracellluar stimulation of the left bag cell cluster (at the arrow). This bag cell burst discharge produced depolarization and activation of the pleural cell.

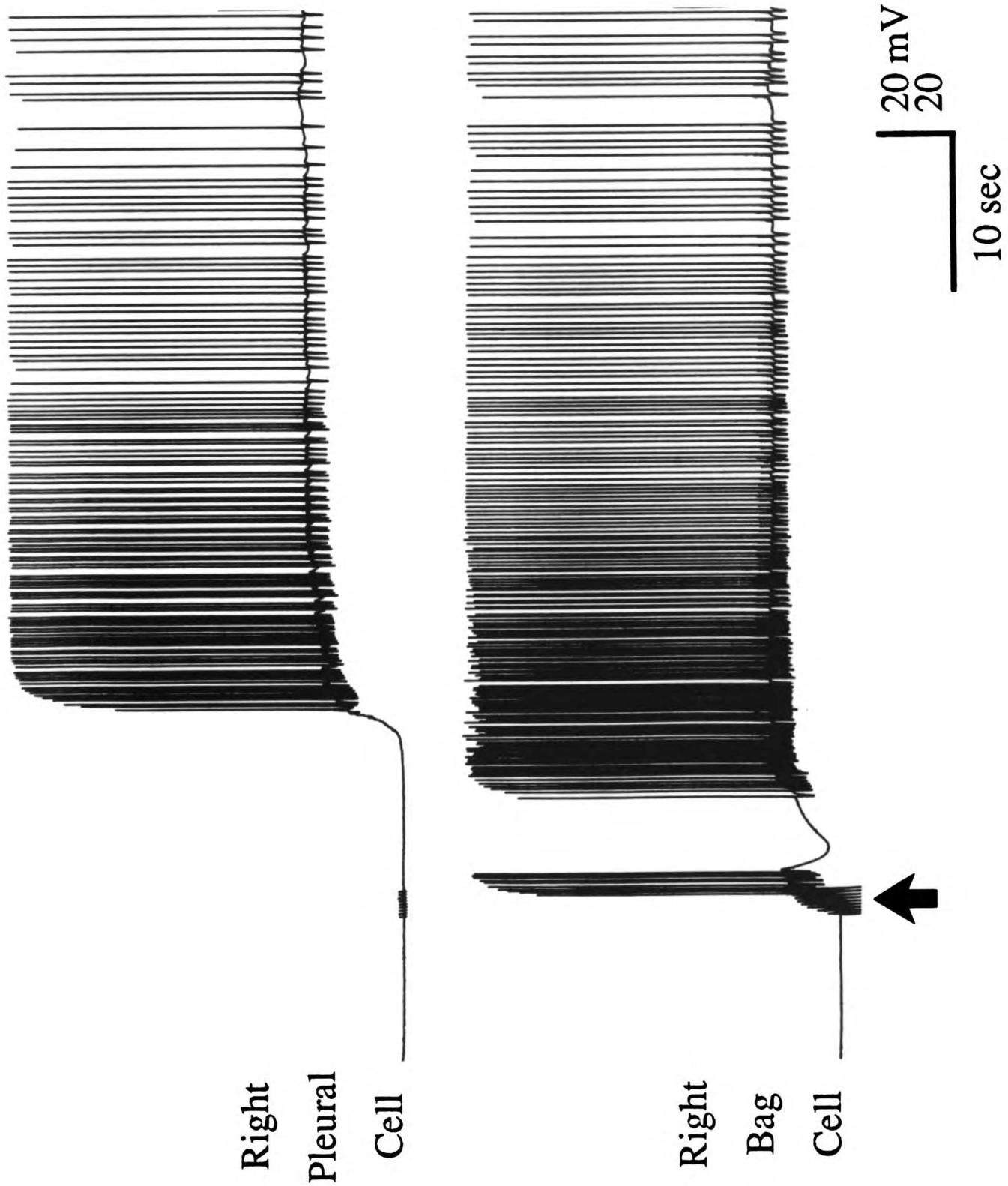
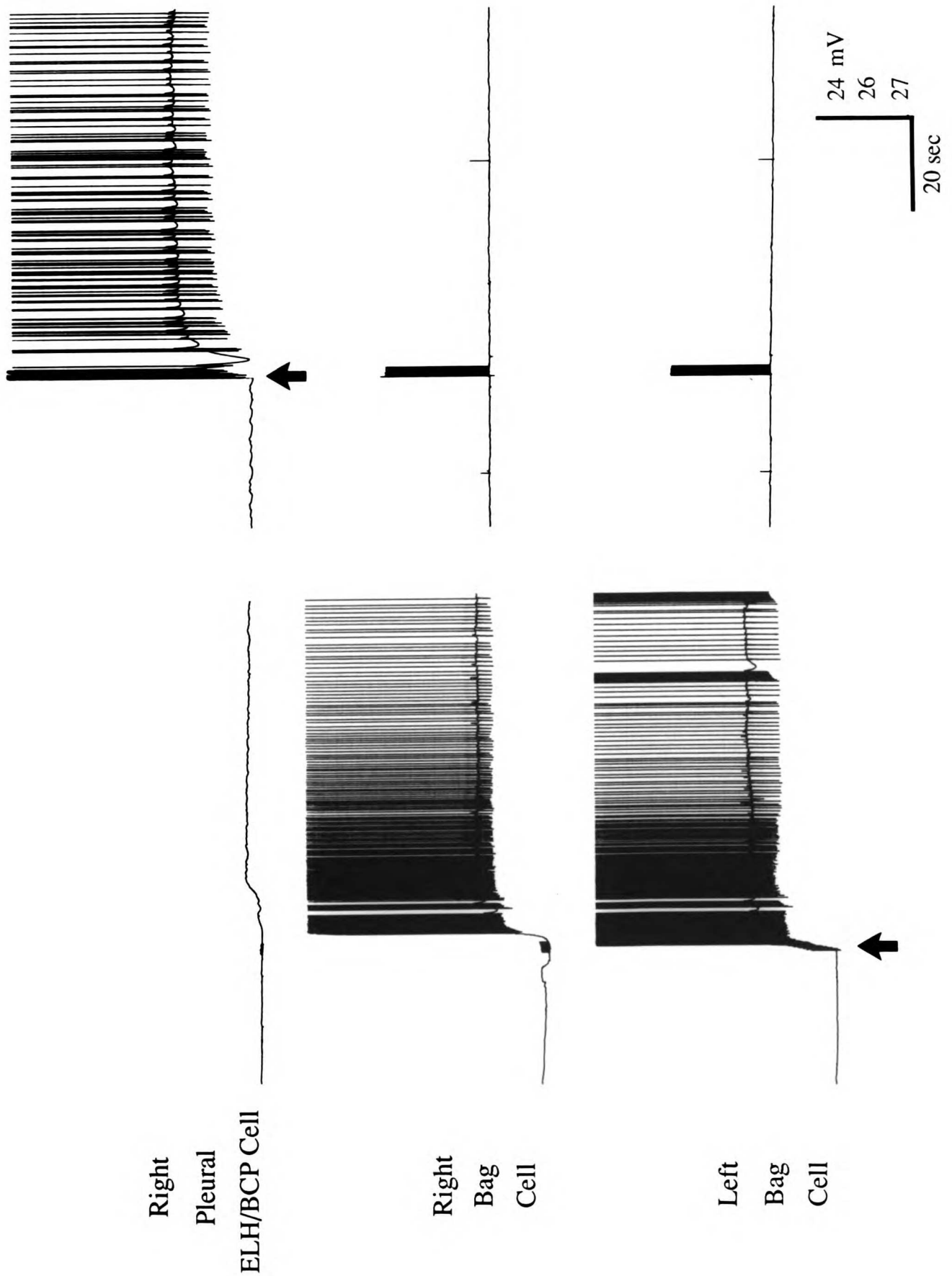

Right
Pleural
Cell
Right
Bag
Cell
20 mV
20
10 sec

Figure 7 Depolarizing response of pleural ELH/BCP cells to bag cell burst discharge. Simultaneous intracellular recordings were made from a right pleural ELH/BCP cell, a right bag cell, and a left bag cell. Extracellular stimulation of the left bag cell cluster (at the arrow, bottom left) produced a bilateral bag cell burst discharge, which caused a slow depolarization of the pleural cell that did not reach threshold for a pleural burst discharge (left side of figure). The bag cell burst discharge lasted 12 minutes. 5 minutes after the bag cell burst discharge ended, during the bag cells' refractory period, the right pleural ELH/BCP cell was stimulated intracellularly (arrow) to initiate a pleural burst discharge. No response was seen in either bag cell. This was the only experiment in which a pleural burst discharge was stimulated while the bag cells were refractory, and was the only instance in which it did not produce depolarization and a burst discharge in the bag cells.


Right
Pleural
ELH/BCP Cell
Right
Bag
Cell
Left
Bag
Cell
24 mV
26
27
20 sec

which is characterized by an inability of electrical or chemical stimulation to elicit depolarizing afterpotentials or discharges following a previous discharge (13,28,29).

The activation of bag cells by the pleural ELH/BCP cells is characterized by fast, depolarizing potentials superimposed on a delayed and slowly developing depolarization. These fast depolarizing potentials potentiated and developed into full action potentials (Fig 8A, middle trace). This activation of bag cells by pleural cells strongly resembles the activation of bag cells by either the contralateral bag cells, as seen in Fig 8A, bottom trace, and as previously described (22,30) or by electrical stimulation of the ipsilateral connective (19). The activation of pleural ELH/BCP cells by the bag cells had these same characteristics (Fig 8B), suggesting these interactions may share a common mechanism. In the case of activation of bag cells by the contralateral cluster, the fast depolarizing potentials represent the progressive invasion of antidromic spike potentials from the cuff neurites into the somata (22).

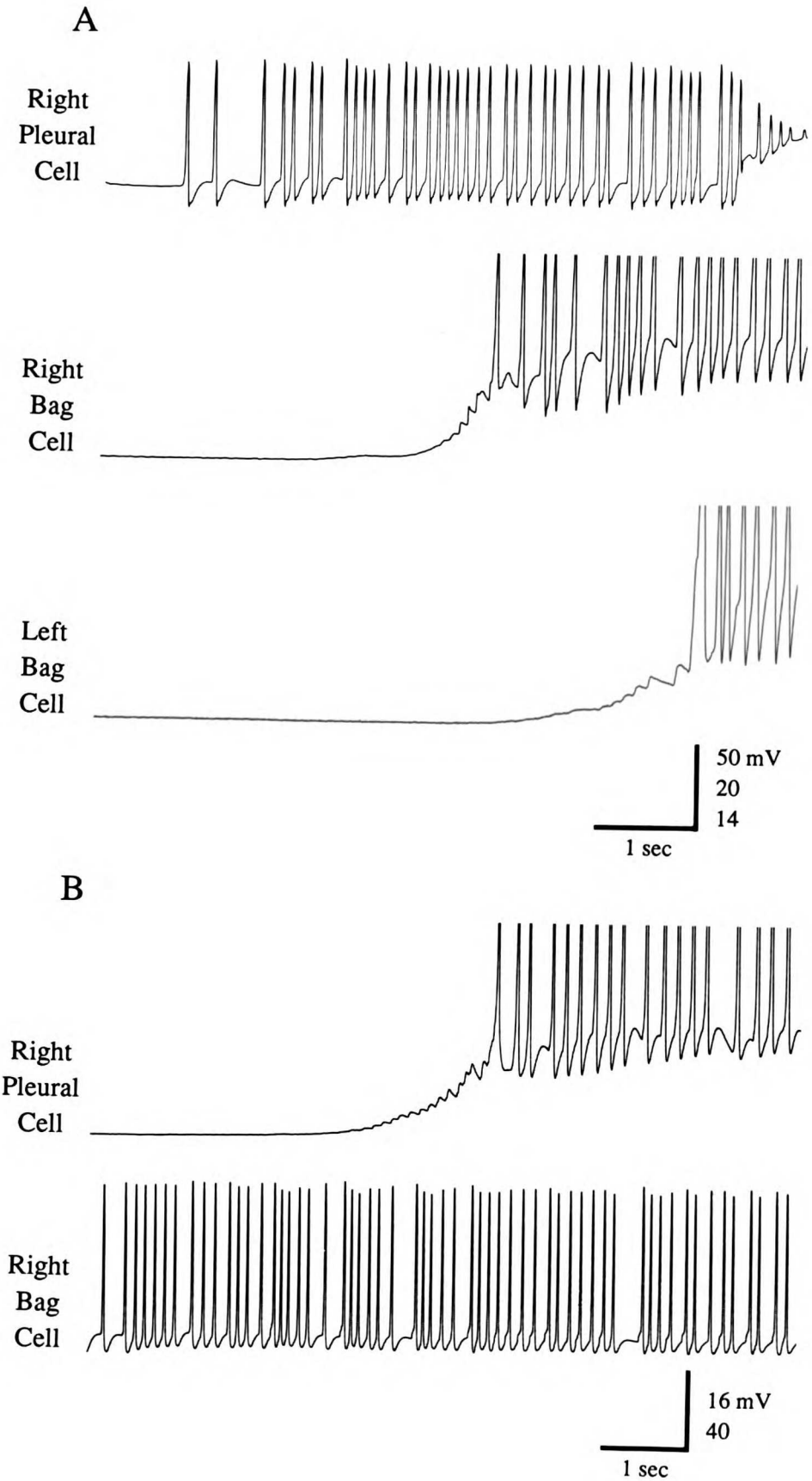
Connections of pleural ELH/BCP cells within the head ganglia

In 5 preparations, we recorded simultaneously from pleural ELH/BCP cells and miscellaneous other right pleural neurons, in an initial search for possible local targets. However, none of these cells had obvious responses to burst discharges in the ELH/BCP cells. (By comparison, strong

Figure 8 Spread of activation between pleural ELH/BCP cells and the bag cells shown at higher speed.

A. Activation of the bag cells. Simultaneous intracellular recordings were made from a pleural ELH/BCP cell, a right bag cell, and a left bag cell. A burst discharge was produced by intracellular stimulation of the pleural cell (top trace) 19 sec prior to the beginning of the traces. The activation of the right bag cell produced by this pleural burst discharge consisted of a slowly developing depolarizaion on which were superimposed quicker depolarizing potentials that potentiated into full action potentials. The right bag cell burst discharge then activated the left bag cells with a shorter delay, but with an otherwise apparently identical process. (The recording from the pleural cell was lost just before the end of this trace.)

B. Activation of the pleural ELH/BCP cells. This is a higher speed trace of the same 2 cells shown in Fig 6, beginning 7 sec after the stimulation of the bag cell burst discharge. The activation of the pleural cell by the bag cells displayed the same characteristics as the activation of the bag cells by the pleural cells shown in A.


A
Right
Pleural
Cell
Right
Bag
Cell
Left
Bag
Cell
50 mV
20
14
1 sec
B
Right
Pleural
Cell
Right
Bag
Cell
16 mV
40
1 sec

responses to bag cell burst discharges are seen in very many abdominal ganglion neurons.)

We were also interested in studying the possible role of pleural ELH/BCP cells in the previously described activation of bag cell burst discharges caused by stimulation of the cerebropleural connectives. In 7 preparations consisting of the abdominal ganglion, right pleural ganglion, right cerebropleural connective, and usually (5/7) the cerebral ganglion, extracellular stimulation of the cerebral end of the right cerebropleural connective failed to activate either the pleural ELH/BCP cells or the bag cells. We used stimulation that is normally very effective when applied to the pleuroabdominal connectives: trains of 1 - 5 msec pulses at 5 Hz, with the current gradually increased for each train up to the level at which physical damage to the nerve was seen, and far above the level at which psps onto other right pleural neurons were activated.

Previous reports of the activation of bag cells by cerebropleural stimulation (22,26) did not specify the stimulation parameters used, nor the rate of success, although Haskins and Blankenship did note that cerebropleural stimulation was less reliable than pleuroabdominal stimulation in activating bag cells (22). Our negative results may reflect either a different stimulation procedure or a difference in the preparations,

DISCUSSION

ELH/BCP cells in the right pleural ganglion

The results support the hypothesis that the pleural ELH/BCP neurons and associated cuff neurites comprise part of the descending neural pathway for activation of the bag cells and initiation of egg-laying behavior. The pleural ELH/BCP cells are the first identified neurons found to activate the bag cells. Direct intracellular stimulation of these neurons reliably initiated bag cell burst discharges. Thus the activation of these pleural ELH/BCP cells would be sufficient to initiate bag cell burst discharges. In contrast, activation of the bag cells was inconsistent in activating the pleural ELH/BCP cells, indicating a functional asymmetry in the interaction.

Clusters of immunoreactive ELH/BCP cells were found in all 8 right pleural ganglia examined, while single ELH/BCP cells were found in just 2 of 5 left pleural ganglia, indicating an asymmetry in the distribution. Two previous studies reported bilateral clusters of pleural ELH/BCP cells (40,51), while a third study reported finding them only in right pleural ganglia, and in at least one instance not even there (36). We had success in finding and recording from pleural ELH/BCP cells only after our attention was shifted to the right pleural ganglion by Painter et al.'s report of the asymmetry (36).

All studies have consistently found ELH/BCP-immunoreactive neuritic cuffs around the pleural ends of the

pleuroabdominal connectives. The bag cell neuritic cuffs at the abdominal ends of the connectives are the critical sites at which bag cell activity normally begins, and are also thought to be the major sites of electrical and chemical interactions among the bag cells (13,22,29). Indeed, bag cell neurites can support apparently normal burst discharge activity even when dissected away from bag cell somata (26). By analogy, the neuritic cuffs at the pleural ganglia may be the critical sites for the control and generation of activity in the pleural ELH/BCP cells.

Although ELH/BCP somata were consistently seen only in right pleural ganglia, bag cell burst discharges can be activated by stimulation of either the right or left pleuroabdominal connectives. The process of bag cell activation by stimulation of either connective strongly resembles that seen following direct activation of right pleural ELH/BCP cells, consistent with the idea that connective stimulation activates projecting ELH/BCP processes. This would suggest that the left pleural cuff neurites (which are invariably present) are also functionally coupled with the bag cells. In thinking about this system, it may useful to focus on the neurites as the critical integrative elements in the initiation and control of egg-laying, with the somata themselves serving mainly metabolic and synthetic functions (while also providing electrophysiologists access to events in the neurites).

Electrophysiological properties of pleural ELH/BCP cells

The basic electrophysiological properties of ELH/BCP cells in isolated right pleural ganglia were strikingly similar to those of the bag cells in the abdominal ganglion. This supports the suggestion that both groups of neurons arise from a common precursor pool during embryogenesis, and migrate to separate locations (16,34). However, we do not favor the characterization of the right pleural ELH/BCP cells as ectopic, or mislocated, bag cells (36), because they are so reliably present, and their physiological properties suggest they comprise an important part of the descending neural pathway that initiates bag cell activity.

One difference found between pleural ELH/BCP cells and bag cells was the strong electrical coupling demonstrated between pleural ELH/BCP somata. The coupling between bag cell somata in *Aplysia californica* or *A. brasiliana* has been too small to directy measure *in situ* (6,29), although there is good electrophysiological (18,22,29) and anatomical (17,27) evidence for bag cell electrical coupling in these species, and weak coupling has been directly measured *in situ* between pairs of bag cells in *A. dactyolema* (22). The greater coupling coefficient in the pleural cells may reflect a shorter electrical distance between the recording sites in the pleural ELH/BCP cell bodies and the sites of electrical coupling (presumably in the neuritic cuff). An alternative explanation is that the much larger number of bag cells per cluster (compared to the pleural clusters)

would be expected to dilute both the absolute number of electrical junctions and the proportion of total electrical conductance between each pair of bag cells, leading to smaller measured coupling coefficients between pairs of bag cells. In either case, for both the bag cells and the pleural ELH/BCP cells, the electrical coupling is sufficient to ensure highly synchronous spike activity.

A second difference between the pleural ELH/BCP cells and the bag cells is that the former were more easily and reliably activated by intracellular stimulation of individual cells. As with the electrotonic coupling, this may also be an effect of a smaller electrical distance between the somata and the site of burst discharge initiation, or of the smaller population of cells to be activated, and need not represent a fundamental biophysical difference. Alternatively, this may represent a genuine lower threshold for activation in the pleural ELH/BCP cells, consistent with the hypothesis that they discharge before the bag cells at the initiation of egg-laying.

Possible circuitry involving pleural ELH/BCP cells

The spread of activity between the pleural ELH/BCP cells and the bag cells strongly resembled the spread of activity between contralateral bag cell clusters (see Fig 8). Since the two bag cell clusters are thought to interact directly, without interneurons, in the neuritic cuffs (22,29), it seems most likely that the pleural ELH/BCP cells

also make direct connections with the bag cells in the neuritic cuffs. This would require that axons of the pleural ELH/BCP cells and/or the bag cells project between the pleural and abdominal ganglia. These projections are presumably bilateral, since the bag cells can be activated by electrical stimulation of either connective (as previously discussed). While the great majority of bag cell axons appear not to extend more than 2 cm along the pleuroabdominal connective (21), a few processes with the morphological characteristsics of bag cell axons have been observed further along the connective and in the vicinity of the pleural ganglion (15,23). Whether these represent ascending bag cell processes and/or descending pleural ELH/BCP cell processes is unknown. The demonstration of this possible pathway probably awaits the development of long range anatomical tracers effective in *Aplysia* neurons.

α-, β-, and γ-BCPs depolarize and activate bag cells, and the all-or-none burst discharge firing pattern in the bag cells is thought to involve positive feedback by these autoexcitatory neuropeptides (11,13). α-BCP, and presumably β- and γ-BCPs, are also expressed in the pleural ELH/BCP cells, and are candidate transmitters, in conjunction with possible electrical synapses, for mediating the descending activation of the bag cells. It is noteworthy in this regard that the only instance in which a burst discharge in the pleural ELH/BCP cells did not depolarize and activate the bag cells was when the bag cells

were refractory, a condition in which they also do not respond to the BCPs (13). The observation that bag cells bathed in low Ca^{++} medium sometimes cannot be activated by stimulation of the pleuroabdominal connective, even when they are still responsive to direct stimulation, was also interpreted as evidence for a chemically-mediated descending activation of the bag cells (19). In addition, the apparent similarity of the positive feedback in the pleural ELH/BCP cells to that in the bag cells suggests a common mechanism, predicting that the pleural ELH/BCP cells also have autoexcitatory responses to the BCPs. The atrial gland peptides A and B, which are structurally related to the BCPs, trigger bag cell activity when applied to pleural ganglia (36), and it is possible that they act by exciting the pleural ELH/BCP cells. This would be in contrast to their apparent lack of activity when applied directly to the bag cells (13,36, but see 25).

In addition to activating the bag cells, the pleural ELH/BCP cells may have effects on other neurons in the head ganglia, particularly if they are the source of ELH/BCP-immunoreactive fibers in the head ganglia (16,36,40). Some buccal ganglion neurons have excitatory responses to ELH (53). While it was initially suggested that these cells respond to ELH released from the bag cells and dispersed hormonally through the hemocoel, it has alternatively been proposed (16,40) that they respond to ELH released locally from the immunoreactive fibers in the head ganglia, in the

same mode of action ELH has in the abdominal ganglion (9,33). It is also possible that the ELH/BCP cells in the head ganglia can release significant amounts of ELH into the blood; this might explain the problematic report that 4 of 15 *Aplysia* with their abdominal ganglia and bag cells removed still displayed spontaneous egg-laying (37). The giant cell LPl_1 in the left pleural ganglion is reportedly inhibited by α-BCP during bag cell burst discharges (48). This is presumably due to locally released α-BCP, since α-BCP is rapidly inactivated by proteolysis in the abdominal ganglion, and would not be expected to diffuse long distances (46,50).

The present demonstration that the pleural ELH/BCP cells activate the bag cells begs the question of what, then, activates the pleural cells. One possibility is that the pleural ELH/BCP cells and associated cuff neurites are the principal components of an endogenous activity cycle, modulated by physiological and environmental stimuli, and constitute the key integrative site for the initiation of egg-laying behavior. However, the evidence that the descending neuronal pathway appears to begin in the cerebral ganglion suggests the alternative possibility that the cerebral ELH/BCP cells may activate the pleural ELH/BCP cells. In this model, all the known ELH/BCP neurons are functionally coupled and comprise a distributed neuroendocrine system controlling egg-laying behavior. This is analogous to what has been proposed for the oxytocin

system in mammals, in which neurons in 4 discrete nuclei fire synchronously during suckling, through a mechanism that may also involve both electrical coupling and the autoexcitatory effects of oxytocin (5).

The idea that neurons sharing a transmitter phenotype might also be functionally related to particular behaviors is an old one that has not generally held up for classical transmitters (see 8), but seems more promising for some of the peptide neurohormones, whose central actions may serve to coordinate complex behaviors with their peripheral neuroendocrine roles. Many mammalian neuropeptides have been associated with specific behavioral functions (35). In the case of the ELH/BCP peptides, this idea was reformulated as "one gene-one behavior" to reflect the idea that multiple neuropeptide cotransmitters processed from a common precursor protein function coordinately to produce egg-laying behavior (33,42,47). A corollary of this hypothesis was the prediction that neurons expressing the ELH/BCP gene are involved in egg-laying behavior. The present finding that pleural ELH/BCP cells are functionally coupled with the bag cells provides further experimental support for this idea.

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Chapter 5

IDENTIFICATION OF *APLYSIA* NEURONS CONTAINING IMMUNOREACTIVE FMRFamide

SUMMARY

Electrophysiological and immunocytochemical techniques were used in the abdominal ganglion of *Aplysia* to identify neurons containing immunoreactive FMRFamide. Large numbers of neurons were immunoreactive for FMRFamide, including R2, L2, L3, L4, L5, L6, 2 cells tentatively identified as L12 and L13, and a previously unidentified cluster on the ventral surface of the right lower quadrant. There was also heavy labelling of fibers, often with beaded varicosities, throughout the neuropil, the cell layers, and the sheath overlying the ganglion This data provides further evidence that FMRFamide is an important neurotransmitter in *Aplysia*. The demonstration of immunoreactive FMRFamide in the giant cholinergic neurons R2 and $LP1_1$ suggests that these well-studied and experimentally convenient cells use acetylcholine and an FMRFamide-like peptide as cotransmitters.

INTRODUCTION

The neuropeptide FMRFamide (Phe-met-arg-phe-amide) was discovered as a cardioexcitatory factor from ganglia of the mollusc Macrocallista nimbosa (1). Immunoreactive- (IR-) FMRFamide has subsequently been described in the nervous systems of coelenterates, insects, fish, chickens, and mammals (2-5), as well as other molluscs (3,6,7), including localization to the identified neuron C3 in Helix (8). The calcium-dependent release of IR-FMRFamide from nervous tissues has been demonstrated in Macrocallista (9). Synthetic FMRFamide has widespread physiological activity in molluscs (7,8,10) and mammals (11-13), including excitatory and inhibitory effects on neurons im molluscs (14-16) and rats (4, 17, 18). [Recent studies from several laboratories of the physiological effects of FMRFamide on *Aplysia* neurons are discussed in Chapter 6 of this dissertation.]

The abdominal ganglion of Aplysia is a convenient preparation for studying cellular communication in a central nervous system. Many of the large neurons are well characterized individuals with known neuronal and peripheral targets (19, 20). In a previous study, synthetic FMRFamide was found to have potent excitatory and inhibitory actions on abdominal ganglion neurons (14). To further investigate the possibility that FMRFamide is a neurotransmitter in this system, we studied the cellular distribution of FMRFamide immunoreactivity in the abdominal ganglion, and identified many of the large neurons which labelled for IR-FMRFamide.

MATERIALS AND METHODS

Materials

Large, sexually mature (0.5 - 2 kg) specimens of *Aplysia californica* were obtained from several *sources* (but primarily Sea Life Supply in Sand City, CA.) and maintained in an open system at Steinhart Aquarium, San Francisco. The anti-FMRFamide serum was generously provided by Dr. Eckard Weber of the University of Oregon; the specificity of these antibodies for the amidated terminus of FMRFamide has been previously described (5). Synthetic FMRFamide was obtained from Peninsula Laboratories. Lucifer yellow CH was obtained from Sigma. Fluorescein- and rhodamine- conjugated goat anti-rabbit antisera (GAR) were obtained from Cappel.

Identification and marking of neurons

Animals were anaetheized by injection of isotonic $MgCl_2$ (1/3 of body weight), and the ganglia dissected out and pinned in a Sylgard dish filled with seawater. Glass microlelectrodes were filled with a filtered solution of 5% Lucifer Yellow in 0.15% LiC1, and intracellular recordings were made to identify individual neurons by their elecrophysiological (and other) charateristics (19). Identified neurons were marked by pressure injection of the Lucifer solution under visual control. The injected Lucifer did not appear to interfere with the subsequent labelling for IR-FMRFamide.

Tissue preparation

The caudal artery was cannulated with polyethylene tubing to allow efficient perfusion of the entire vascular space of the abdominal ganglion (as in 22). The ganglion was rinsed thoroughly with filtered fresh seawater, then fixed by perfusion with 4% paraformaldehyde in 0.1M Na phosphate buffered saline at pH 7.6 (PBS). After overnight incubation at 4°C, the ganglion was transferred to a 30% sucrose solution for 24 hours at 4°C. The tissue was cut in 20 µm serial sections on a cryostat, and transferred to wells containing 0.05 M PBS.

Immunohistochemical procedure

All washes and incubations were carried out at room temperature in wells with a stock solution of 1% normal goat serum and 0.3% Trition X-100 in 0.05 M PBS, except as noted. Sections were preincubated for 30 minures with 3% normal goat serum, then incubated for 48 hours at 4°C in rabbit anti-FMRFamide serum (preabsorbed with rat liver powder) at a dilution of 1:4000. Sections were rinsed for 30 minutes, incubated with filtered fluorescent-conjugated GAR for 30 minutes, and rinsed for 30 minutes. When Lucifer-injected cells were being studied, rhodamine GAR (at 1:50) was used to permit easy discrimination of the two signals; otherwise fluorescein GAR (at 1:40) was generally used. To control for nonspecific staining, some sections were reacted with anti-FMRFamide antisera which had been preincubated for 24

hours at 4°C with 100 μM synthetic FMRFamide; this preincubation eliminated the labelling. Sections were mounted on slides with Gelvatol, cover-slipped, and photographed. Magnifications given in the figures indicate the scale of the linear dimensions of the final figure relative to the dimensions of the mounted tissue.

RESULTS

Widespread IR-FMRFamide in the abdominal ganglion

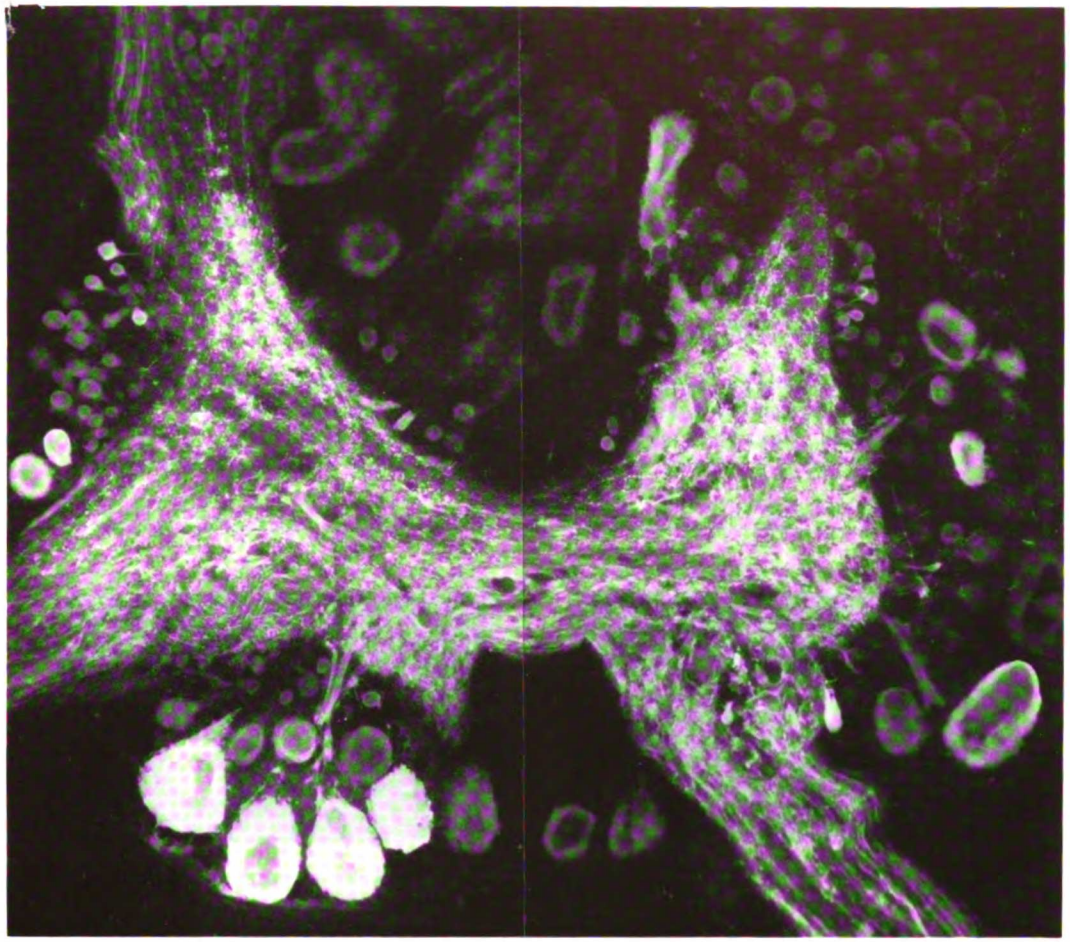
The abdominal ganglion contains large amounts of IR-FMRFamide (Figure. 1). Many neurons of all sizes and in most regions of the ganglion labelled for IR-FMRFamide, with a considerable range in the intensity of labelling. Labelled neurons often occurred in clusters. The neuropil and all major nerves were densely packed with labelled fibers. The cell layers and the connective tissue sheath overlying the ganglion also contained numerous FMRFamide-immunoreactive fibers with beaded varicosities.

Identified cells containing IR-FMRFamide

Individual neurons were identified electrophysiologically and filled with Lucifer Yellow dye prior to immunocytochemistry. Fig. 2 illustrates the use of this technique with neuron L5. Fig. 2A shows a section containing L5, which had been injected with the Lucifer dye. Fig. 2B shows the same section labelled for FMRFamide-like immunoreactivity, with L5 and two other large cells (probably L12 and L13) clearly labelled. L12 and L13 were not positively identified as FMRFamide-immunoreactivity; however, two large cells in the expected locations of L12

Figure 1 FMRFamide immunoreactivity in the abdominal ganglion

Photographic montage of a 20 µm section through the abdominal ganglion, cut just to the ventral side of the midline. Many individual neurons and clusters of neurons are labelled for IR-FMRFamide. The neuropil and the nerves exiting the ganglion are also heavily labelled. (X 40)



and L13 consistently labelled. (L10, the other large cell in this region of the ganglion, did not label for IR-FMRFamide.) L2, L3, L4, L6, and R2 were also found to contain IR-FMRFamide; these 5 large labelled cells can be seen in Fig. 3A. $LP1_1$, the homolog of R2 in the left pleural ganglion (20), also labelled for IR-FMRFamide (data not shown). In addition, a cluster of about 10 medium-sized white cells on the ventral surface of the right lower quadrant of the abdominal ganglion labelled for IR-FMRFamide (figure 3B). These cells are in the same position as the ventral RB cells described by Frazier et al. (19), but can be distinguished from RB cells by morpological and electrophysiological criteria, and because they do not label for immunoreactive serotonin (data not shown). These cells were usually silent or firing tonically at a low rate. They received depolarizing inputs from L10, and appeared to have biphasic excitatory-inhibitory responses to bursts of interneuron II activity. They probably constitute a previously unidentified cell cluster.

FMRFamide-immunoreactive processes

IR-FMRFamide-labelled processes with beaded varicosities were seen throughout the abdominal ganglion. Some of these processes appeared to envelop individual neuronal somata, as shown in fig. 3C. Fig. 3D illustrates the rich supply of IR-FMRFamide fibers seen in the sheath overlying the bag cell clusters.

Figure 2 Identification of an FMRFamide-immunoreactive processes neuron as L5.

A. L5, identified by morphological and electrophysiological criteria (19), and stained by pressure injection of Lucifer Yellow. (X 25)
B. FMRFamide immunoreactivity in the same section, labelled with a Rhodamine-conjugated second antiserum. L5 is shown to contain immunoreactive FMRFamide. The two other large labelled cells in this section are probably L12 and L13.

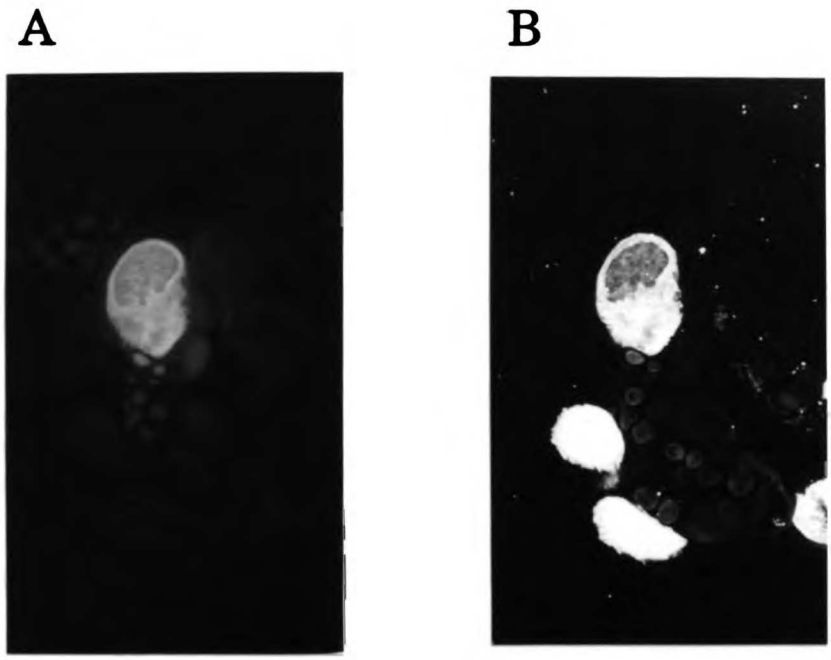

A
B

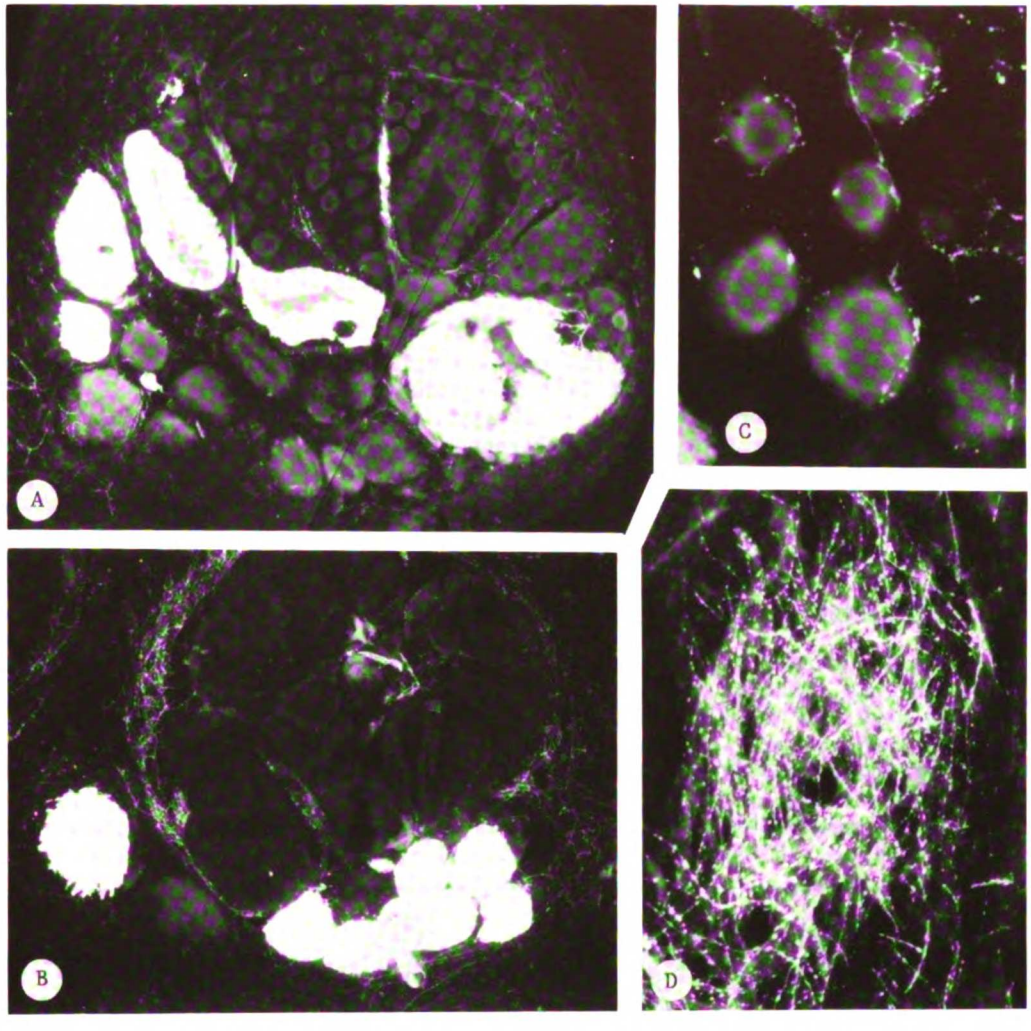
Figure 3 Immunoreactive FMRFamide neurons in the abdominal ganglion.

A. IR-FMRFamide in R2 and the large left upper quadrant neurons. R2 is the giant cell on the right. The four large labelled cells on the left are L2, L3, L4, and L6. (X 50)

B. A cluster of neurons in the ventral right lower quadrant containing IR-FMRFamide. The large labelled neuron on the left is probably L12 or L13. (X40)

C. IR-FMRFamide fibers with beaded varicosities in close apposition to unidentified somata. (X 200)

D. Section through the sheath overlying the bag cells, showing very rich supply of FMRFamide-immunoreactive fibers with beaded varicosities. (X 100)


A
B
C
D

DISCUSSION

FMRFamide-like immunoreactivity in the abdominal ganglion

These results demonstrate that large amounts of IR-FMRFamide are present in the central nervous system of Aplysia, including several large, identified cells. Immunocytochemical methods cannot unambiguously identify the antigenic substance(s) responsible for the labelling, and indeed several distinct peptides have been recognized by anti-FMRFamdie antisera in other systems (2,4,6,12). However, FMRFamide has been isolated from head ganglia of A. brasiliana (23) and from abdominal ganglia of A. californica (35); thus much of the labelling we see probably represents authentic FMRFamide. [This issue is discussed briefly below, and in further detail in Chapter 8.]

Many Aplysia abdominal ganglion neurons respond to arterially perfused synthetic FMRFamide (14). The Ca^{++}-dependent ganglionic release of IR-FMRFamide has been demonstrated in a bivalve mollusc (9) (though not, to our knowledge, in Aplysia or other gastropod molluscs). From these considerations, and given the abundance of IR-FMRF-amide in the abdominal ganglion and its localization to neurons and nervous processes (including presumed release sites), it seems probable that FMRFamide is an important central neurotransmitter in the abdominal ganglion of Aplysia.

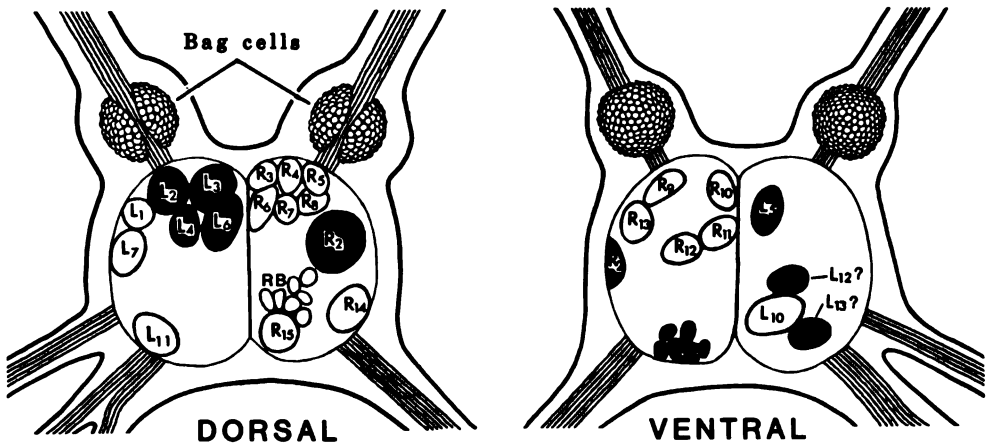
Identified neurons containing IR-FMRFamide

The identified cells found to contain IR-FMRFamide are shown in Fig. 4. With previous findings for cells L10, R2, L11, and R3-R13, candidate transmitters have now been identified for most of the large identified cells in the ganglion, as well as the bag cells and the RB and LD clusters (22,24-26). Except for LP11 and R2 (discussed below), little is known about the functions of the identified cells containing IR-FMRFamide. The finding that a FMRF-amide-like peptide is likely to be a major secretory product of these cells will contribute to the eventual understanding of their physiological roles.

Tissue specific +/- screening has been used to isolate presumed neuropeptide precursor genes from several groups of abdominal ganglion cells (26). This identification of FMRFamide-immunoreactive neurons enables the selection of cells for cloning of the gene encoding FMRFamide-like peptides in Aplysia. Using this approach, a candidate FMRFamide precursor gene was isolated and cloned (R. Scheller, personal communication). [One gene, expressed in R2, L12, and L13, encodes 24 copies of FMRFamide and 1 of FLRFamide (36). A second gene, expressed in L5, encodes a distinct peptide with a carboxy terminal RFamide (37). Thus the immunocytochemical labeling reported in this paper represents multiple neuropeptides, though all of them presumably share the carboxy terminal RFamide recognized by the antisera.]

Figure 4 Identified FMRFamide-immunoreactive neurons in the abdominal ganglion.

Schematic diagram of the abdominal ganglion shows identified neurons which contained immunoreactive FMRFamide. The blackened cells labelled for IR-FMRFamide, while the white cells did not. The identification of two of the large labelled neurons as L12 and L13 has not been positively confirmed.


Bag cells
L1
L2
L3
L4
L5
L7
L11
R1
R2
R3
R4
R5
R6
R7
R8
RB
R14
R15
DORSAL
R9
R10
R11
R12
R13
L10
L12?
L13?
VENTRAL

IR-FMRFamide processes in the abdominal ganglion

The presence of immunoreactive fibers with beaded varicosities in close apposition to neuronal somata suggests that FMRFamide is released directly onto somata to act via somatic receptors. A similar relationship of immunoreactive serotonin fibers to somata has been described (27,28), and many or most abdominal ganglion neurons do have somatic FMRFamide receptors (14). In addition, the abundance of IR-FMRFamide processes in the ganglionic sheath raises the possibility that FMRFamide is released into the vascular spaces, and may diffuse to act nonsynaptically on target cells, as is the case with egg-laying hormone (22). Another possibility, given the generally potent effects of FMRFamide on molluscan smooth muscle (10) and in particular its strong vasodilatory effects in the abdominal ganglion (14), is that FMRFamide acts directly on the vasculature to regulate circulation through the abdominal ganglion.

The sheath surrounding the bag cells contains many IR-FMRFamide fibers with beaded varicosities. This region is also densely packed with bag cell processes (19, 28, 29), suggesting possible axo-axonic relationships. Since FMRFamide has direct electrical effects on bag cells (L. Stone, personal communication), these IR-FMRFamide processes may be involved in the modulation of bag cell impulse activity or secretion.

IR-FMRFamide in the giant cholinergic neurons R2 and $LP1_1$

The giant neurons R2 and $LP1_1$ are the largest neurons known, with diameers of up to 1 mm (19), and they have been highly favored for single cell biochemical, physiological and developmental studies. Their size permits even microdisseciton of individual isolated somata into parts (30). By several criteria, including the conversion of microinjected {^{3}H} choline to acetylchloine (24), R2 and $LP1_1$ are considered cholinergic. R2 and $LP1_1$ are also known to innervate the body wall and to control the secretion of mucus (31). R2 and $LP1_1$ synapse onto each other, though this is apparently a purely electrical interaction (32, 33). While no neuronal targets of chemical transmission have been described for these cells in vivo, in primary cell culture R2 does make chemical synapses which have both cholinergic and non-cholinergic properties, implying the release of both acetylcholine and a cotransmitter (34). The present finding that R2 and $LP1_1$ contain an FMRFamide-like peptide in addition to acetylcholine makes them attractive models for studies of cotransmission.

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Chapter 6

PHARMACOLOGY OF FMRFamide AND RELATED PEPTIDES ON NEURONS IN THE ABDOMINAL GANGLION OF APLYSIA

SUMMARY

The neuronal responses to FMRFamide* and structurally related peptides were studied in the abdominal ganglion of *Aplysia*. FMRFamide had widespread excitatory, inhibitory, and biphasic effects when arterially perfused at concentrations of 0.1 to 10 μ*M*. The stereoisomer FMR*d*Famide mimicked only the excitatory effects of FMRFamide, while the heptapeptide YGGFMRFamide had only inhibitory effects, suggesting that the actions of FMRFamide in the abdominal ganglion involve at least two distinct receptor subtypes. No responses were seen to met-enkephalin (YGGFM), met-enkephalin-arg-phe (YGGFMRF), or γ_1-MSH (), and the bag cell peptides were shown to act on separate receptors. The neuronal responses were used as a bioassay to identify a fraction containing native FMRFamide in abdominal ganglion extract.

*The standard one-letter notation for amino acids (IUPAC/IUB Commission on Biochemical Nomenclature, 1968) is used throughout this paper.

INTRODUCTION

The "molluskan cardioexcitatory neuropeptide" FMRFamide (phenylalanyl-methionyl-arginyl-phenylalaninamide) is likely to be an important neurotransmitter in *Aplysia*. FMRFamide-like immunoreactivity is very widespread in the central nervous systems of *Aplysia brasiliana* (Lehman, Price and Greenberg 1984) and *A. californica* (Brown et al. 1984, 1985; McCaman and Ono 1985; Schaefer et al. 1985). Excitatory and inhibitory effects of synthetic FMRFamide on abdominal ganglion neurons were found by Linda Padgett and Mayeri in 1980 (unpublished), and first reported by Stone and Mayeri (1981). FMRFamide has been shown to have a wide variety of physiological effects on *Aplysia* neurons (Stone and Mayeri 1981; McCaman and Ono 1985; Ocorr and Byrne 1985; Ruben, Johnson and Thompson 1986; Goldberg et al. 1987; Brezina, Eckert and Erxleben 1987a, 1987b; Belardetti, Kandel and Siegelbaum 1987; Kramer et al. 1988).

A number of neuropeptides structurally related to FMRFamide have been described in mollusks (reviewed in Price 1986). Based on molecular genetic studies, the FMRFamide-like immunoreactivity in *Aplysia* represents heterogeneous neuropeptides (Schaefer et al. 1985; Shyamala, Fisher and Scheller 1986). Heterogeneity of FMRFamide receptors was first described in molluskan peripheral tissue (Greenberg et al. 1983). The central nervous system of *Helix* manifests at least four different receptor subtypes (Cottrell and Davies 1987), and McCaman and Ono (1985) reported evidence for

distinct excitatory and inhibitory FMRFamide receptors in *Aplysia* cerebral ganglion neurons, suggesting the likelihood of multiple FMRFamide receptors in the abdominal ganglion as well. The work reported here was undertaken to clarify the effects of FMRFamide and related peptides on abdominal ganglion neurons, and to search by bioassay for native FMRFamide-like peptides in abdominal ganglion extracts.

METHODS

Experiments were performed at room temperature on abdominal ganglia dissected from anaesthetized, sexually mature specimens of *Aplysia californica*. Conventional electrophysiological recording techniques were used as previously described (Mayeri et al. 1979, 1985). Peptides were applied either by arterial perfusion through the caudal artery with the sheath intact, or by pressure ejection onto desheathed neurons. All the reported results were repeatable, with $N \geq 2$. Synthetic peptides were obtained from Peninsula Laboratories. HPLC fractions of abdominal ganglion extracts were prepared by Brad Fowler and Barry Rothman (the details of this purification have been published in Rothman et al. 1985).

RESULTS

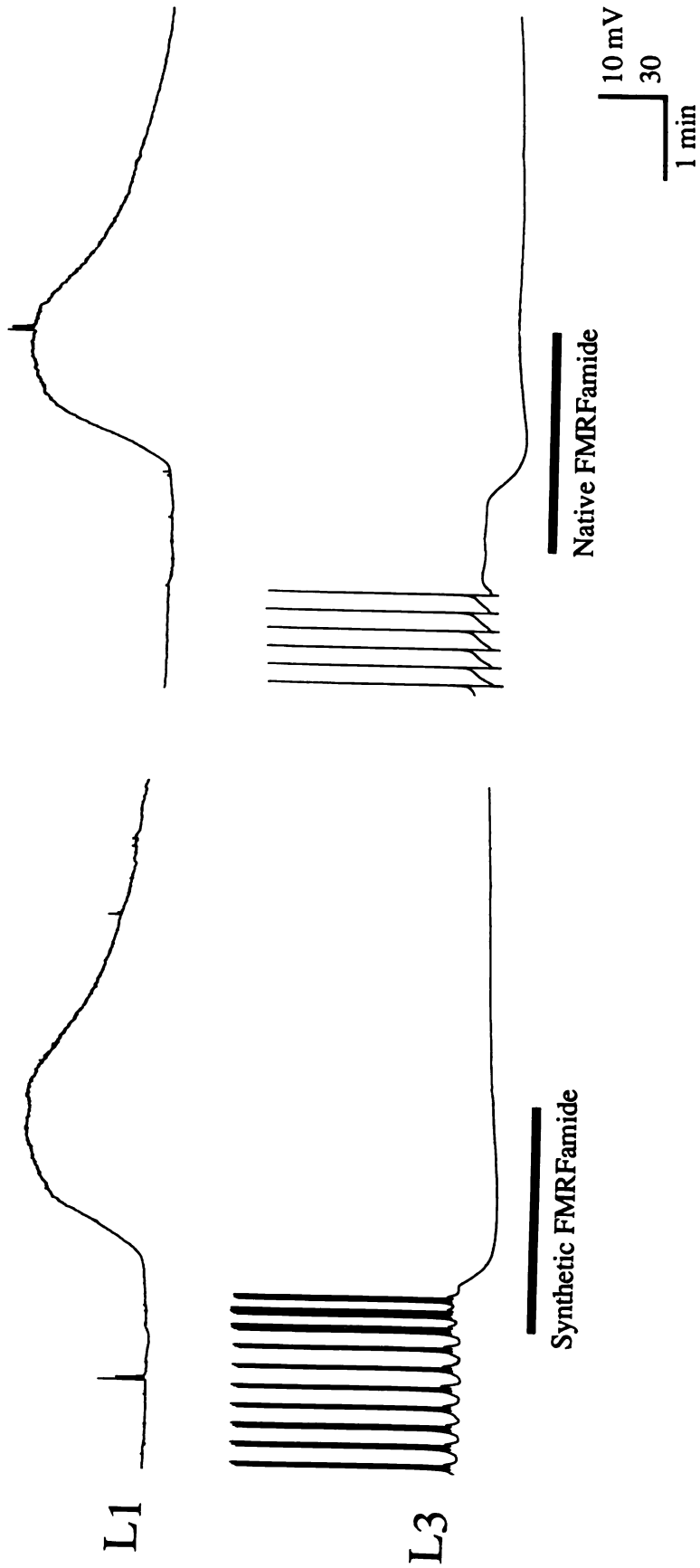
When arterially perfused into the abdominal ganglion, FMRFamide had widespread excitatory, inhibitory and biphasic effects. The simultaneous inhibition of the LUQ neurons and excitation of L1 and R1, as first reported by Stone and Mayeri (1981), is shown in figure 1A in response to synthetic FMRFamide. The applied concentration shown (20 μM) was rather high, being about 20 and 200 times higher than the response thresholds for L1 and L3, respectively. These responses were used to bioassay HPLC fractions of abdominal ganglion extracts (prepared by Brad Fowler and Barry Rothman) for a native FMRFamide-like peptide. One major fraction displayed the characteristic conjoint excitatory/inhibitory effects of FMRFamide. Figure 1B shows the effects of this fraction in the same preparation. Further purification of this fraction and amino acid sequence analysis positively identified this peak as authentic FMRFamide (Rothman et al. 1985). No other fractions were found with the conjoint excitatory and inhibitory effects of FMRFamide, and the fractions with other neuronal effects were identified as peptides from the bag cell and R_{3-14} precursors. Thus FMRFamide appears to be the major FMRFamide-like peptide in the abdominal ganglion.

To look for possible heterogeneity in the receptors mediating the excitation of L1/R1 and the inhibition of the LUQs, we arterially perfused FMRFamide and the FMRFamide analogs shown by McCaman and Ono to discriminate *Aplysia*

Figure 1. Conjoint excitatory and inhibitory effects of FMRFamide on L1 and L3.

A. Arterial perfusion of 20 μM synthetic FMRFamide produces excitation of L1 and inhibition of L3.

B. Arterial perfusion of 20 μM native FMRFamide has apparently identical effect. (The weak response in L3 which began just befor the perfusion was due to the presence in the cannula of a previous dose at a lower concentration, which was pushed into the ganglion by the perfusion tube.)


10 mV
30
1 min
Native FMRFamide
Synthetic FMRFamide
L1
L3

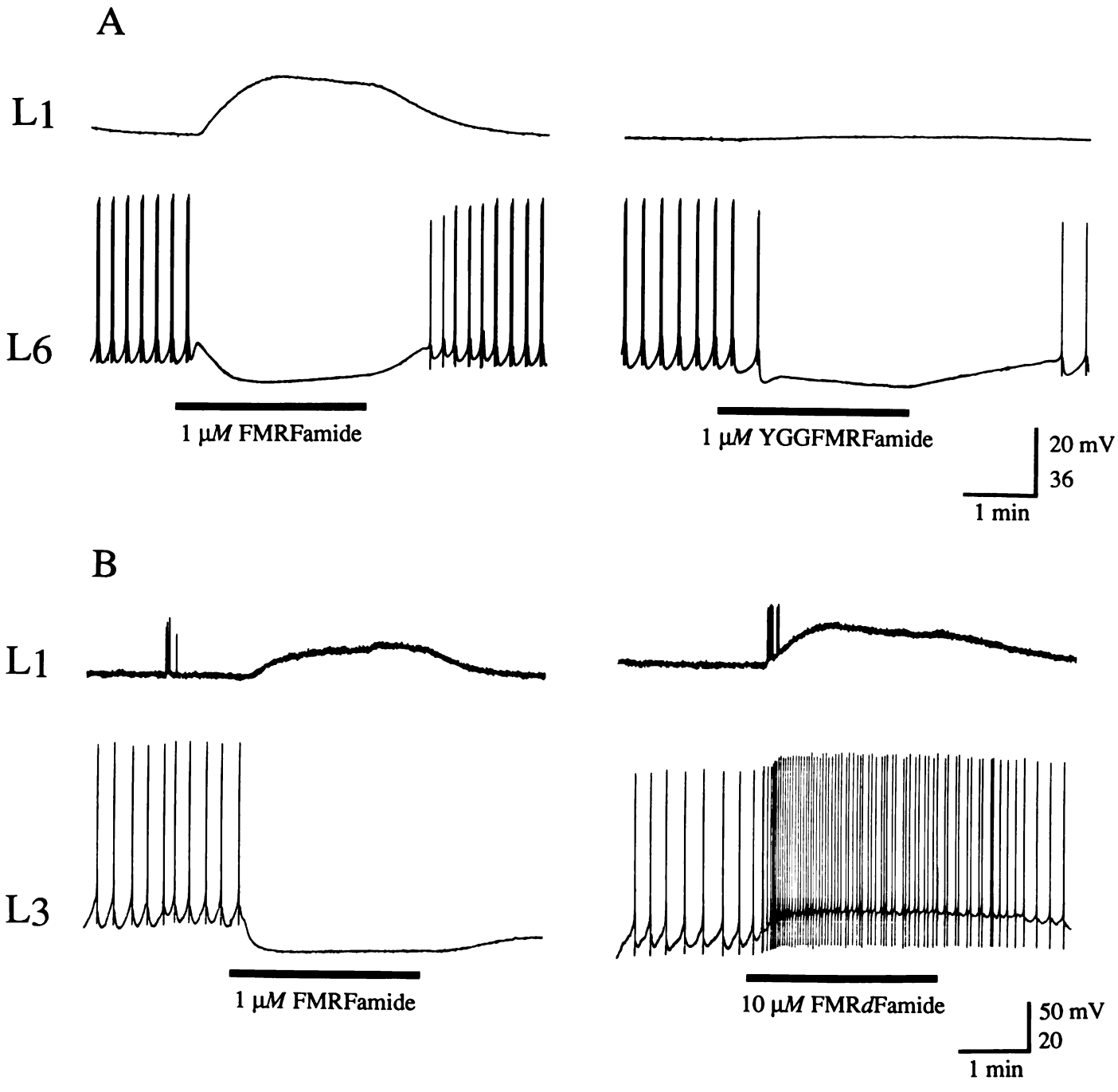
central FMRFamide receptors. Figure 2A shows that the heptapeptide YGGFMRFamide is at least as potent as FMRFamide in producing inhibition of L6, but has only a slight depolarizing effect on L1. Figure 2B (data from a different preparation) shows that the stereoisomer FMR*d*Famide, at substantially higher concentrations, mimics the excitation of L1 by FMRFamide, but also produces excitation of L3. The most parsimonious explanation for these results is that FMRFamide acts through two receptor subtypes, each of which responds to authentic FMRFamide but is selective for only one of the two analogs. The excitatory response of L3 to FMR*d*Famide suggests that this neuron expresses both receptor subtypes, but the excitatory response to FMRFamide may usually be obscured by a stronger inhibitory response.

While the response of LUQs to arterially perfused FMRFamide was predominantly monophasic inhibition (Stone and Mayeri 1981), an early transient excitatory phase was occasionally seen in the LUQs, especially at high concentrations. Ruben, Johnson and Thompson (1986) reported that pressure ejection of FMRFamide onto LUQ somata reliably produced a biphasic response consisting of a Na-dependent inward current lasting about 10 sec, followed by a K current lasting about 1 min. Brezina, Eckert and Erxleben (1987a) reported that the early phase was seen inconsistently when FMRFamide or YGGFMRFamide were pressure ejected onto LUQ somata, though it was not clear whether this variability might reflect systematic differences between the two

Figure 2. Comparison of effects of arterially perfused FMRFamide and two analogs on cells L1, L3, and L6.

A. Simultaneous recordings from L1 and L6. YGGFMRFamide mimics the hyperpolarization of L6 by FMRFamide, but not the depolarization of L1.

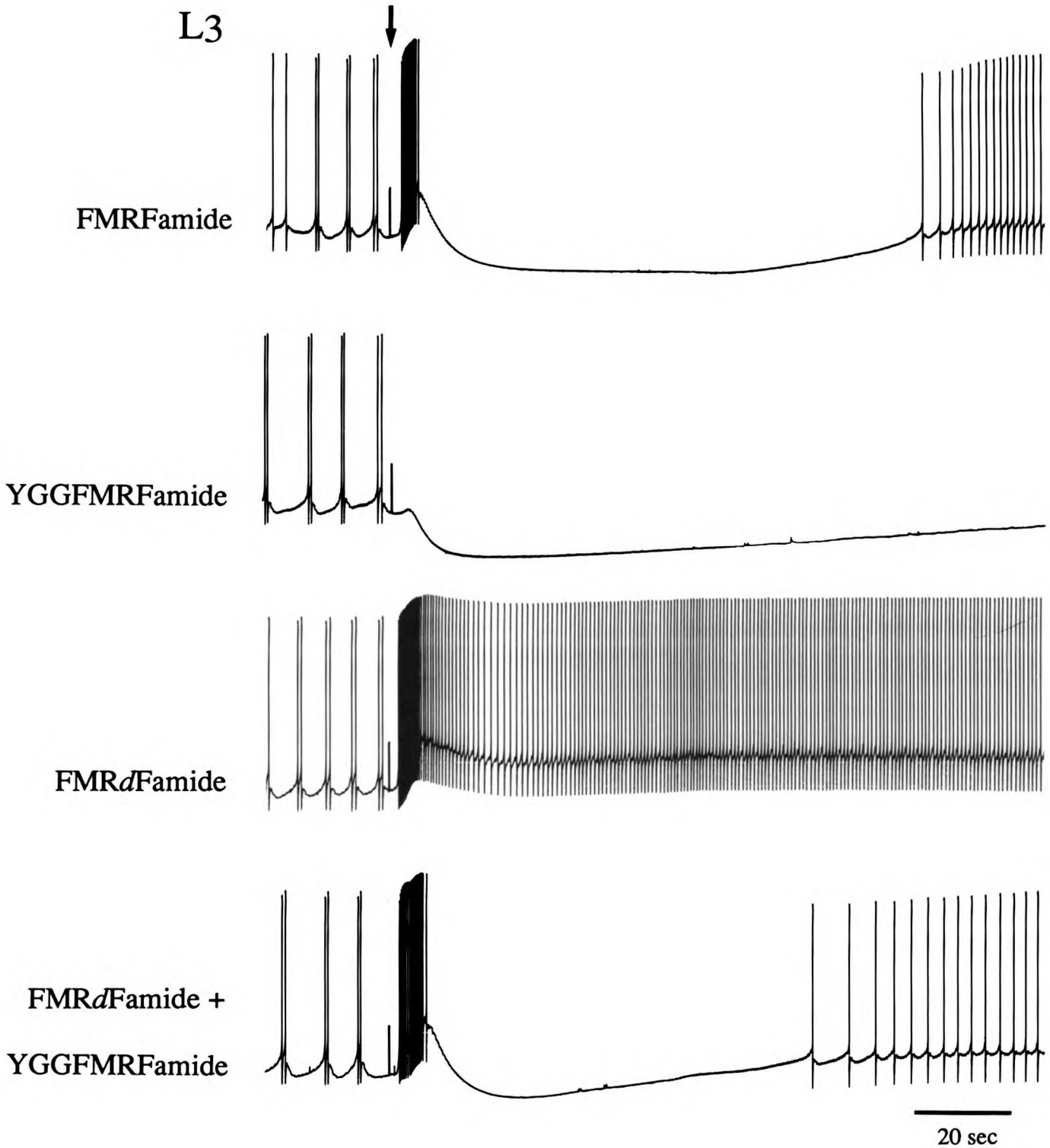
B. Simultaneous recordings from L1 and L3. FMRdFamide mimics the depolarization of L1 by FMRFamide, but also has excitatory effects on L3.


A
L1
L6
1 μM FMRFamide
1 μM YGGFMRFamide
20 mV
36
1 min
B
L1
L3
1 μM FMRFamide
10 μM FMRdFamide
50 mV
20
1 min

ligands used in their experiments. It seemed likely that some of these apparent discrepancies may be due to differences in the methods of peptide application (Ruben, Johnson and Thompson 1986).

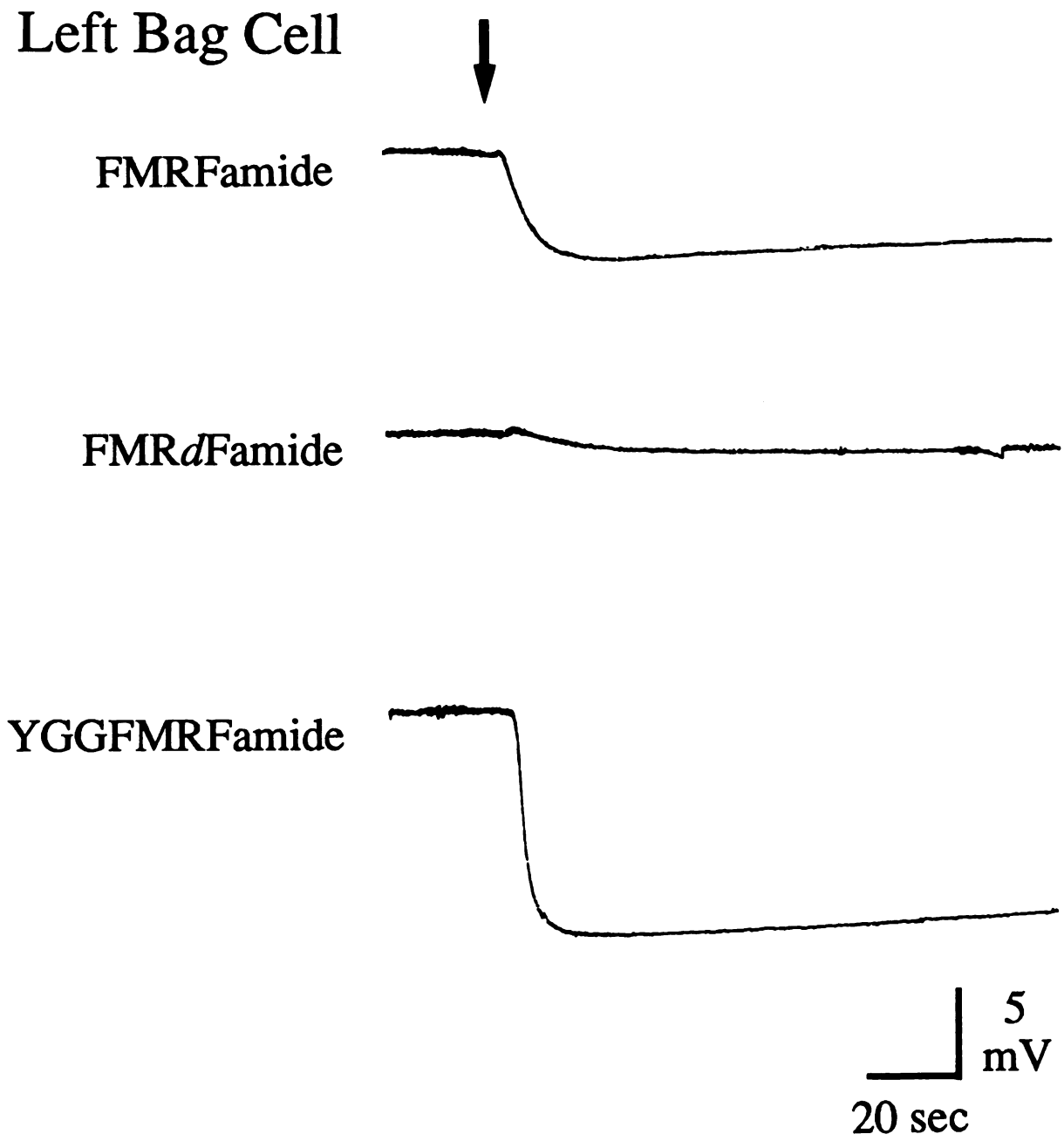
To address this issue, FMRFamide and analogs were applied directly to desheathed LUQ neurons, rather than being arterially perfused into the ganglion as in the previous experiments. This method of application also provides evidence for direct actions of the peptides on the neurons, since only the target neuron, glia, and possibly neuronal processes in the immediate vicinity are exposed to the peptides. Figure 3 (top trace) shows a biphasic response in membrane potential of one such LUQ neuron to pressure ejection of FMRFamide. YGGFMRFamide at the same concentration produced a monophasic inhibitory response (fig 3, second trace). FMR*d*Famide, at a 10-fold higher concentration, produced a monophasic excitatory response (fig 3, third trace). Finally, the biphasic response to FMRFamide was qualitatively simulated by combined application of YGGFMRFamide and FMR*d*Famide (fig 3, fourth trace). This supports the hypothesis that the LUQ neurons express two receptor subtypes, which each mediate one component of the biphasic response to FMRFamide. This is consistent with the finding that the two phases of the LUQ response apparently involve distinct second messenger systems (V. Brezina, personal communication).

Figure 3. Comparison of effects of FMRFamide and analogs pressure ejected onto desheathed LUQ soma. 0.5 µl of each peptide was ejected directly onto the soma of L3 (a 20 mV calibration pulse marks the application in each trace, below arrow). FMRFamide produced a biphasic response. The excitatory response was mimicked by FMRdFamide, and the inhibitory response by YGGFMRFamide. The two analogs applied together produce a biphasic response similar to that seen to FMRFamide. The concentrations of peptide inside the pipette were 10 µM for FMRFamide and YGGFMRFamide, and 100 µM for FMR*d*Famide.


L3
FMRFamide
YGGFMRFamide
FMRdFamide
FMRdFamide +
YGGFMRFamide
20 sec

Since our laboratory's focus of interest has been the bag cell neuroendocrine system, we were especially interested in the effects of FMRFamide and its analogs on the bag cells. Arterially perfused FMRFamide produces primarily an inhibition of the bag cells, which may sometimes be followed by a small depolarization (Stone and Mayeri, unpublished observations). The sheath overlying the bag cells was cut away, and peptides were applied to the bag cells by pressure ejection. The responses of a desheathed bag cell to FMRFamide and its analogs are shown in fig 4. FMRFamide produced a monophasic hyperpolarization (top trace), FMR*d*Famide had little or no effect (middle trace), while YGGFMRFamide produced a very strong hyperpolarization (bottom trace). Bag cell burst discharges terminated immediately after application of FMRFamide (n=2, data not shown), although a controlled study would be necessary to verify this was an effect of FMRFamide. FMRFamide has been shown by Brussard et al. (1988) to have two inhibitory effects on the *Lymnaea* caudo-dorsal cells (the homologs of the bag cells in that pond snail), consisting of direct membrane hyperpolarization and modulation of excitability. These results, combined with the immunocytochemical demonstration of strong FMRFamide-like immunoreactivity in fibers around the bag cells (Brown et al. 1985), suggest that FMRFamide may function as a potent inhibitory modulator of bag cell activity.

Figure 4. Effects of FMRFamide and analogs pressure ejected onto bag cell soma. FMRFamide hyperpolarized the bag cell. Subsequent application of FMRdFamide had no effect, while YGGFMRFamide had a powerful hyperpolarizing effect.


Left Bag Cell
FMRFamide
FMRdFamide
YGGFMRFamide
5
mV
20 sec

There is a weak structural similarity between FMRFamide and the bag cell peptides α-, β-, and γ-BCP, which all contain RF near the carboxy terminal. Thus it was possible that they act on common receptors. α-BCP has strong hyperpolarizing effects on the LUQs and many other abdominal ganglion neurons (Sigvardt et al. 1986), and is a putative transmitter for the inhibition of these cells by the bag cells (Rothman et al. 1983; Sigvardt et al. 1986). While the hyperpolarizing response to FMRFamide displays little or no desensitization (Stone and Mayeri 1981), the response to α-BCP is almost completely desensitized by prolonged exposure to 1 mM concentrations (as shown in Chapter 2, and Sigvardt et al. 1986). Fig 5A shows that the response to FMRFamide persists after desensitization to α-BCP, indicating that they act at different receptors.

A second BCP receptor subtype mediates the autoexcitation of the bag cells by α-, β-, and γ-BCPs (Brown and Mayeri 1986,1988). β-BCP was also suggested as a candidate transmitter for the excitation of L1/R1 by the bag cells, based on the identity of its amino acid composition with that of a fraction of bag cell extract that excited L1/R1 (Mayeri et al. 1985). Fig 5B shows that FMRFamide excites L1 and weakly hyperpolarizes a bag cell, while β-BCP has strong autoexcitatory effects on the bag cell, but no effect on L1. The response of L1 to a bag cell burst discharge elicited by intracellular bag cell stimulation (arrow) is also shown in fig 5B. This shows that β-BCP

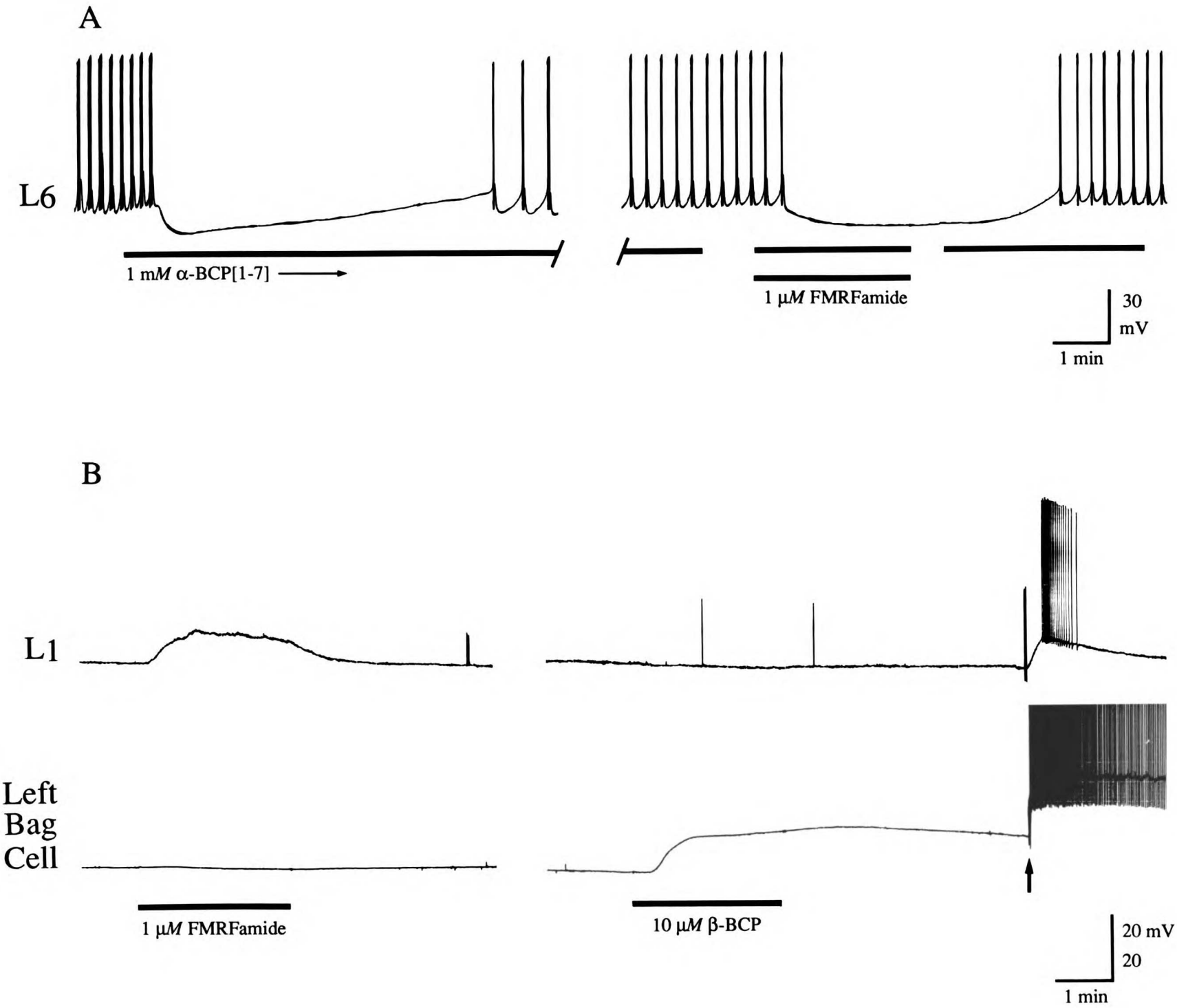
cannot account for the bag cell excitation of L1/R1. The combined results shown in fig 5A and 5B demonstrate that the two BCP receptor subtypes are separate from the FMRFamide receptor subtypes.

Besides the BCPs, other peptides with structural similarities to FMRFamide include the *Aplysia* peptides SCP_A and SCP_B, and the vertebrate peptides met-enkephalin (YGGFM), non-amidated YGGFMRF, gastrin/CCK, and γ_1-MSH. As has been previously shown in other preparations (Morley, Painter and Price 1982), FMRFamide and YGGFMRFamide were not acting at enkephalin receptors, since met-enkephalin (YGGFM) and the non-amidated peptide YGGFMRF were inactive on these cells (and on all other cells we've examined). However, the apparently stronger inhibitory response of the bag cells to YGGFMRFamide than to FMRFamide (fig 4), which was also observed in L11 and RUQ cells (data not shown), suggests that YGGFMRFamide may activate an additional inhibitory receptor subtype in abdominal ganglion neurons, as has been demonstrated in *Helix* (Cottrell and Davies 1987). γ_1-MSH had no effects, despite its RFamide carboxy terminus. The effects of the SCPs are generally distinct from those of FMRFamide (Lloyd et al. 1987), though there is no direct evidence that it does not act on the same excitatory FMRFamide receptor. While gastrin/CCK is generally ineffective on *Aplysia* neurons (Ono 1986), effects of CCK-8 which were distinct from FMRFamide actions have been seen in

Figure 5. Comparison of FMRFamide responses with bag cell peptide responses.

A. Continuous arterial perfusion of 1 mM α-BCP(1-7) produces a strong inhibitory response in an LUQ neuron which soon starts to desensitize (left trace). 20 minutes later (right trace) the response to α-BCP is almost completely desensitized, but the response to FMRFamide remains.

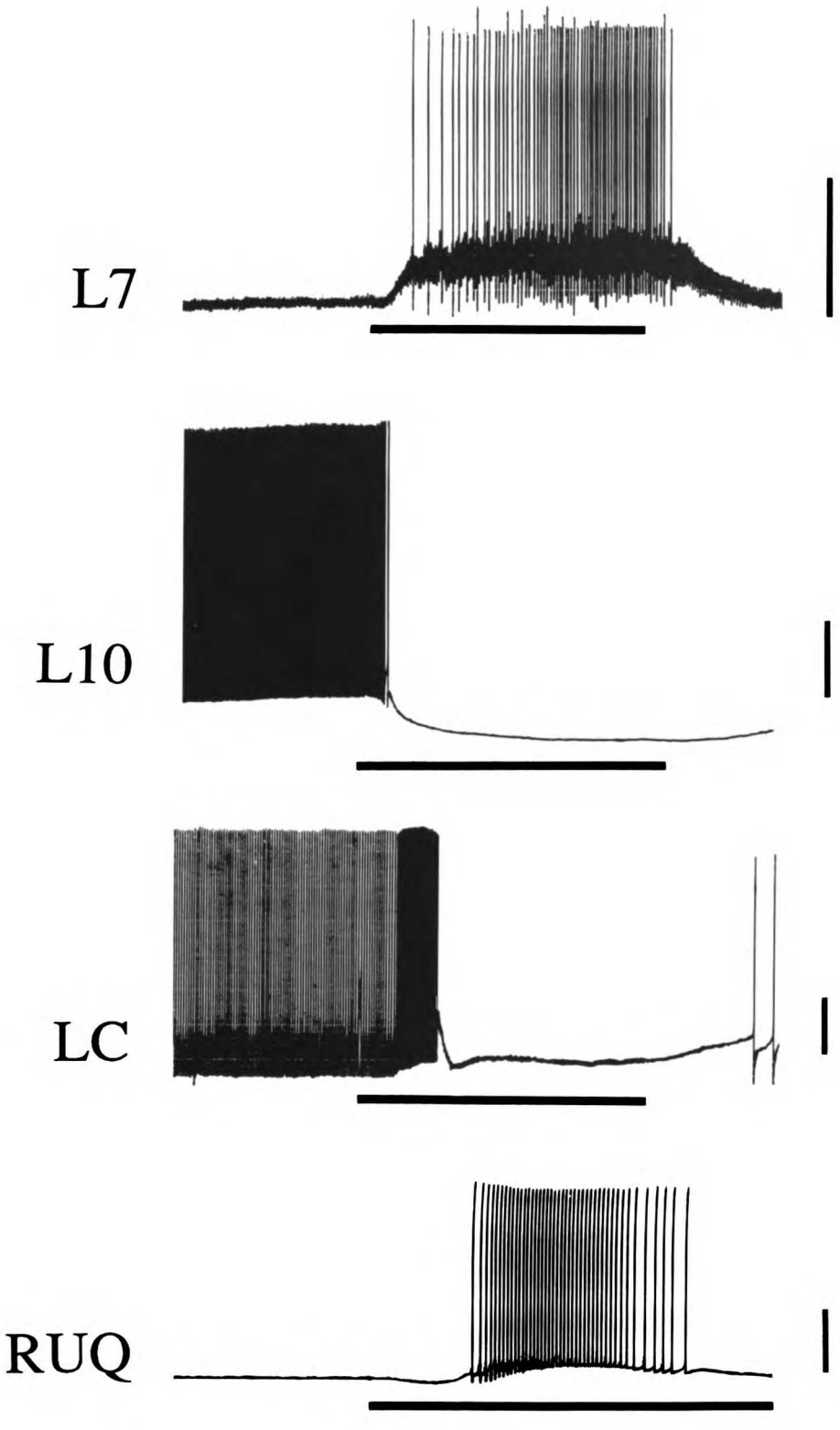
B. FMRFamide inhibits a bag cell and excites L1, while β-BCP excites a bag cell and has no effect on L1. A bag cell burst dischage was selectively stimulated at the arrow by brief intracellular electrical stimulation, producing the characteristic excitatory response in L1.


A
L6
1 mM α-BCP[1-7]
1 μM FMRFamide
30
mV
1 min
B
L1
Left
Bag
Cell
1 μM FMRFamide
10 μM β-BCP
20 mV
20
1 min

Helix neurons (Gerschenfeld, Hammond and Paupardin-Tritsch 1986).

Besides the responses in L1/R1, the LUQs, and the bag cells which were presented above, arterial perfusion of 1-10 μ*M* FMRFamide into the abdominal ganglion had effects on most of the other neurons we recorded from. Representative responses in four other identified abdominal ganglion neurons (L7, L10, an LLQ cell and an RUQ cell) are shown in fig 6. It is not known whether these are direct or indirect (i.e. polysynaptic) effects, since peptide application by arterial perfusion reaches every neuron in the ganglion. The reponse of L7 in particular obviously involves the activation of presynaptic inputs.

Figure 6. FMRFamide responses in other abdominal ganglin neurons. Data from 4 different preparations shows the responses of L7, L10, an LLQ and an RUQ neuron to arterially perfused FMRFamide (bar). The concentrations of FMRFamide were 1 μM in the top three traces, and 10 μM in the bottom trace.


L7
L10
LC
RUQ
1 min

DISCUSSION

The finding that multiple FMRFamide responses occur in many abdominal ganglion neurons, coupled with the previous demonstration of immunoreactive FMRFamide in many abdominal ganglion neurons, support the hypothesis that FMRFamide functions as a neurotransmitter in the abdominal ganglion of *Aplysia*. Further analysis of a possible transmitter role for FMRFamide has been hampered by the lack of known chemical synaptic target neurons to the identified FMRFamide-immunoreactive neurons in the abdominal ganglion. R2 and LP11 are coupled by electrical synapses (Meunier and Tauc 1973; Rayport and Kandel 1980), and R2 makes apparently FMRFamidergic synapses onto other identified abdominal ganglion neurons *in vitro* which have not been found *in situ* (Schacher, Rayport, and Ambron 1985). Recently Mackey et al. (1987) used combined immunocytochemistry and fluorescent dye backfilling to identify an FMRFamide-immunoreactive neuron in the left pleural ganglion which projects to the abdominal ganglion. This cell (LPL16) is activated by tail shock, and presynaptically inhibits siphon sensory neurons. Since the presynaptic inhibitory effects of FMRFamide on the senory neurons have already been extensively studied (Occor and Byrne 1985; Belardetti, Kandel, and Seigelbaum 1987; Piomelli et al. 1987), it is likely that this pathway will serve to establish a critical role for FMRFamidergic transmission in a defined behavioral function.

The existence of multiple receptor subtypes on *Aplysia* neurons was first demonstrated for ACh receptors (Gerschenfeld and Tauc 1962), and the *Aplysia* CNS is now thought to express at least 3 ACh receptors (Kehoe 1972), 4 5-HT receptors (Gerschenfeld and Paupardin-Tritsch 1974) and 4 histamine receptors (McCaman and Weinrich 1985). In addition to the two or more FMRFamide receptor subtypes, there are also at least two α-BCP receptor subtypes (Brown and Mayeri 1986,1988). The functional implications may be different in the case of the multiple peptide receptor subtypes, since there are also families of related peptides that act differentially on the recptor subtypes. (It would be interesting in this regard to determine the pharmacological activity of the L5 peptide, which cross-reacts with antisera directed at the carboxy terminus of FMRFamide (Brown, Basbaum and Mayeri 1985; Schaefer et al. 1985). However, the L_{29} cells apparently contain a transmitter which is related to, but distinct from, 5-HT (E. Kandel, personal communication), so it is also possible that multiple forms of the classical transmitters occur in *Aplysia*.

While at least some of the FMRFamide-like immunoreactivity in the *Aplysia* nervous system is authentic FMRFamide, the known heterogeneity of FMRFamide-like peptides requires caution in interpreting immunocytochemical results. This is especially important given the heterogeneity in FMRFamide receptors, and the likelihood

that they distinguish between natural ligands as well as between the artificial ones used in this study. It would seem well advised to investigate the pharmacological properties of a particular FMRFamide response before conducting extensive analysis of the second messengers and ion channels involved. As a case in point, the sensory neurons which are normally inhibited by FMRFamide sometimes show excitatory responses to FMRFamide (S. Seigelbaum, personal communication); a judicious choice of ligands, while arguably less biologically relevant, would reduce the complexity and variability in this preparation and may expedite the mechanistic studies, even if FMRFamide itself is actually the bioogically relevant molecule.

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Chapter 7

CENTRAL ACTIONS OF R15, A PUTATIVE PEPTIDERGIC NEURON IN *APLYSIA*

SUMMARY

We report that the bursting pacemaker neuron R15 has central actions on other identified neurons in the abdominal ganglion of *Aplysia californica*. The follower cells are located on the dorsal surface of the left lower quadrant of the ganglion and include members of the LC cell cluster. A spontaneous burst of impulses in R15 produces a slow, graded, excitatory potential of up to 8 mV in follower cells. The response begins about 2-3 s after the first impulse in an R15 burst, and reaches its peak at about 4-6 s (corresponding approximately to the end of the R15 burst). In some preparations a biphasic response was seen composed of the early depolarization followed by a slower excitatory or inhibitory phase. All the responses were blocked when R15 was hyperpolarized to prevent spiking. The magnitude of the response was reduced in a graded fashion by prematurely terminating the R15 burst with hyperpolarizing current and was increased when depolarizing current was injected into R15 during a burst. Central actions of R15 were observed in only 28% of our preparations, and their presence may depend on unknown physiological factors. The effects are likely to be mediated by R15 neuropeptides. The acessibility of both R15 and its targets in this preparation should facilitate further analysis of this interaction.

INTRODUCTION

R15 is an extensively studied, identified neuron in the abdominal ganglion of *Aplysia*. Its large size and characteristic bursting pacemaker activity have made it a favorite model for cellular biophysical studies, which have contributed much to our basic understanding of the ionic currents underlying bursting pacemaker activity in neurons (Lewis, 1984; Thomas and Gorman, 1977; Adams and Benson, 1985; Adams 1985; Adams and Levitan 1985) and the modulation of these currents by transmitters and intracellular messengers (Adams and Levitan, 1982; Lewis et al., 1984; Kramer, Levitan, and Levitan 1988; Levitan and Levitan 1988; Lotshaw and Levitan 1988). R15 is considered a neurosecretory cell on the basis of its white appearance and content of dense core vesicles (Frazier et al., 1967). Evidence that R15 is peptidergic includes biochemical studies that have established that it synthesizes, processes, and transports large amounts of low molecular weight peptides (reviewed in Gainer et al., 1977) and the discovery of a biologically active peptidic factor in R15 homogenates (Kupfermann and Weiss, 1976). A gene encoding the presumptive precursor for R15 neuropeptides has been isolated, cloned, and sequenced (Buck et al. 1983; Buck, Bigelow, and Axel 1987).

Possible presynaptic terminals of R15 onto unidentified neuronal processes in the neuropil of the abdominal ganglion have been described in ultrastructural studies (Frazier et

al., 1967), but no neuronal responses to R15 activity have been previously described. We report here that R15 has central actions on identified clusters of neurons in the left lower quadrant of the abdominal ganglion, with significant effects on their firing patterns.

A preliminary report of this work has appeared in an abstract (Brown and Mayeri, 1982).

MATERIALS AND METHODS

Large (400-3000 g) animals were collected from Elkhorn Slough or obtained from Sea Life Supply in Sand City, CA. There were stored in an open aquarium system at the Steinhart Aquarium in San Francisco or for short periods in our own recirculating aquaria. They were regularly fed fresh or dried red seaweed.

Animals were anesthetized by injetion of isotonic $MgCl_2$ (one-third of body weight). Abdominal ganglia were dissected out and pinned in a Sylgard-lined plexiglass dish containing one-third isotonic $MgCl_2$ and two-thirds bathing solution (fresh seawater with 0.25 mg/mL dextrose, buffered to pH 7.6 with 10 mM HEPES). Ganglia were continuously perfused during experiments with the bathing solution. Experiments were carried out at room temperature (19-24^{o}C).

Conventional electrophysiological recording and stimulating techniques were used to make up to four simultaneous intracellular recordings, as previously described (Mayeri et al., 1979). Single- or double-barrelled microelectrodes were filled with filtered 1.5 *M* potassium acetate. Recordings were stored on magnetic tape for playback and analysis.

RESULTS

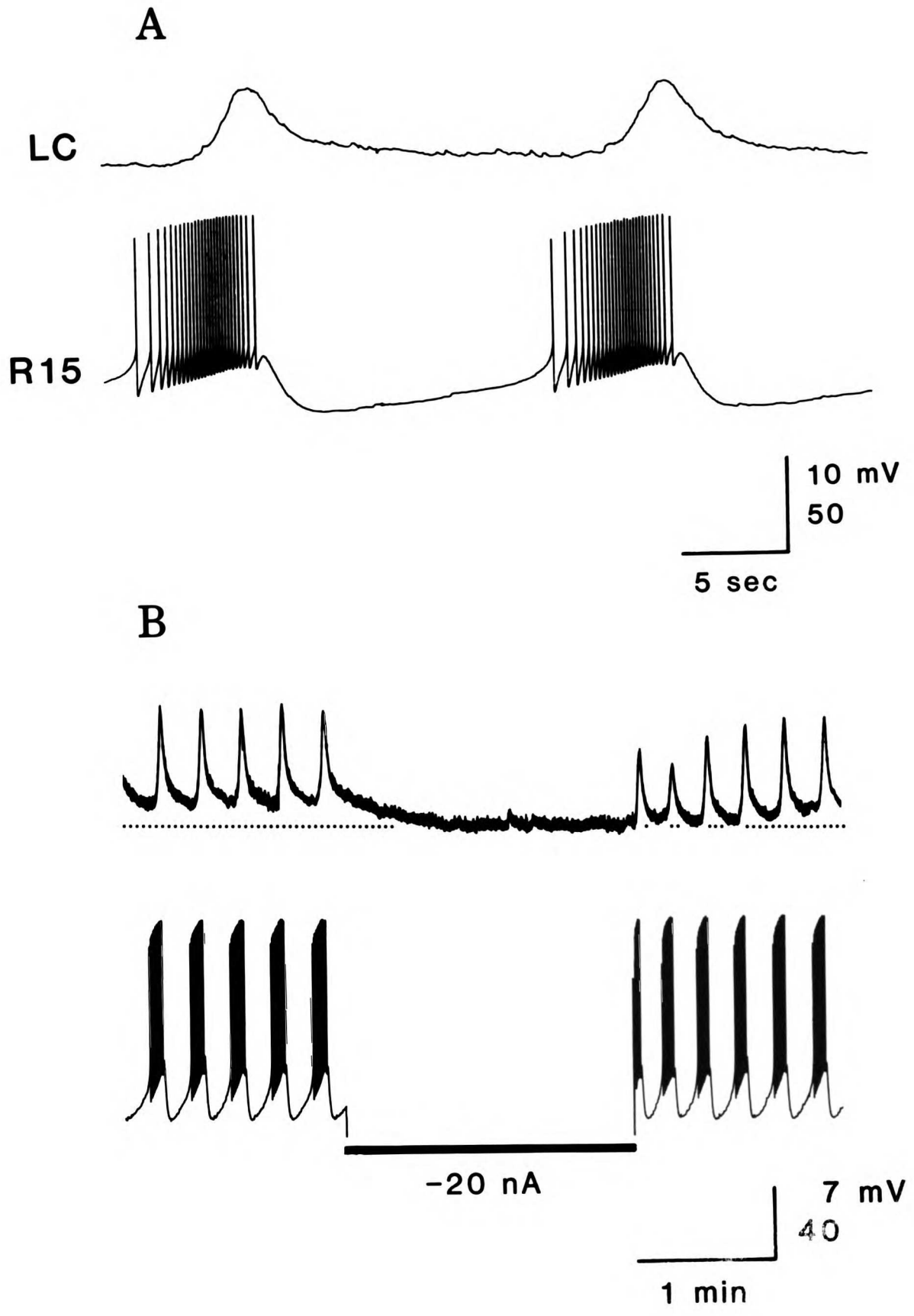
Types of Responses

Responses to bursting pacemaker activity in R15 were observed in neurons located caudally and medially on the dorsal surface of the left lower quadrant (LLQ), including (but not limited to) members of the identified LC cluster (Frazier et al., 1967). The most commonly seen response was a slow, smooth depolarizing wave during each burst of impulses in R15. An example of this response in an LC cell is shown in Fig. 1 (A). When R15 was hyperpolarized to prevent spiking, the depolarizing waves were eliminated, as shown in Fig. 1(B). In addition to the depolarizing waves in phase with R15, a second phase of sustained depolarization due to R15 activity was also revealed by this manipulation. It is seen in Fig. 1 (B) as a very slow decay of membrane potential to a more negative value during the period when R15 was hyperpolarized, and a slow return to the initial, more positive membrane potential when R15 resumed firing. The membrane potential during the falling phase for this cell was well fit by a double exponential with time constants of 3.1 and 28 s, corresponding to the decays of the phasic and sustained components, respectively. The late phase was smaller than the early phase of the response and was only detected in cells in which the depolarizing waves were unusually large.

The latency from the start of an R15 burst to the onset of the response was typically 2-3 s, and the time to the

Figure 1 Excitatory responses produced by R15 in an LC cell in the left lower quadrant (LLQ) of the abdominal ganglion.

A. Slow depolarizing waves in the LC cell are in phase with each R15 burst.
B. When R15 was hyperpolarized to prevent spiking (bar), the depoolarizing waves were eliminated. In addition to the phasic depolarizations of 7-8 mV, a sustained depolarization of 4-5 mV was revealed by the slow decay to baseline (dotted line) during the period when R15 was hyperpolarized.


A
LC
R15
10 mV
50
5 sec
B
-20 nA
7 mV
40
1 min

peak of the response was about 4-6 s. For each individual follower cell, there was little variation in the latency and time to peak of the response, as shown in Fig. 2 by the superposition of five successive traces. For this LC cell, the time from the first impulse in each R15 burst to the peak of the response was 6.14 $\pm$ 0.11 s (mean $\pm$ S D) during 40 consecutive R15 bursts. (The average latency to the apparent onset of the response over the same period was approximately 2.8 s, but this value was difficult to measure with precision due to the shallowness of the early response.) In contrast, there was considerable variability in the response times between preparations and even between individual cells in the same preparation. In one preparation, 2 LLQ cells had significantly different times to peak response of 4.53 $\pm$ 0.18 and 5.02 $\pm$ 0.13 s ($p<0.001$, Student's *t*-test).

The phasic input from R15 was often sufficient to drive the target cells' potentials past the firing threshold. Figure 3(A) shows 2 LLQ cells in the same preparation whose firing patterns were being driven by R15. Each cell fired 0, 1, or 2 spikes during or immediately following each R15 burst and was silent between R15 bursts. These cells also became silent when R15 was hyperpolarized to prevent firing (data not shown). Thus the bursting pacemaker activity in R15 can produce synchronous, phasic electrical activity in multiple follower neurons. Figure 3(B) shows another cell, which was spontaneously active, in which R15 caused phasic

Figure 2. Constancy of the response to R15

Superimposed traces of five successive R15 bursts and the responses in an LC cell demonstrate the constancy of the response. Oscilloscope sweeps were triggered off the first spike in each burst.

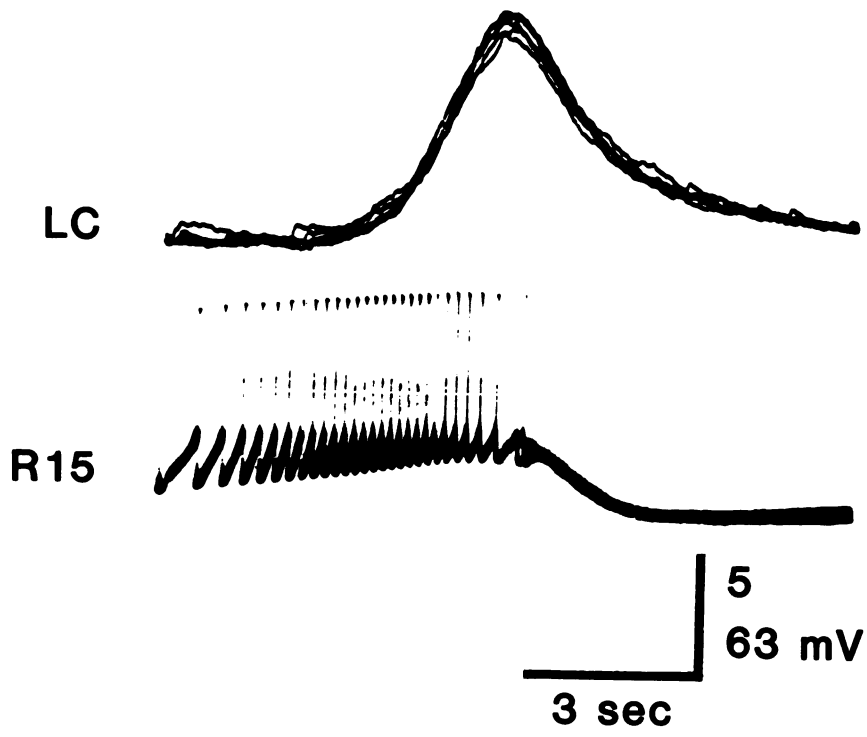
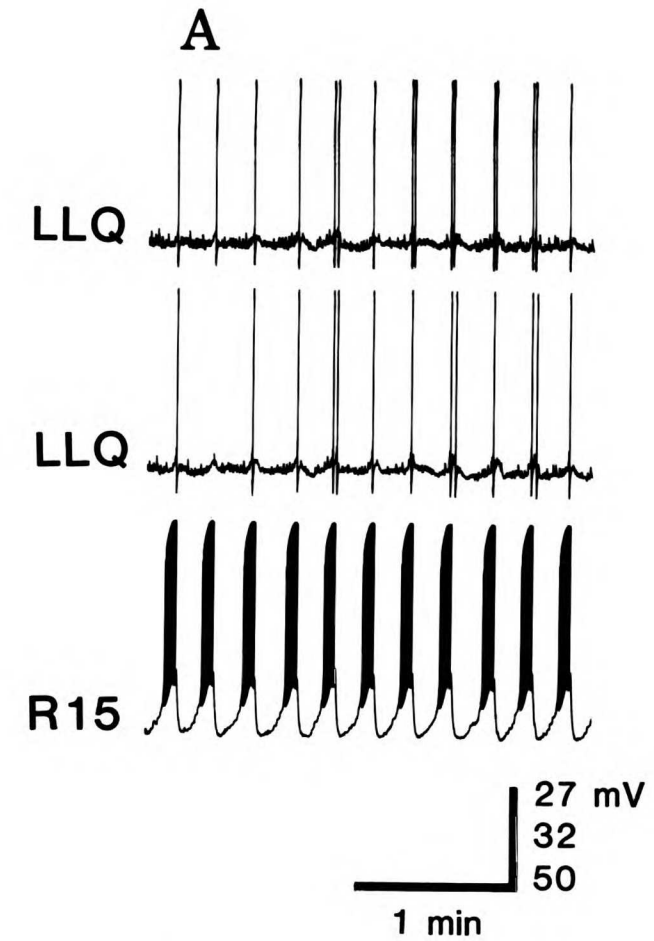
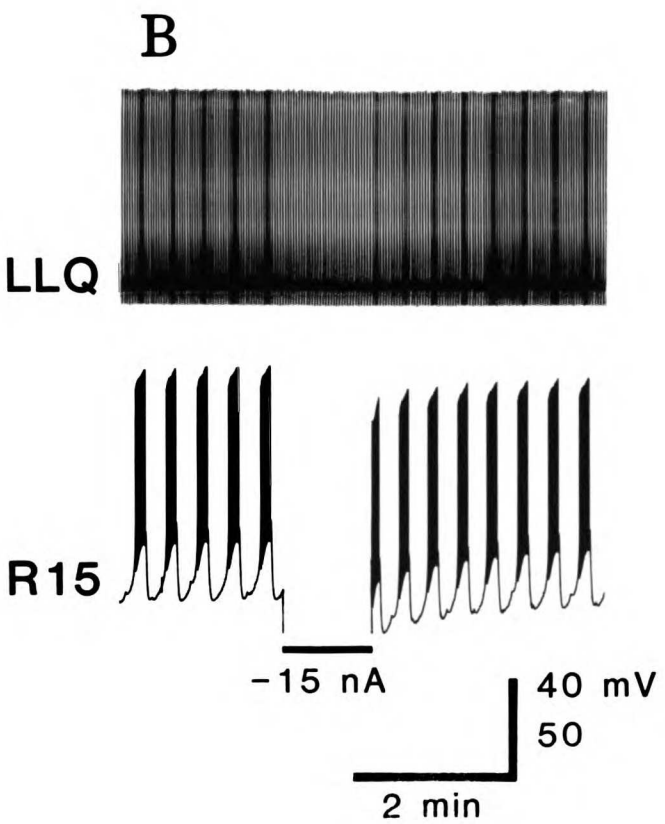

LC
R15
5
63 mV
3 sec

Figure 3. R15 modulation of impulse activity in follower cells.

A. R15 activity entrained the two LLQ follower cells to fire in synchrony with the R15 pacemaker. B. R15 bursts produced transient increases in the firing rate of a spontaneously active LLQ cell, seen as fusion of successive spike traces. When R15 was silenced by hyperpolarizing current (bar), the firing rate of the LLQ cell steadied and slowly declined.


A
LLQ
LLQ
R15
27 mV
32
50
1 min


B
LLQ
R15
-15 nA
40 mV
50
2 min

increases in its firing rate (in addition to a weak sustained excitatory effect). The instantaneous firing rate of this cell rose from 0.55/s between R15 to 0.75/s during each burst. The firing rate fell slowly to 0.45/s when R15 was hyperpolarized, indicating that R15 had both phasic and sustained excitatory actions on this cell.

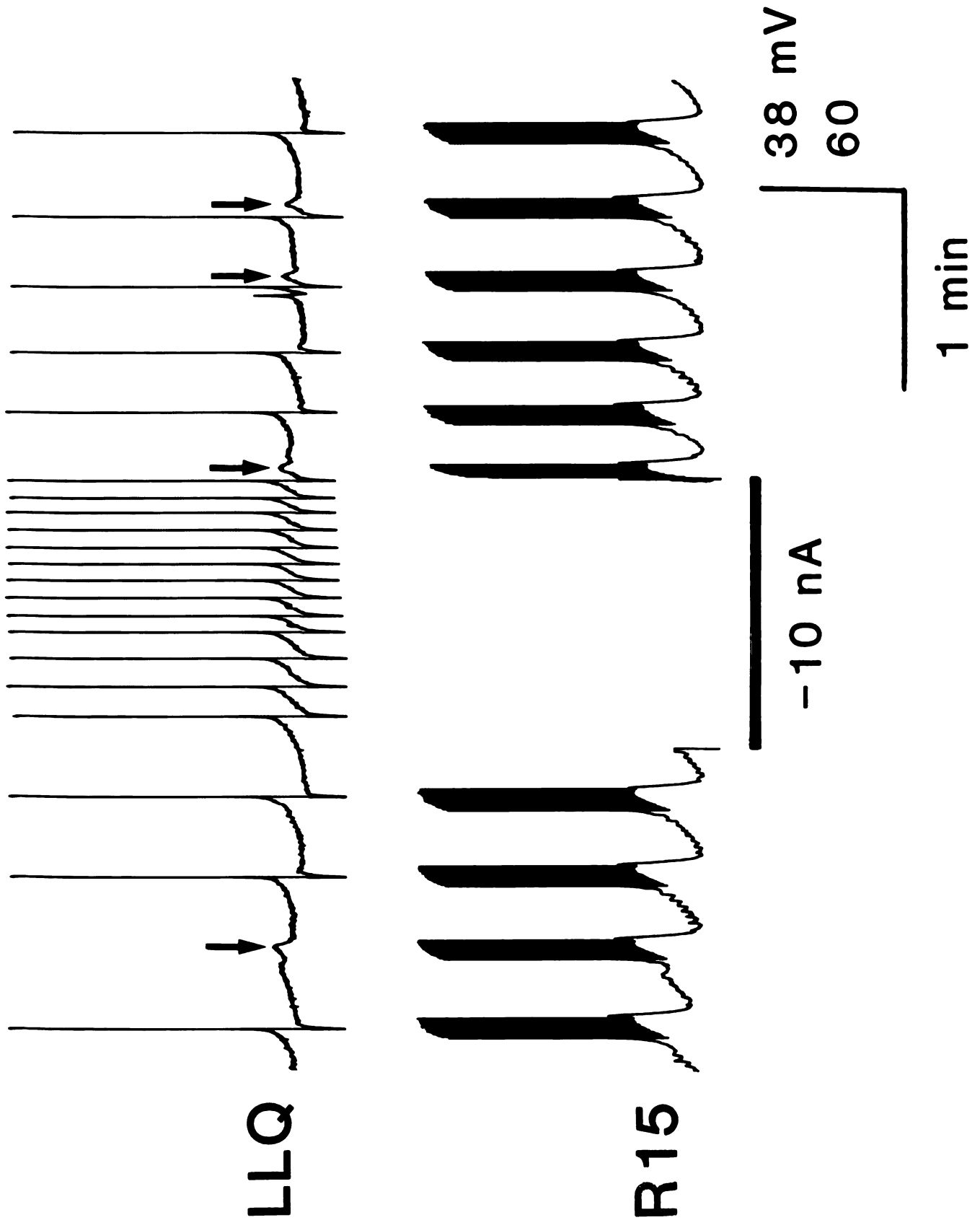
Although all the observed R15 actions included the early slow depolarizing wave, and some included a second phase of depolarization, an inhibitory second phase was seen in other preparations. Figure 4 shows a cell whose sustained inhibition by R15 was revealed when R15 was hyperpolarized. The early depolarizing component that was also seen in this cell (arrows) may serve in combination with the later inhibition to entrain the follower cell to the R15 pacemaker.

Effects of Presynaptic Manipulations of R15 on the Responses

To investigate the relationship between the number of spikes in an R15 burst and the response in its followers, hyperpolarizing current was injected into R15 to prematurely terminate spiking at various points during the burst. As illustrated in Fig. 5(A), this manipulation reduced the size of the follower cell's response. The magnitude of the response varied in a graded fashion with the number of spikes in the R15 burst and the threshold for a response was about 10 R15 spikes [Fig. 5(C)]. The magnitude of the response could also be increased by injecting depolarizing

Figure 4. Biphasic excitatory-inhibitory effects of R15 on an LLQ cell.

R15 bursts produced slow, small depolarizing waves (arrows) in the LLQ cells, which fired no or one spike during each burst. However, when R15 was hyperpolarized (bar), the LLQ firing rate increased dramatically, indicating that R15 was both transiently exciting and tonically inhibiting this cell.


LLQ
R15
−10 nA
38 mV
60
1 min

current into R15 during its burst [Figure 5(B)]. Although this manipulation had little effect on the number of spikes per burst, it did increase the spike frequency of R15 during bursts, and the response grew with increasing levels of depolarizing current [Figure 5 (C)].

Variability in the Ocurrence of the Interaction

In most preparations, R15 effects on follower neurons could not be found, even when R15 appeared white and healthy and was bursting normally. We verified LLQ responses to spontaneous R15 activity in 14 preparations. In nine other preparations we observed periodic phasic modulation of LLQ cells that were presumed to be R15 effects, but R15 was not impaled and hyperpolarized to confirm this. In 59 other preparations, no R15 actions were seen. In the preparations in which R15 effects were present, however, they could be seen in many or most of the dorsal LLQ cells and persisted throughtout experiments lasting several hours.

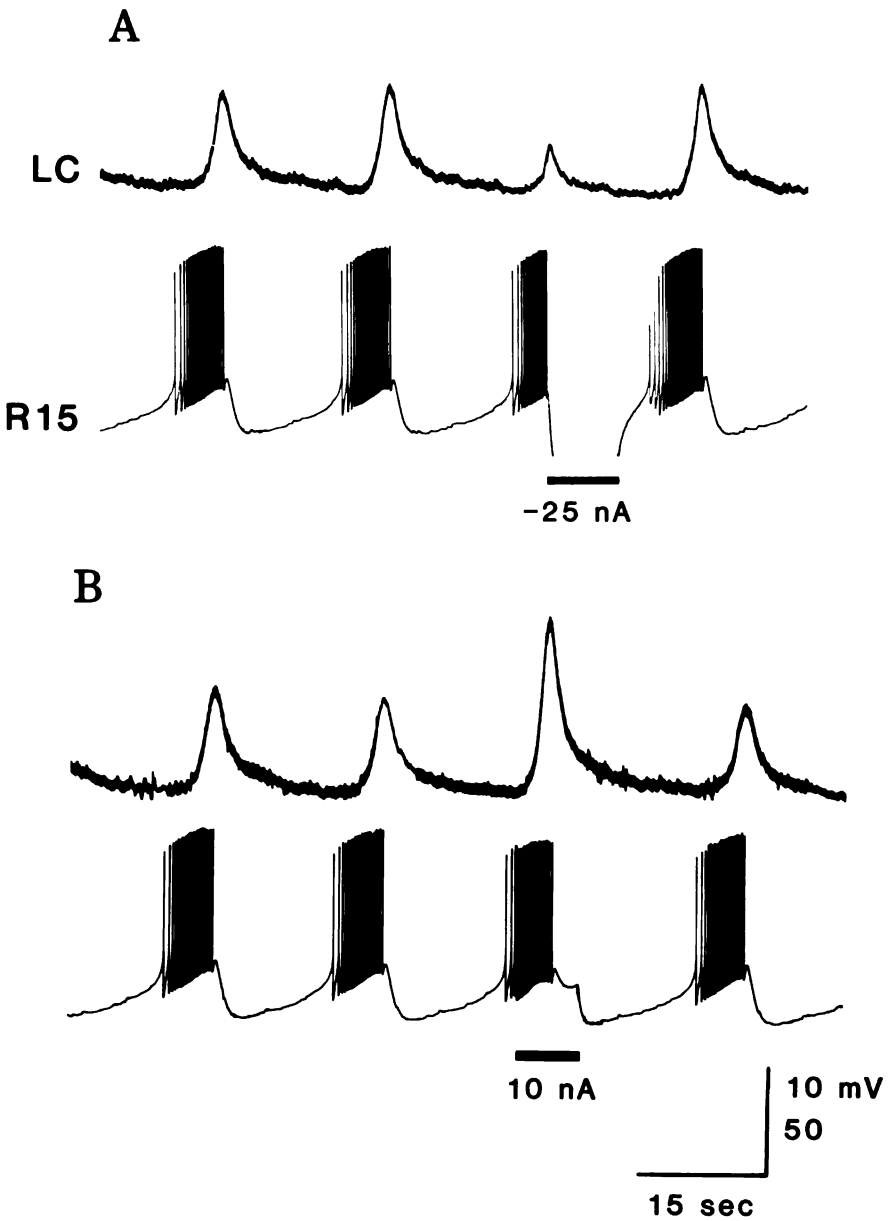
The variability in the appearance of R15 actions did not appear to represent a seasonal variation. It seemed to be necessary, but not sufficient, for these actions that the experimental animal was recently fed. We have not identified any other factors that correlate with R15 actions.

Figure 5. Graded modulation of the response by current injections into R15.

A. The depolarizing response in an LC cell was reduced when the burst in R15 was prematurely terminated by injection of -25 nA (bar).

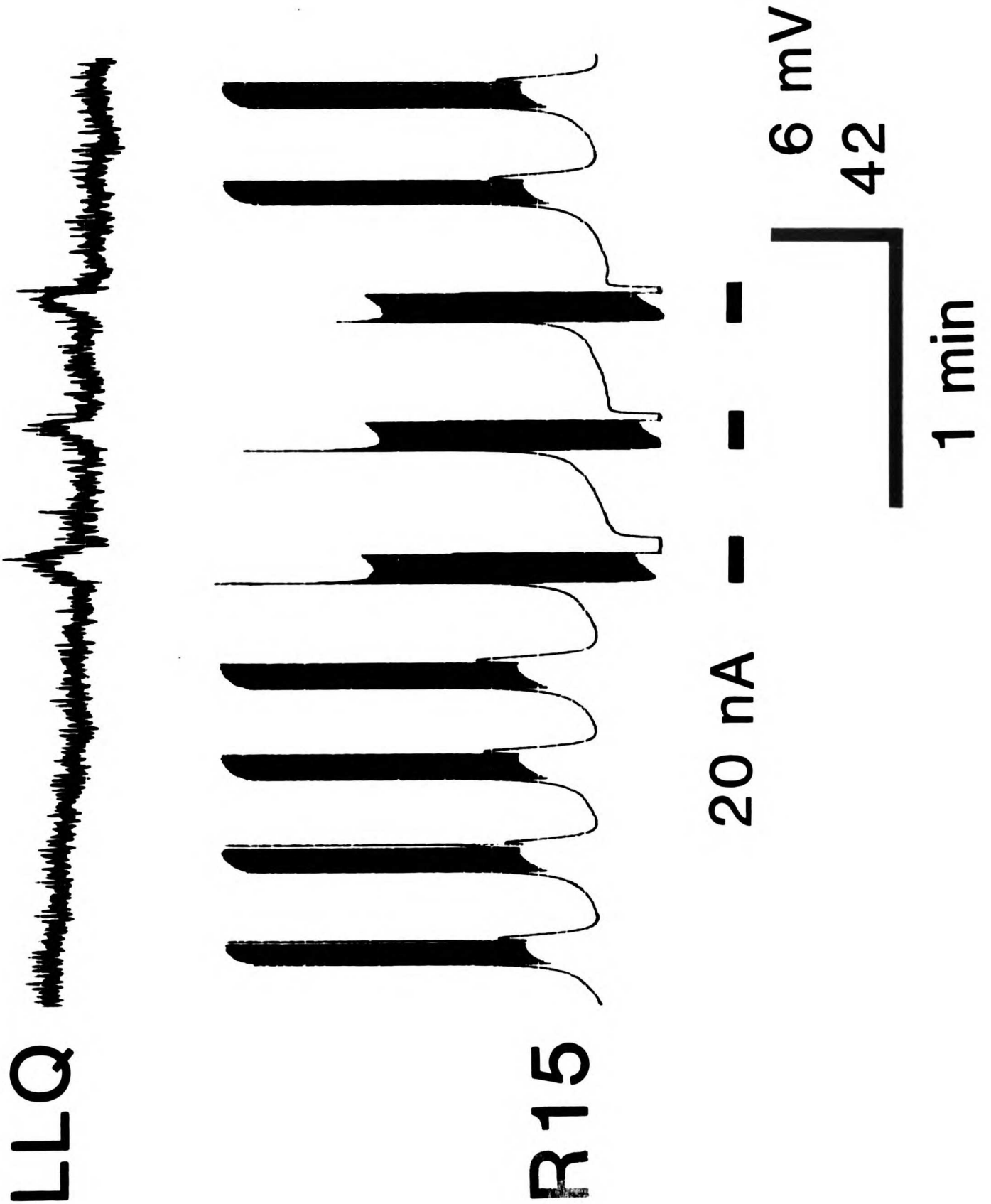
B. The response in the same cell was augmented when 10 nA depolarizing current was injected into R15 during a burst (bar).

C. The effects of these manipulations on the response in this LC cell are plotted against the number of spikes in each R15 burst. Asterisk: control point, average when R15 was not manipulated (n = 20). Solid circles: the R15 burst was terminated after a variable number of spikes, as in A. Open squares: Variable amounts of depolarizing current, displayed to the right of each point (in nanoamps), were injected during the R15 burst, as in B. This manipulation increased the intensity of R15 bursts, as measured by peak firing rate, with little effect on the total number of spikes per burst. In one instance (not plotted), injection of 10 nA into R15 during the burst caused a response in the LC cell that was suprathreshold and produced an inpulse.


A
LC
R15
-25 nA
B
10 nA
10 mV
50
15 sec
C
15
mV
10
5
0
LLQ Peak Depolarization
9.0
10
10
5.4
5.4
3.0
0
10
20
30
40
Spikes in R15 Burst

Since injections of depolarizing current into R15 during bursts augmented the responses in follower cells [Figure 5(B)], we tried this manipulation on some of the preparations in which normal R15 activity had no apparent effects on LLQ cells. In three of these preparations injection of depolarizing current into R15 during the burst produced a response in LLQ cells, indicating that the substrate for R15 actions was intact, but latent. An example of this is shown in Fig. 6. In five other preparations, no R15 effects were uncovered by this manipulation.

Figure 6. A latent response to R15 is revealed by injection of depolarizing current into R15. In this LLQ cell, no responses to normal R15 activity were seen. When R15 was injected with 20 nA during bursts to increase its burst intensity (bars), a response appeared in the LLQ. (An artifactual displacement of the potential recording in R15 occurred during current injections through the recording electrode.)


LLQ
R15
20 nA
6 mV
42
1 min

DISCUSSION

Neuronal Actions of R15

The present results demonstrate that R15 has central neuronal actions within the abdominal ganglion. The effects are excitatory, biphasic excitatory-excitatory, or biphasic excitatory-inhibitory. The transient depolarizing waves that were the predominant response may act not only to raise the firing rate of followers but to synchronize the activity of a population of neurons with the R15 pacemaker. The biphasic effects are unusual in that the sustained components, which are slower than the interburst intervals of R15, can contribute significantly to the apparent resting potential and tonic activity of follower cells.

The responses in LLQ cells required that the R15 bursts contained at least 10 spikes [Figure 5 (C)], suggesting that the early part of an R15 burst is relatively ineffectual. In this regard, Thomas and Gorman (1977) reported that the free Ca++ in R15 (measured with arsenazo III in the soma) increased substantially throughout this early phase of its bursts. It is therefore possible that the levels of free Ca++ required for effective transmitter release were only reached after about 10 spikes. Most of the unusually long latency from the start of an R15 burst to the onset of the response could then be accounted for by this period of the first 10 spikes in the burst during which free Ca++ is accumulating in R15, and thus need not involve diffusion, recruitment of interneurons, or slow postsynaptic response

mechanisms. [In fact, the time course of arsenazo signals in somata is generally not a useful indication of the time course of free Ca^{++} at release sites in nerve terminals. Simon and Llinas (1985) and Fogelson and Zucker (1985) have developed detailed models of the Ca^{++} signal based on the squid giant synapse, in which Ca^{++} entry and vesicular release occur at discrete active zones in the terminal membranes, and facilitation of transmitter release is very fast compared to the development of the R15 response. However, peptidergic neurons in general do not have active zones, and peptide secretion may depend on a slower and more diffuse Ca^{++} signal than is the case for classical transmission (DeCamilli and Navone 1987; Hirning et al. 1988).]

In a preliminary investigation of the ionic mechanism of the response, we found that injections of hyperpolarizing current into the follower cell decreased the size of the depolarizing waves caused by R15, whereas injections of depolarizing current increased it (data not shown). This suggests a mechanism of action involving either a conductance decrease (e.g. to potassium or chloride ions), or an effect on a voltage-dependent conductance (e.g., pacemaker currents). Further work will be necessary to characterize the actual ionic mechanism of the responses. It has not been determined whether the actions of R15 on its followers are direct or mediated by interneurons. We favor the former possibility for the phasic depolarizing

responses, based on the constancy of the time course and magnitude and their graded dependence on the duration and intensity of R15 bursts. The conventional tests for a direct interaction (see Berry and Pentreath, 1976) are problematic, however, because of the requirement for a burst of many R15 impulses and the very slow kinetics of the response. Should an R15 transmitter candidate be identified (see below), an alternative approach would be to show that this substance is released from R15 and has direct actions on the follower cells.

The apparent presynaptic terminals of R15 in the neuropil described by Frazier et al. (1967) provide a possible anatomical substrate for its central actions, although the specific relationship of R15 processes to LLQ processes in the neuropil is unknown. Given the slow onset and long-lasting nature of the responses, and the likelihood that these are peptidergic actions, the R15 transmitter may be secreted not at conventional synapses onto its targets but instead at distant release sites from which it diffuses to the follower cells. Such a mode of aciton, termed nonsynaptic or parasynaptic, has been postulated for the effects of the neuroendocrine bag cells on their targets in the abdominal ganglion (Branton et al., 1978; Mayeri et al., 1985).

Possible Role of R15 Central Actions in the Regulation of Water Balance

On the basis of its responses to stimulation of the osphradium (Jahan-Parwar et al, 1969; Stinnakre and Tauc, 1969) it was proposed that R15 is involved in osmoregulation. This hypothesis was further supported by the demonstration that R15's processes project to the pericardium, heart, and hepatopancreatic sheath (Rittenhouse and Price, 1985), the discovery of a pronase-sensitive factor in R15 which produces rapid water uptake when injected into *Aplysia* (Kupferman and Weiss, 1976), and the report that hyperpolarization of R15 in intact preparations affects the concentrations of osmolytes in the hemolymph (Bablanian and Treistman, 1985).

Although hormones released by R15 may act on peripheral tissues to mediate some of its effects, it is also possible that the control of water balance, presumably a vital function in this intertidal mollusc, involves additional neuronal components. Many of the dorsal LLQ cells are motor neurons, innervating the kidney, gill, siphon, pericardium, heart, and vasculature (Koester and Kandel, 1977). It is possible that the modulation of these LLQ cells by R15 is part of a physiological or behavioral program involved in regulating water balance. In this respect, R15 neuropeptides would act analogously to bag cell neuropeptides, which have both hormonal effects on

peripheral tissues and central actions on abdominal ganglion neurons and serve to coordinate the complex egg-laying behavior in *Aplysia* (Branton et al., 1979; Mayeri and Rothman, 1985). The coordination of central and peripheral aspects of complex behaviors may be a central role played by neuropeptides (Iversen, 1981). We hypothesize that R15 neuropeptides perform such a dual function.

Variability of R15 Actions

An unusual aspect of this interaction is that it is only occasionally seen in our preparations. It is possible that this variability reflects a developmental process, or represents an intraspecific polymorphism, and it might be related to the considerable variability in the morphology of R15 which has been reported (Winlow and Kandel, 1976). A basic difference in the functional connections of R15 would, however, stand in contrast with most of the previously reported properties and interactions of abdominal ganglion neurons, which display remarkable constancy from preparation to preparation (Frazier et al., 1967). It seems more likely that the substrate for this interaction is usually present in *Aplysia*, but is regulated by (as yet unknown) environmental or physiological factors. The implication of R15 in the regulation of water balance suggests that environmental osmotic stimuli might have important effects, although this has not been determined. The site of regulation could be presynaptic (e.g. peptide processing or

release), postsynaptic (e.g., receptors or intracellular messenger systems) or elsewhere (e.g., interneurons or extracellular proteases).

Although considerable work has been done on the ionic conductances underlying the electrical activity of R15, and their modulation by pharmacological agents and intracellular second messengers, it has not been possible to assess the relative importance of these elements to the normal physiological function of R15. The accessibility of both R15 and its follower cells in the abdominal ganglion, and the large electrical length constant of R15's processes that could permit direct manipulations of its terminals (Graubard, 1975), provides a good opportunity for such studies. The gene encoding a presumptive prohormone from R15 has recently been cloned and sequenced (Buck, Bigelow and Axel 1987), and neuropeptides predicted by this molecular genetic data are candidates for the transmitter mediating the central actions of R15. Identification of the transmitter will facilitate studies on the response mechanisms in the follower cells and help detemine the source of the variability of these actions.

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Chapter 8

EPILOGUE AND CONCLUSIONS

The specific results of this study have been discussed in the previous chapters. In this chapter I will briefly review the principal findings, relate some more recent relevant results, interpret the studies in the broader context of neuropeptide functions, and indulge in further speculations about their general significance.

α-BCP as a bag cell cotransmitter

Chapter 2 reported physiological studies that, in conjunction with studies by others in our laboratory and our collaborators, established α-BCP as a putative bag cell cotransmitter. α-BCP and ELH are coreleased during bag cell burst discharges to mediate separate components of the central neuronal actions of the bag cells. α-BCP and ELH each meet almost all of the strict criteria considered necessary to identify neurotransmitters in the CNS (see Werman 1966), although the lack of specific antagonists for these peptides prevents the final critical test. α-BCP is the transmitter responsible for the transient inhibition of many bag cell target neurons, while ELH mediates the prolonged excitation of other target neurons. Evidence was also presented that α-BCP and ELH may act conjointly to mediate the biphasic inhibtory/excitatory response in right upper quadrant neurons.

This provides the first direct demonstration of the long hypothesized idea that two neuropeptides derived from a

common precursor may function as cotransmitters in the central nervous system. A single polyprotein is processed to yield multiple active neuropeptides which act on independent, but overlapping, sets of central and peripheral targets to coordinate a complex behavior pattern. In addition each neuropeptide is itself capable of a multitude of complex effects (Mayeri, Jansen, and Brown 1988). For instance, ELH acts on at least four different currents in LLQ target neurons (Jansen and Mayeri 1988, 1989a, 1989b; Mayeri, Jansen and Brown 1988), and acts on different currents in B_{16} (Kirk and Scheller 1986) and R_{15} (Levitan, Kramer and Levitan 1987), as well as causing egg release from the ovotestis (Rothman et al. 1983b), acceleration of heart rate (Sigvardt and Mayeri, unpublished) and probably vasoconstriction of certain blood vessels (Ligman and Brownell 1985).

Including ELH and α-BCP, the bag cell precursor encodes at least 8 neuropeptides which are coreleased during burst discharges (Rothman et al. 1985, 1989; B. Rothman, personal communication). β-BCP and γ-BCP, as well as α-BCP, have autoexcitatory activities. A fifth bag cell neuropeptide, δ-BCP, is homologous to the peptide calfluxin, from the homologous ovulation hormone precursor in the pond snail *Lymnaea stagnalis*. δ-BCP (like calfluxin) is reported to stimulate Ca^{++} uptake in the albumen gland (Dictus et al. 1987; Nagle et al. 1988). The other three bag cell neuropeptides might be involved in mediating some of the

central bag cell actions not yet accounted for (e.g. excitation of L_1 and R_1, or long-lasting inhibition of LUQs). Thus 5 or more of the 8 neuropeptide products of the ELH/BCP precursor protein have biological activity and are thought to function in the generation of egg-laying behavior. The known actions of the bag cell peptides, and the putative functions of their targets, are summarized in Table 1 (modified and updated from Mayeri et al. 1979b and Scheller et al. 1983b). Specific predictions about the contributions of these bag cell actions to behavior can be made from inspection of this table, but the necessary behavioral studies of egg-laying that would test most of these predictions have not been performed. It will be especially important to identify components of egg-laying behavior which are not produced by ELH, reflecting the central actions of the other bag cell peptides.

Approximately equimolar amounts of ELH and α-BCP are synthesized in the bag cells (Sigvardt et al. 1986), as expected since they arise from a common precursor. This would apparently restrict the flexibility to independently regulate the amounts of the cotransmitters, as is possible in other types of cotransmitter systems. However, only about 10% of α-BCP appears to be processed to the most potent (1-8) form (Pulst et al. 1987), suggesting that regulation of this alternative processing step may affect the spectrum of bag cell actions. It has also been

Table 1. Central and peripheral bag cell targets

Target	Putative function of target	Response of target	Bag Cell Transmitter
Central			
Bag cells	Trigger egg-laying	Activation and autoexcitation	α-, β-, γ-BCPs
R_{15}	Neurosecretory (Water retention)	Burst augmentation	ELH
LB, LC cells	Motorneurons (Siphon, gill, pericardium, etc.)	Prolonged excitation	ELH
R_2	Mucus release (defensive)	Slow inhibition	α-BCP
L_{14} A,B,C	Ink release (defensive)	Slow inhibition	α-BCP
RUQs (R_{3-14})	Cardiovascular regulation?	Biphasic inhibition/excitation	α-BCP + ELH
L_{10}	Cardiac command neuron	Biphasic inhibition/excitation	α-BCP + ELH?
LUQs (L_{2-6})	Renal pore constriction	Slow and prolonged inhibition	α-BCP + ?
L_1, R_1	Mechanoreceptor (foot)	Transient excitation	?
Peripheral			
Ovotestis	Egg production	Release of eggs	ELH
Albumen gland	Albumen secretion	Mitochondrial Ca^{++} uptake	δ-BCP
Vasculature	Blood flow	Vasoconstriction	ELH
Heart	Cicrulation	Acceleration	ELH

suggested that ELH and other bag cell peptides may be packaged into separate populations of secretory vesicles after cleavage from the common precursor (Molloy, Bruns and Arch 1987; Fisher et al. 1988). One of the most exciting recent developments in studies of cotransmission is the idea that the release of cotransmitters contained in different types of secretory vesicles within individual neurons may be independently regulated by coupling to distinct populations of Ca^{++} channels (see De Camilli and Navone 1987; Perney et al. 1986; Hirning et al. 1988), potentially allowing for local and dynamic control of the ratios of released cotransmitters.

α-, β-, and γ-BCP as autoexcitatory transmitters

The third chapter presented evidence that the episodic, all-or-none bursting behavior of the bag cells involves positive feedback by autoexcitatory transmission. α-BCP, as well as the structurally related pentapeptides β-BCP and γ-BCP, have direct excitatory actions on the bag cells and meet most of the strict criteria to be considered the autoexcitatory transmitters. The autoexcitatory responses of the bag cells were pharmacologically distinct from the inhibitory responses of other cells to α-BCP, indicating a second receptor subtype is involved.

Pulsatile hormone release during episodic bursts of action potentials is apparently a common feature in neuroendocrine systems (see Lincoln et al. 1985; Leng 1988).

The bag cells are a convenient model system for studies at the cellular level of the role of autoexcitatory transmission in pulsatile hormone release. The hypothesis that autoexcitatory transmission provides a general mechanism for generating this pattern of activity remains to be widely tested, although evidence for similar mechanisms in other systems was discussed in chapter 3.

Besides autoexcitatory transmission, several other mechanisms have also been proposed to underly bag cell activation. Electrotonic coupling is certainly important for synchronizing the bag cells, but passive electrotonic coupling among a population of neurons, in the absence of a specific regenerative mechanism, cannot be expected to produce prolonged all-or-none activation (see Merickel, Kater and Eyman 1978). Cyclic AMP has frequently been suggested to play an important role in the activation of bag cells, based largely on the evidence that cAMP levels in bag cell clusters rise transiently during the first minutes of burst discharges (Kaczmarek et al. 1978). However, the same study also found that 5-HT produced a much larger (513% vs 133%) and more statistically significant ($P<0.01$ vs $P<0.025$) rise in bag cell cAMP levels than burst discharges, even though 5-HT has strong inhibitory effects on bag cell activity (Jennings et al. 1981). The other key foundation on which the cAMP hypothesis rests is the report that cAMP analogs "are capable of" initiating bag cell burst discharges (Kaczmarek et al. 1978). This cannot be properly

evaluated because, to my knowledge, it has never been documented. Kauer and Kaczmarek (1985) have additionally observed that forskolin in the presence of theophylline can trigger bag cell discharges in intact bag cell clusters (about 50% of the time, J. Kauer, personal communication). If cAMP does play an essential role in bag cell activation, it still must be coupled to some kind of positive feedback mechanism. It has also been suggested that protein kinase C is involved in bag cell activation (DeRiemer et al. 1985; Kaczmarek 1986). The demonstration of a causal role for any of these mechanisms, including autoexcitatory transmission, will probably require specific inhibitors or antagonists to dissect out its functional contribution.

Besides the autoexcitatory effects of the three BCPs on bag cells, α-BCP may have other actions on the bag cells (Kauer, Fisher and Kaczmarek 1987), suggesting a more complicated autoregulatory scheme. In addition, it is possible that the secreted BCPs also exert positive feedback regulatory effects on their own synthesis by the bag cells. This hypothesis arises from two lines of evidence: (1) Releasing hormones, in general, activate second messengers which act concomitantly at the membrane level to increase secretion and at the nuclear level to increase synthesis of the released hormone (Murdoch et al. 1982, 1983; Barinaga et al. 1983; Reisine et al. 1985; Montminy et et al. 1986), and the BCPs may be regarded as auto-releasing hormones; and (2) the synthesis of bag cell peptides is increased by bag cell

activity, and this increase has been suggested to depend on secretion from the bag cells (Berry and Arch 1981). Alas, this attractive hypothesis has recently been challenged by the report that α-BCP in fact reduces synthesis of the ELH/BCP precursor (Berry 1988).

Pleural ELH/BCP cells and the initiation of egg-laying

In the fourth chapter it was shown that the small cluster of neurons in the right pleural ganglion which express the bag cell ELH/BCP peptides are functionally coupled to the bag cells. These pleural cells have electrophysiological properties very similar to those of the bag cells, and activation of these cells leads to bag cell burst discharges. This interaction presumably involves the autoexcitatory bag cell peptides α-, β-, and γ-BCPs released from the pleural cells. The pleural ELH/BCP cells are the only identified neurons which can initiate bag cell activity, and they are likely to comprise part of the descending pathway that triggers bag cell burst discharges, and thus egg-laying behavior, *in vivo*. It remains to be determined whether these right pleural cells actually fire before the bag cells *in situ*, as predicted by the hypothesis that they are part of the descending pathway. The implication of the BCPs as transmitters in this descending pathway provides a third identified function for α-BCP in the bag cell system, that of acting to trigger bag cell activity, in addition to acting as an autoexcitatory

transmitter to provide positive feedback in the bag cells following activation, and acting as an inhibitory neurotransmitter onto many target neurons in the abdominal ganglion.

While this finding may bring us one level closer to the site of the initial event that triggers egg-laying, it still remains to be determined what triggers activation of the right pleural ELH/BCP cells. A logical extension of these results, in line with the "one gene-one behavior" hypothesis for the ELH/BCP gene, suggests that the small clusters of ELH/BCP neurons in the cerebral ganglion may be the next earlier stage in the regulation and initiation of egg-laying.

The initiation of egg-laying in behaving *Aplysia* involves other internal and external signals that are not yet accounted for. One of these is a distinct diurnal rhythm to egg-laying, with most episodes occuring near dawn (Siciliano, Nonomura and Mayeri, unpublished observations; see Thompson 1976). This is also the time of peak activity in the circadian pacemaker in the eye (Jacklet 1969). The circadian pacemaker projects axons throughout the central nervous system which are apparently dopaminergic (Olson and Jacklet 1985). Since dopamine is reported to increase the excitability of the bag cells (Kaczmarek et al. 1978), the possibility arises that these circadian pacemaker axons may provide an important input to the system. It is also thought that pheromonal cues may serve to synchronize egg-

laying within populations of *Aplysia* (Audesirk 1977; Rothman et al. 1986). Experiments testing the hypothesis that atrial gland peptides may function as water-borne pheromones mediating this effect have generally yielded negative results (Painter et al. 1986; N. Levin and Mayeri, unpublished; G. Fisher and Mayeri, unpublished). However, in other mollusks, sexual pheromones require direct contact (the head wart of *Euhadra*, Takeda and Tsuruoka 1978) or injection into the hemocoel (the love darts of *Helix*, Chung 1986) to function. Given the well known social aggregation of *Aplysia* in the wild, it remains possible that the atrial gland peptides function as contact pheromones. An alternative is that they may be secreted into the egg string to function in the development of *Aplysia*, similar to the role suggested for LHRH secreted in milk in fetal ovarian development (Smith (White) and Ojeda 1984).

The neuropeptide FMRFamide as a possible transmitter in Aplysia

FMRFamide, the "molluscan cardioexcitatory neuropeptide", was studied as a possible neurotransmitter in the abdominal ganglion of *Aplysia*.

Chapter 5 reported immunocytochemical studies demonstrating the widespread distribution of FMRFamide-like immunoreactivity in the abdominal ganglion. Many of these cells were identified by electrophysiological and other criteria. One of these was the giant neuron R_2, the largest

neuron known in any species, which is also a well-established cholinergic neuron. R_2 has subsequently been shown to have both cholinergic and FMRFamidergic-like actions on other identified neurons in primary cell culture, and is promising as a model cotransmitter neuron (Schacher, Rayport and Ambron 1985). Native FMRFamide was identified in abdominal ganglion extracts, suggesting that at least some of the observed immunoreactivity represents authentic FMRFamide, although many forms of this peptide have been reported in a variety of species. The immunoreactive cells identified by this study were used in cell-specific mRNA screening by Shaeffer at al. (1985) and Shyamala et al. (1986) to isolate two distinct neuropeptide genes. One gene, expressed in R_2, L_{12}, and L_{13}, encodes 24 copies of FMRFamide and 1 of FLRFamide. The second gene, expressed in L_5, encodes a distinct peptide with a carboxy terminal RFamide but which is otherwise apparently unrelated. Thus multiple FMRFamide-like peptides occur in the abdominal ganglion. In addition to coexisting with ACh in R_2 (and R_2's homolog LPl_1), FMRFamide has also been reported to coexist with the SCP peptides in buccal ganglion neurons (Lloyd et al. 1987). The LUQ neurons, which all contain FMRFamide-like immunoreactivity (presumably representing the L5 peptide), also express the L11 peptide (Taussig et al. 1985). The FMRFamide-like immunoreactive cells in the ventral right lower quadrant appear to be identical to the cluster of Gastrin/CCK-like immunoreactive cells reported by

both Vigna et al. (1984) and Ono (1986), though this may represent a single species of peptide which reacts with both antisera. Thus the FMRFamide peptides seem to frequently coexist with other transmitters, although the functional implications of this are not yet clear.

Chapter 6 reports the pharmacological effects of FMRFamide and structurally related synthetic peptides. As first reported by Stone and Mayeri (1981), FMRFamide had diverse inhibitory and excitatory effects on many identified abdominal ganglion neurons. The stereoisomer FMR*d*Famide mimicked just the excitatory effects of FMRFamide, while the extended peptide YGGFMRFamide had only inhibitory effects, suggesting at least two FMRFamide receptor subtypes are found in the abdominal ganglion, as was previously shown in the cerebral ganglion using the same analogs (McCaman and Ono 1985). The demonstration of multiple FMRFamide receptor subtypes on the LUQ neurons may explain apparent conflicts between several studies of the FMRFamide responses in these neurons (Stone and Mayeri 1981; Ruben, Johnson and Thompson 1986; Brezina et al. 1987a, 1987b).

While the wide distribution of multiple FMRFamide peptides and multiple receptor subtypes in abdominal ganglion neurons are evidence for an important central function, a specific neuronal interaction mediated by FMRFamide has yet to be identified. Some of the abdominal ganglion neurons identified in the immunocytochemical study have known peripheral functions, but none of these are known

to be (chemically) presynaptic in the central nervous system. One possibilty is that the overall levels of FMRFamide-like peptides in the abdominal ganglion, reflecting the integrated activity levels of the many neurons releasing them, act nonsynaptically to tonically modulate the responsive neurons. It is interesting in this regard that the circulating levels of FMRFamide in some opisthobranch molluscs are approximately 10 nM (Price 1986), the threshold for some of the neuronal effects (Stone and Mayeri 1981). It seems probable that the levels within the abdominal ganglion, where FMRFamide is being released, could be significantly higher than in the blood. The specific association of FMRFamide-immunoreactive fibers with the bag cells, and the inhibitory effects of FMRFamide on the bag cells, also suggest a possible role for FMRFamide in the regulation of bag cell activity.

Interest in FMRFamide as a transmitter in *Aplysia* has been intensified by recent reports that it antagonizes the effects of 5-HT on potassium channels in sensory neurons (Ocorr and Byrne 1985; Belardetti, Kandel, and Seigelbaum 1987), and produces presynaptic inhibition through an arachidonic acid mechanism (Piomelli et al. 1987; but see Brezina 1987c). Mackey et al. (1987) have recently found that the left pleural ganglion neuron LPl_{16} is immunoreactive for FMRFamide, is activated by strong tail shocks, and has presynaptic inhibitory effects on the synapse from siphon sensory neurons to motor neurons. The

giant neuron R_2 is also activated by nonspecific mechanical stimulation (Cobbs and Pinsker 1978), and causes mucus release from the body wall (Rayport, Ambron and Babiarz 1983), suggesting it may play a role in defensive behavior.

Central actions of R15

Chapter 7 showed that R15, a putative peptidergic neuron which has been studied intensively for 20 years, has both phasic and tonic central actions on target neurons in the left lower quadrant of the abdominal ganglion. The identified target neurons were members of the LB and LC clusters, which include motorneurons innervating the gill, siphon, pericardium, and related tissues. The R15 actions served to entrain the activity in these cells with the endogenous rhythm of R15, and Alevizos and Koester (1986) have recently reported phasic contractions in the siphon caused by R15 acting on these motorneurons. Very recently, they have also observed tonic effects of R15 on L7-mediated respiratory pumping (Alevizos and Koester, personal communication). These central actions may act in conjunction with the putative hormonal effects of R15 on water uptake to integrate the physiological and behavioral components of water balance.

The modulation of R15 activity by a variety of transmitters and pharmacological agents has been widely studied. The identification of R15 target neurons within the abdominal ganglion may allow for the direct analysis of

the functional consequences of this modulation. It is expected that R15 transmitter release increases during the ELH-mediated burst augmentation response to bag cell activity, but the target neurons identified so far also have strong responses to ELH which may have masked this effect.

A neuropeptide gene expressed in R15 has recently been cloned and sequenced (Buck et al. 1983, 1987). The precursor encoded by this gene yields two major predicted cleavage products, α- and β- R15 peptides, both of which have been identified in R15 extracts. Synthetic α-R15 peptide was found to produce water retention when injected into *Aplysia*. While this peptide was also reported to have activity on abdominal ganglion neurons, these effects were apparently indirect, and did not include actions on the identified R15 target neurons (A. Alevizos, personal communication). The effects of β-R15 peptide have not been reported, but it is possible that R15 uses both cotransmitters to mediate its central and peripheral actions, in a fashion similar to the bag cells. While expression of the R_{15} mRNA is apparently restricted to R_{15} in the abdominal ganglion and a small cluster of pleural ganglion cells, an alternatively spliced mRNA yielding distinct neuropeptides was expressed in many abdominal ganglion neurons (Buck et al. 1987).

Conclusions- Functions of neuropeptides in the CNS of Aplysia

It is now apparent that neuropeptides play important roles in the function of the central nervous system of *Aplysia*. When this thesis work was begun, the only identified neuropeptide in *Aplysia* was ELH in the bag cells. Since then, there has been an explosion of *Aplysia* neuropeptides, and it seems increasingly to be the rule that most identified *Aplysia* neurons express at least one neuropeptide. The properties of these neuropeptides and their associated identified neurons are summarized in Table 2. In addition to these neuropeptides, there is immunochemical evidence for several vertebrate peptides in the CNS of *Aplysia*, including met- and leu-enkephalin (Hopkins et al. 1982; Leung et al. 1986; Pulst, Rothman and Mayeri, unpublished), arginine-vasotocin (Moore et al. 1981), atrial natriuretic factor (Castellucci and Gutowska 1988), and vasoactive intestinal polypeptide (Soinila and Mpitsos 1988). The ELH/BCP peptides are the only ones for which there is yet compelling evidence of a neurotransmitter function. However, since most of these peptide-containing neurons have known central or peripheral targets, it should be possible to conduct detailed cellular studies of the physiological functions of these other peptides.

In general, the common properties of *Aplysia* neuropeptide systems are the same as those of vertebrate neuropeptide systems, validating the use of this model

Table 2. Neuropeptides in identified *Aplysia* Neurons - 1988

Neuropeptide	Native Struct.	Cloned mRNA	Bioactivity Neural	Bioactivity Other	Mimicry	Identified Neuron	Putative function of neuron	Cotransmitter
ELH	✓	✓	✓	✓	✓			
α-BCP	✓	✓	✓		✓	Bag cells	Control of egg-laying	(ELH/BCPs)
β-, γ-BCPs	✓	✓	✓		✓	RPl. cells	Activation of bag cells	(ELH/BCPs)
δ-BCP	✓	✓		✓		Cereb. cells	?	(ELH/BCPs)
ε-BCP, Acidic Peptide	✓	✓						
α-, β- R15 peptides	✓	✓	✓	✓	✓	R15	Water balance	(α and β)
R3-14 peptides I-III	✓	✓	✓?	✓		R3-14	Cardiovascular regulation	(I-III), Glycine?
FMRFamide	✓	✓	✓	✓	✓	R2, LPl1	Mucus secretion	ACh
						L12, L13	?	
						LPl16	Presynaptic inhibition	
L5 peptide		✓				L2-6	Renal pore constriction	L11 peptide
L11 peptide		✓				L2-6	Renal pore constriction	L5 peptide
						L11	Innervates mantle/gill	ACh
SCPA, SCPB	✓	✓	✓	✓		R20	Respiratory pumping	(unidentified)
						B1, B2	Gut peristalsis	
						B7, B9	?	
						B11	Swallowing	
						B15	Biting	ACh, Buccalins
Buccalin	✓		✓			B15	Biting	ACh, SCPs
Parabuccalin	✓		✓			B16	Biting, rejecting food	ACh, Myomodulin
Myomodulin	✓		✓	✓		B16	Biting, rejecting food	ACh, Buccalins
						L10	Cardiac command neuron	ACh
Pedal Peptide	✓		✓			Pedal cells	Innervate body wall	
Gastrin/CCK-like						B13	Interneuron (↑feeding?)	ACh
						B18	Interneuron (↓feeding)	

system to uncover new principles of neuropeptide function. These common properties include a diversity of the peptides and their receptors, wide distribution and diffuse release in the central nervous system, complex and long-lasting effects on the physiological properties of target neurons, and conjoint central and peripheral actions which may relate to common behavioral functions. However, the three neuropeptide systems I have concentrated on represent a considerable range of functions, suggesting that further generalizations about the functions of neuropeptides in the *Aplysia* CNS, as in the vertebrate CNS, may be unwarranted.

It is interesting to consider each of these systems in relation to a well known vertebrate peptide system. This comparison suggests that despite the great diversity of neuropeptide systems in both *Aplysia* and vertebrate CNS, there may be parallels in the strategies employed for similar types of functions:

ELH/BCP and Oxytocin. The ELH/BCP peptides are expressed in distributed clusters of neuroendocrine cells, most or all of which are functionally coupled. They are activated episodically, in an all-or-none fashion, to secrete large amounts of peptides and generate the prolonged, complex egg-laying behavior of *Aplysia*. This system is most reminiscent of the oxytocin neurosecretory cells in the vertebrate hypothalamus. These also are found in discrete, functionally coupled clusters, which are episodically activated (with a mechanism also involving

autoexcitation and electrical coupling) to secrete large pulses of oxytocin. While ELH/BCP peptides are only known to be involved in egg-laying, the pulsatile release of oxytocin is involved in several all-or-none reproductive behaviors, including parturition, milk letdown, and orgasm. There is a family of ELH genes in *Aplysia* which also includes genes encoding homologous peptides (Scheller et al. 1982; Mahon et al. 1985; Rothman et al. 1986). These peptides, which can trigger egg-laying, are secreted in the atrial gland, and apparently have an exocrine function (Arch et al. 1980; Painter et al. 1985). Oxytocin secreted by the fetus is thought to play a key role in the initiation of parturition (Fuchs et al. 1982), and LH-RH, another pulsatilely-released hypothalamic neuropeptide involved in reproductive behavior, may be released into semen to serve an exocrine function in receptive females (van Minnen 1988). The exciting possibility of important exocrine functions for neuropeptides is only beginning to be studied, and the atrial gland peptides in *Aplysia* could provide a valuable model system.

R15 and vasopressin. R15 displays continuous endogenous bursting activity, with its activity susceptible to graded up and down modulation, in contrast to the episodic all-or-none burst discharge pattern of the bag cells. R15 is apparently involved in the homeostatic regulation of water balance, with R15 being activated by hyperosmotic stimuli at the osphradium to secret a hormone

with antidiuretic activity. The activity patterns and putative function of R15 are quite similar to those of vasopressin neurons in the vertebrate hypothalamus. Kupfermann (1986) likened the activity patterns of vasopressin and oxytocin cells to the generation of graded reflex responses and fixed action patterns, respectively; this relation holds equally well for R15 and the bag cells. While vasopressin has widespread effects on central neurons, it seems unlikely that the phasic effects of R15 on central targets have an analog in the central effects of vasopressin cells, which apparently fire out of phase, and by force of numbers would not be expected to produce net phasic actions.

FMRFamide and opioids. FMRFamide peptides are expressed in a very large number of neurons throughout the CNS. There does not appear to be any general similarity in the electrophysiological properties of FMRFamide-immunoreactive neurons, whose spontaneous activities range from silence (R_2, VRLQ cells) to bursting pacemaker activity (LUQs) to fairly high tonic firing (L12, L13), nor is there an obvious simple unifying principle to the functions of these cells. This is reminiscent of the vertebrate opioid peptides, which are expressed in a very large and diverse set of neurons throughout the nervous system, and are involved in a multitude of functions (reviewed in Akil 1984). FMRFamide and the opioids are both represented by a family of genes, expressed in different neurons and often coexisting with other classical or peptide transmitters.

Both systems also have multiple receptor subtypes expressed on different targets. It is provocative, given these other similarities, that one common function appears to be the presynaptic inhibition of sensory transmission following activation by noxious stimuli. The possible evolutionary relationship between FMRFamide and the enkephalins has been much discussed (Greenberg et al. 1983; Taussig and Scheller 1986; Greenberg 1986), and was additionally supported by the discovery of the peptide YGGFMRF on the enkephalin precursor (Noda et al. 1982), and the controversial report of YGGFMRFamide in the octopus nervous system (Voigt and Martin 1982). Curiously, the actions of FMRFamide-like peptides in the vertebrate CNS are apparently opposite to opiate actions (Tang, Yang and Costa 1984).

Neuropeptides and neural processing

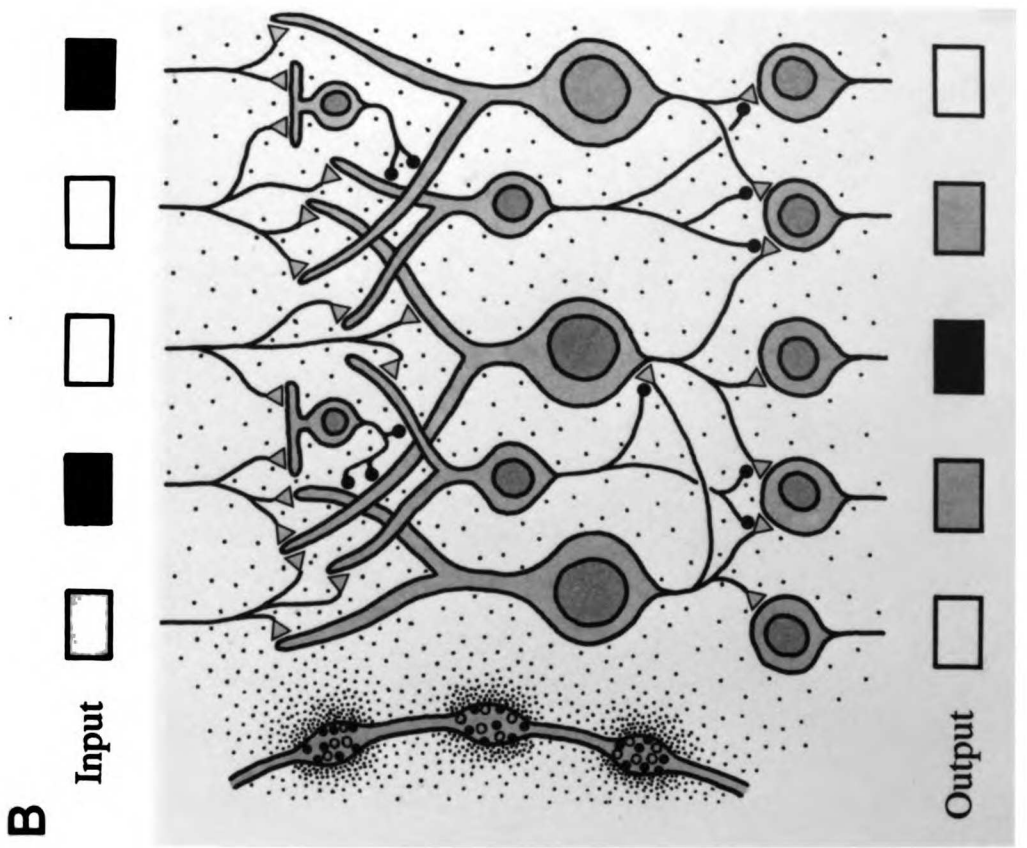
One generalization that can be made about neuropeptides is that they introduce considerable complexity into the local circuit analysis of nervous system processing. The natural milieu of central neurons is likely to be a sea of nonsynaptic neuropeptides (and nonpeptidic neuromodulators), whose aggregate actions produce complex and long-lasting changes in the basic properties of the neurons and networks. These actions may serve to modify the local circuits for the purposes of higher level processes. However, this increased complexity from a local perspective may represent an enormous simplification from the global perspective, if

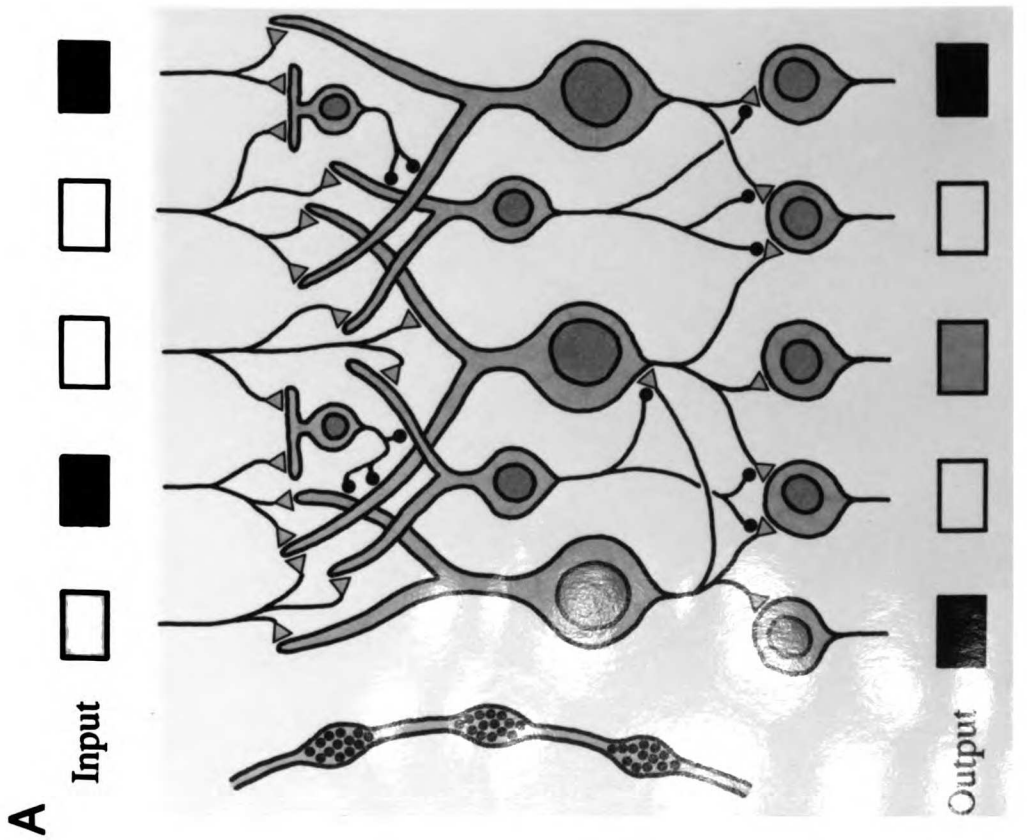
complex behaviors and motivations can in fact be represented by a relatively small number of parameters (e.g. the concentrations of released neuropeptides across the CNS) rather than an unimaginably large set (e.g. the distribution of synaptic activities across the CNS).

Figure 1 illustrates the implications of this view of neuropeptide actions for neural network-style representations of the nervous system. An external stimulus provides an input to the arbitrary neural network shown, left side of figure, which then produces a particular output (which may be likened to a behavior or a perception). Following the diffuse release of neuropeptides, right side of figure, there are widespread and profound changes in the properties of the elements of the neural network. Now the same network, under otherwise identical circumstances, produces a completely different output (which may be likened to the different behaviors and perceptions which are produced during different motivational states). The presence of neuropeptides in this case corresponds conceptually to the hidden variables of motivation. If such a system is modelled as a "Hopfield" neural network (Hopfield 1982; Hopfield and Tank 1986), it would also be likely to display state-dependent learning (J. Hopfield, personal communication). Metaphorically, the hardwired local synaptic connections may be thought of as providing the neural syntax, while the diffusely acting modulators may be thought of as providing the semantics of neural function.

Figure 1 Effects of neuropeptides on neural processing

On the left is shown a typical neural network-style representation of a neural circuit, with neurons communicating through excitatory and inhibitory synapses. The intrinsic properties of the network, in combination with the inputs to the network, will generate a particular activity pattern, or output. On the right, the same neural network is shown following activation of a nonsynaptic neuropeptidergic input which alters the intrinsic properties of the neural elements. Now the network will generate a very different output, even with identical input.


B
Input
Output


A
Input
Output

(A related idea of the "functional rewiring" of neural circuits, developed from studies in crustaceans, has been discussed by Marder and Hooper (1985) and Selverston (1988)).

Positive feedback in the nervous system

Perhaps the most important result in this thesis is the implication of a role for positive feedback by autoexcitatory transmission in the generation of instinctive behavior patterns. This provides a possible cellular mechanism for the independence of a fixed action pattern from the characteristics of its trigger, and can also explain how the identical behavior pattern may sometimes be produced even in the absence of an external stimulus (Lorenz 1937). The threshold which characterizes positive feedback allows the intrinsic (positive feedback) properties of neurons to dominate all other influences once the threshold is crossed, independent of how the threshold was initially reached.

Positive feedback may be a valuable unifying concept for understanding many of the unique properties of the nervous system. The distinguishing feature of the nervous system is that it actively generates activities, behaviors, and perceptions which are not continuous responses to the environment. Stability and homeostasis, the outcomes of negative feedback systems, are characteristic of many physiological processes, but not generally of nervous

activity. Indeed, positive feedback mechanisms are found at every level of the nervous system. Well-known examples of neural positive feedback mechanisms include the action potential produced by transient voltage-dependent sodium channels, the bursting and pacemaker activity produced by voltage- and Ca^{++}-dependent inward currents, the voltage-dependent unblocking of glutamate receptors, the autophosphorylation-dependent activation of protein kinase, and the activity-dependent strengthening of synapses. Other neural mechanisms involving positive feedback have been proposed for such phenomena as the vestibuloocular reflex (Robinson 1981), binocular rivalry (Sejnowski and Lehkey 1988), the filling-in of visual images (Cohen and Grossberg 1984), content-addressable memory (Hopfield 1982), visual hallucinations (J. Cowan, personal communication), the ovulatory luteinizing hormone surge (Knobil 1974), and epilepsy (Ayala et al. 1973). In general, processes of differentiation and generation, which superficially appear to conflict with the second law of thermodynamics, involve positive feedback (or more general nonlinear feedback with positive and negative components- see Prigogine 1983; Gleick 1987). Several writers have urged modelers of biological and social processes to focus on understanding positive feedback mechanisms as an alternative to the more analytical negative feedback-based control theory (Maruyama 1963; Stanley-Jones 1969; May 1976; Milsun 1968; De Angelis, Post, and Travis 1986). Further exploration of the mechanisms and

functions of positive feedback in the nervous system may provide new insights into some of the outstanding basic questions of neurobiology, perhaps even advancing our understanding of the relationship between mind and matter.

"I don't believe a word of it!"

- Max Delbruck

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