The Bag Cell Neurons of *Aplysia*

A Model for the Study of the Molecular Mechanisms Involved in the Control of Prolonged Animal Behaviors

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Abstract

Egg laying in *Aplysia* involves a well-characterized series of behaviors that can last for several hours. The behaviors are controlled by two bilateral clusters of peptidergic neurons in the abdominal ganglion. Following brief stimulation, these neurons, which have been termed the bag cell neurons, undergo a sequence of changes in their excitability lasting many hours. The bag cell neurons have served as a model system for studying the molecular mechanisms involved in the synthesis, processing, and release of neuroactive peptides and in the regulation of prolonged changes in neuronal excitability.

Index Entries: *Apylsia*; abdominal ganglion; bag cell neurons; molecular mechanisms.

Introduction

The marine mollusk, *Aplysia*, provides an excellent model system for studying the molecular and neurophysiological mechanisms involved in the control of animal behavior. Unlike the complex network of cells that comprise the mammalian brain, many of the individual neurons that make up the *Aplysia* nervous system have been identified. Furthermore, the relatively large size of *Aplysia* neurons allows biochemical, electrophysiological, and molecular biological analysis of individual neurons that are responsible for modulating a given behavioral pattern. These advantages have allowed advances to be made in our understanding of the cellular basis of the modification of behavior in response to environmental stimuli, such as conditioning of the *Aplysia* gill withdrawal reflex (Castellucci et al., 1986; Kandel and Schwartz, 1982). In addition, *Aplysia* has been useful in elucidating the neurobiological basis of innate behavior patterns, such as reproduction and feeding, that do not depend on the prior experience of the animal. Of particular note in this last category is the set of behaviors that comprise egg laying in *Aplysia*. Egg laying in this animal is controlled by a group of cells called the bag cell neurons. The bag cell neurons comprise two bilaterally-situated, bag-shaped clusters of about 400 identical neurons that are located on the rostral end of the abdominal ganglion (Fig. 1). In many ways, these cells are analagous to neurons in the vertebrate hypothalamus, in that they release peptides locally to act on other neurons and also directly into the circulation. Bag cell neurons provide an ideal model neuroendocrine system, in that they are anatomically separated from the other cells of the abdominal ganglion and lend themselves well to the traditional techniques of biochemistry, electrophysiology, and molecular biology. Investigations of the peptidergic bag cell neurons are providing insights into the mechanisms of the long-term regulation of electrical excitability, as well as the regulation of synthesis, processing, transport, and secretion of neuropeptides, including the role of second messengers in these processes. Finally, an understanding of the mechanisms by which neuropeptides can initiate and maintain a fixed series of behaviors is beginning to emerge from the study of the effects of the bag cell peptides on various target cells.

Reproductive Behavior in *Aplysia*

Courtship and Mating

Like other opisthobranch mollusks, *Aplysia* is hermaphroditic. Unlike some sexually dimorphic molluskan species, *Aplysia* eggs are not fertilized externally, but are stored in the "female" and deposited after fertilization. Although one case of autocopulation has been reported (Susswein et al., 1984), this appears to be extremely rare. However, a pair of animals can inseminate each other simultaneously.

Reproductive behavior in *Aplysia* has been studied in the field and in the laboratory (Eales, 1921; MacGinitie, 1934; Audesirk, 1979; Cobbs and Pinsker, 1982a,b; Blankenship et al., 1983; Susswein, 1984; Susswein et al., 1984; Switzer-Dunlap et al., 1984; Painter et al., 1988; Leonard and Lukowiak, 1989). It consists of a fairly complex, but highly stereotyped, series of behaviors that include courtship, mating, and egg laying. Courtship consists of the "male" approaching and crawling over and around an animal acting as a female. The "male" then places its head between the parapodia of the "female" and inserts its penis into the vagina. The animal serving as the female may reciprocate and act as a male with its partner, thereby forming a pair, or act as a male to a third animal. Mating chains of up to 20 animals have been seen. Furthermore, a single animal can serve as a female to two "males," thereby allowing the formation of complex branched chain networks. Although the neuroendocrine mechanisms of control of

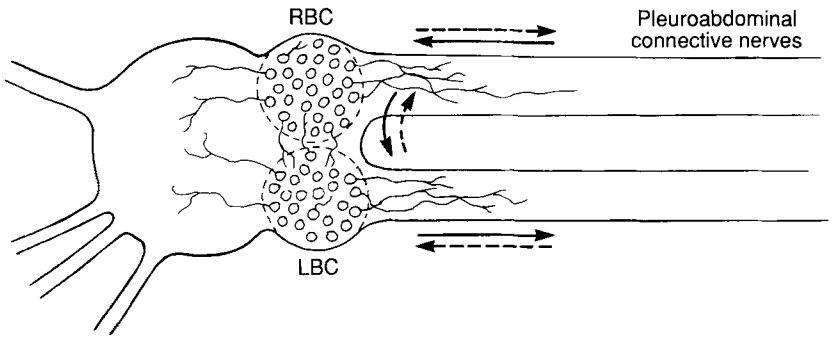

Fig. 1. Diagram of an abdominal ganglion showing the positions of the left and right bag cell clusters (LBC and RBC). Solid and dashed arrows indicate two directions of propagation of bag cell action potentials.

foreplay and copulatory behavior are not well understood, this behavior in *A. dactylomela* is known to come under the influence of a circadian rhythm and is increased as a result of food deprivation in *A. fasciata*. The latter adaptive response may serve to ensure survival of the species in situations when food is scarce.

Egg Laying Behavior

At some point after fertilization, the eggs are extruded as a string that is covered with a sticky mucus. The mucus functions to attach the egg string to any surface with which it comes into contact. While laying the eggs, the animal moves its head back and forth in a very stereotyped fashion, resulting in the formation of a sticky egg mass that adheres to substrates such as eel grass, seaweed, or rock. A single egg mass may contain well over 100 million eggs (MacGinitie, 1934; Susswein et al., 1984). The average rate of egg extrusion is on the order of 40,000/min, and a single animal in captivity has been reported to lay as many as 478 million eggs during a period of just over four months (MacGinitie, 1934).

The sequence of behaviors that accompanies egg laying follows a very predictable pattern and has been well characterized in the laboratory (Arch and Smock, 1977; Strumwasser et al., 1980; Cobbs and Pinsker, 1982a,b; Blankenship et al., 1983). Prior to the beginning of egg laying, animals discontinue eating and often locomote to a vertical surface. Perhaps the first behavior that predicts that egg laying will soon occur is an unusual puckering of the labia surrounding the mouth. This constriction of oral musculature could effectively prevent food intake and thereby ensure that the animal does not inadvertently eat its own eggs. Shortly after the beginning of these puckering mouth movements, a swelling of the common genital groove occurs, locomotion ceases, and head weaving movements and frequent twitching of the rhinophores become apparent. Head weaving becomes the predominant behavior measured and is coupled with a tucking movement as the egg strand is extruded from the common genital groove. Together, these movements allow the strand to be deposited as a compact egg mass. Within 10 min after laying a string of eggs, the animal may resume feeding.

Although *Aplysia* are one of the most prolific egg producers of the marine bottom-dwelling invertebrates (Kandel, 1979), egg laying consumes a relatively small proportion of their time (Susswein et al., 1984). Egg laying is a seasonal activity, and its frequency can be enhanced by increases in the seawater temperature (Pinkser and Parsons, 1985). As with copulation, egg laying behavior increases during periods of food deprivation (Susswein, 1984), which is

likely to serve as an adaptive response to ensure survival of the species. Egg laying is also influenced by the composition of diet and is dramatically increased when the animals eat certain types of seaweed (Carefoot, 1967).

The natural stimulus that leads to the initiation of egg laying behavior is unknown. Although *Aplysia* are often observed laying eggs during copulation, evidence suggests that copulation is not a necessary or sufficient stimulus for triggering egg laying (Blankenship et al., 1983). *Aplysia* often lay eggs in synchrony (Susswein et al., 1984), and it has been suggested that egg laying animals release a pheromone that induces other animals to lay eggs (Audersirk, 1977).

The development of an understanding of the neural mechanisms involved in the control of egg laying in *Aplysia* was greatly facilitated by the discovery of Kupfermann (1967) that an extract taken from the abdominal ganglion of *Aplysia* is capable of stimulating egg laying when injected into the hemocele of recipient animals. Kupfermann localized the egg laying activity to a group of peptidergic cells called bag cell neurons. Since that time, it has been shown that this activity is contained in a 4400 dalton polypeptide called egg laying hormone (ELH) that is secreted from actively-firing bag cell neurons (Chiu et al., 1979; Stuart et al., 1980). These data and the data discussed below suggest that the bag cell neurons respond to an appropriate stimulus by secreting ELH, thereby triggering the series of behaviors that comprise egg laying. Whatever the natural stimulus is that causes the release of ELH, it seems that ELH secretion may be an "all or none" event that is tightly regulated. Once egg laying has commenced, the associated behaviors prevent the animal from engaging in other activities for a period of at least 1–2 h. Therefore, it is likely that ELH is not secreted at times when the animal cannot devote full time to egg laying activities (such as during attack or copulation as a male). As discussed below, the electrical and morphological properties of the bag cell neurons make them particularly suitable for the task of releasing ELH in such a tightly-controlled "all or none" fashion.

Interestingly, egg laying in the freshwater pond snail, *Lymnaea stagnalis*, is controlled by a similar group of neurons called the caudodorsal cells. The electrical properties, neuromodulation, and secretory products of these cells are very similar to those of the bag cell neurons, and the caudodorsal cells have also served as a useful system for studying neuronal regulation of animal behavior (Buma et al., 1986; Geraerts and Hagenes, 1985; terMaat et al., 1988; Vlieger et al., 1980).

Developmental, Morphological, and Electrical Properties of Bag Cell Neurons

Development

The timing of the development of individual *Aplysia* depends on species, temperature, and environmental factors, such as the availability of suitable substrates for metamorphosis (Ger et al., 1984). In laboratory culture, after being released from the egg case, immature *Aplysia californica* enter into a larval stage of development that lasts approximately 34 d (Kriegstein et al., 1974, 1977). The animal then undergoes a metamorphosis before entering the juvenile phase, in which it appears similar to the adult but is not yet sexually mature. The animal gradually grows into the larger, sexually mature adult within 2–3 mo of metamorphosis (Kriegstein et al., 1974, 1977). During the larval stage of development, the cells that will eventually make up the bag cell cluster are located in the ectoderm of the body wall (McAllister et al., 1983). During and after metamorphosis, the cells migrate along fibrous connective tissue strands into the body cavity and toward the central nervous system. The bag cell neurons first appear on the

pleuroabdominal connective nerve (the afferent connective to the abdominal ganglion from the head ganglia), at 10–20 d after metamorphosis, as a primitive cluster of about 10 small neurons that are located somewhat distal to the abdominal ganglion (McAllister et al., 1983). The number of cells increases, and the cluster moves closer to the abdominal ganglion to form clusters of bag cell neurons that are connected primarily on the ventral side of the abdominal ganglion at the base of each pleuroabdominal nerve (Coggeshall, 1967; Frazier et al., 1967; Haskins et al., 1981). In younger animals, there are probably less than 100 small (about 10 μm) bag cell neurons in each cluster. These cells are not yet in contact with glial cells, and their processes extend only slightly from the soma. Furthermore, unlike bag cell neurons of larger animals, there is no morphological evidence that these cells contain neurosecretory granules. As the animals develop into sexually mature adults, the cells become more numerous (about 400/cluster), larger in size (40–100 μM), and extend dense networks of processes (Coggeshall, 1967; Frazier et al., 1967; Haskins et al., 1981).

Morphology

The bag cell neurons are typically multipolar and send their processes in every direction into the surrounding connective sheath. Typically, two or three processes extend from a single soma and branch extensively into smaller, minor processes. The processes contain numerous varicosities that are more common near axon endings (Haskins et al., 1981). A great number of the processes extend as bundles in the connective tissue sheath of the pleuroabdominal connective nerve that connects to the head ganglia. These axons wrap around the proximal portion of the pleuroabdominal nerve after leaving the bag cell cluster to form a "cuff" around the connective nerve. The number of processes contained within the cuff decreases progressively with increasing distance from the cluster, and none of these processes appear to extend more than 10–16 mm in *A. californica* and even less in other species. A small population of bag cell axons do not form a cuff, but extend up the pleuroabdominal connective among the axons of other cells that form the core of the nerve (Haskins et al., 1981). These axons extend well beyond those located in the connective sheath, but it is not known if they reach as far as the head ganglia. In addition, most bag cell neurons extend one or more branches that extend caudally over the abdominal ganglion or toward the contralateral bag cell cluster.

Based on early electron and light microscopic studies, it was suggested that bag cell neurons in mature animals are neurosecretory cells (Frazier et al., 1967; Coggeshall, 1967). They contain peptidergic granules that are round and have electron-dense cores. A subpopulation of very large dense core granules is not detected in the processes, suggesting that some peptide-containing organelles are soma specific (Kreiner et al., 1986). In addition, bag cell neurons may also contain a population of small clear vesicles that are not found in the soma or along the neurites of bag cell neurons, but are generally clustered along with dense core vesicles near the terminal axolemma (Haskins et al., 1981).

Many of the bag cell processes extend out of the bag cell clusters, where they end in a highly vascularized connective tissue sheath. This could allow release of the vesicular contents into the general circulation, where they could be transported to other central ganglia and peripheral organs (Frazier et al., 1987). Morphological evidence for the exocytotic release of vesicular contents includes the finding of omega-shaped profiles in plasma membrane in stimulated tissues that were fixed immediately after dissection (Haskins et al., 1981).

Bag Cell Neurons Can Generate a Prolonged Afterdischarge

The first description of the electrophysiological properties of the bag cell neurons was by

Frazier et al. (1967) and was followed three years later by a more extensive study by Kupfermann and Kandel (1970). Bag cell neurons have resting potentials of –40 to –65 mV and are generally silent. Action potentials can be evoked in response to intracellular depolarizing current injection, with the spike duration ranging from 30 to 150 ms. Although long depolarizing pulses sometimes produce repetitive firing in bag cell neurons, the cells soon become inactivated and cannot sustain prolonged periods of firing in their normal resting state.

A brief electrical stimulus to either of the two pleuroabdominal nerves causes the bag cell neurons to undergo a dramatic change in their electrical properties, which results in a period of repetitive firing that can last up to 60 min, but more often lasts on the order of 30 min (Fig. 2). Under some conditions, afterdischarges also can be triggered by intracellular stimulation of bag cell neurons (Brown and Mayeri, 1986). This afterdischarge is accompanied by a sustained depolarization and preceded by a series of small prepotentials. Kupfermann and Kandel (1970) noted that the afterdischarge resembled an all-or-none event in that there was no correlation between the number of stimuli used to initiate the response and the duration of the afterdischarge. This could provide a mechanism for triggering an all-or-none behavioral response. No hormone would be released under normal conditions, but when the afterdischarge is initiated, it could result in release of sufficient ELH to induce a full egg-laying response.

Bag Cell Neurons Are Electrically Coupled

By impaling multiple cells within a bag cell cluster, it was found that all cells within the cluster fire in tight synchrony during the afterdischarge. Although such close synchronization could be achieved if each bag cell neuron receives innervation by a common interneuron, it now appears likely that synchronization results from electrical coupling among the bag cell neurons. Since Kupfermann and Kandel (1970) were unable to detect the presence of electrical synapses among bag cell neurons of *A. californica*, using electrophysiological techniques within intact bag cell clusters, they suggested that such coupling may exist at a remote site that is distal to the somata of the bag cell neurons. Blankenship and Haskins (1979) later successfully demonstrated electrotonic coupling between the bag cell neurons in *Aplysia dactylomela*. The postjunctional responses that they measured across the electrical synapses of bag cell neurons were of small amplitude, long latency, and had a prolonged time course, suggesting that the coupling was remote to the cell bodies. Not only are the bag cell neurons within a cluster electrically coupled, but coupling also exists between cells in contralateral clusters of bag cell neurons. Thus, one cluster can serve as a pacemaker for the other so that initiation of an afterdischarge in one cluster results in recruitment an afterdischarge in the contralateral cluster. Kaczmarek et al. (1979) provided morphological evidence for electrotonic coupling of the bag cell neurons by showing that injection of lucifer yellow into a bag cell soma can result in labeling of adjacent cells. Furthermore, they reported freeze-fracture studies that indicate that the processes of bag cell neurons are joined by numerous gap junctions. Cross dye labeling can also be observed in closely adjacent bag cell neurons grown in primary culture, and, in this preparation, electrotonic connections between the cells are easily measured (Fig. 3) (Kaczmarek et al., 1979). The strong degree of electrical coupling in cell culture may result from the formation of soma to soma gap junctions that would not normally form in the intact ganglion. Tight coupling among the bag cell neurons, and the resultant synchrony of firing during the afterdischarge, could ensure that each cell within the cluster participates in the afterdischarge, thereby allowing for maximum output of secretory product.

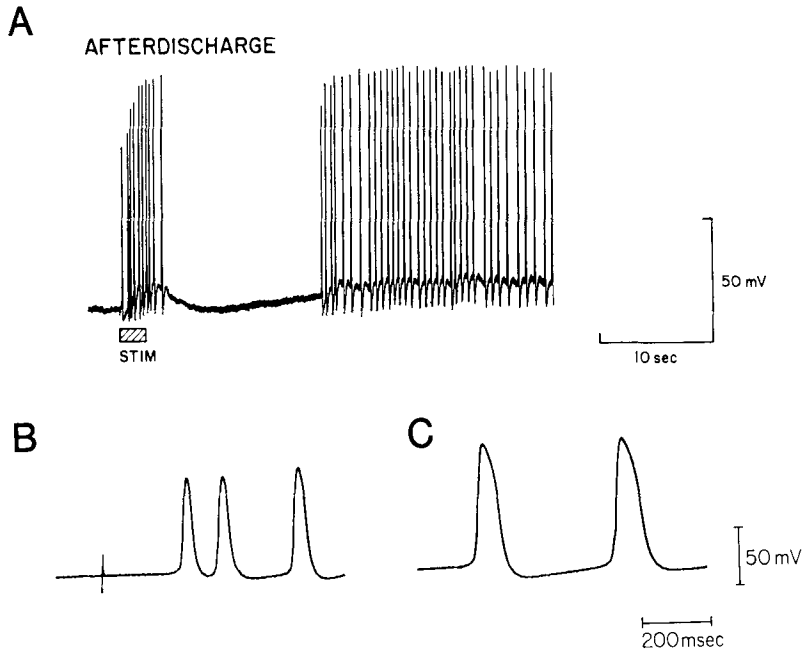

Fig. 2. (A) Onset of afterdischarge in a bag cell neuron following stimulation of a pleuroabdominal connective nerve (STIM). (B and C) Enhancement of action potentials during a discharge: B shows intracellularly recorded action potentials at the onset of stimulation, whereas C shows action potentials recorded 10 min after the onset of discharge (Strong et al., 1987b).

Multiple Excitability States Within the Afterdischarge

The afterdischarge begins with a high frequency firing of action potentials. The early phase of rapid firing (2–6 Hz) lasts less that 1 min and is followed by a prolonged second phase of relatively low frequency firing (<0.5 min) that lasts for the remainder of the discharge (Kupfermann and Kandel, 1970; Dudek and Blankenship, 1977a; Kaczmarek et al., 1982). During the initial phase of the afterdischarge, the spikes are primarily sodium dependent (Kaczmarek, 1982), have a high conduction velocity, and are of short duration (Dudek and Kossatz, 1982). During this period, there is a frequency-dependent spike heightening and broadening that apparently results from the inactivation of a voltage- and time-dependent potassium current (Acosta-Urquidi and Dudek, 1981; Kaczmarek et al., 1982). This frequency-dependent spike potentiation may play a role in augmenting the release of egg laying hormone. During the second slower phase of firing, the properties of the spikes undergo further changes. The second phase appears to be associated with an enhancement of the calcium component of the action potentials (Kaczmarek et al., 1982). During this phase, the action potentials have a relatively slow conduction velocity (Dudek and Kossatz, 1982). Furthermore, the action potentials become enhanced in both height and width early in the second phase of firing and remain enhanced for the duration of the afterdischarge (Fig. 2) (Kaczmarek et al., 1982). Unlike the spike enhancement that is seen shortly after the onset of the afterdischarge, this spike enhancement is not frequency-dependent and appears to be owing to a more basic change in the electrical properties of the bag cell

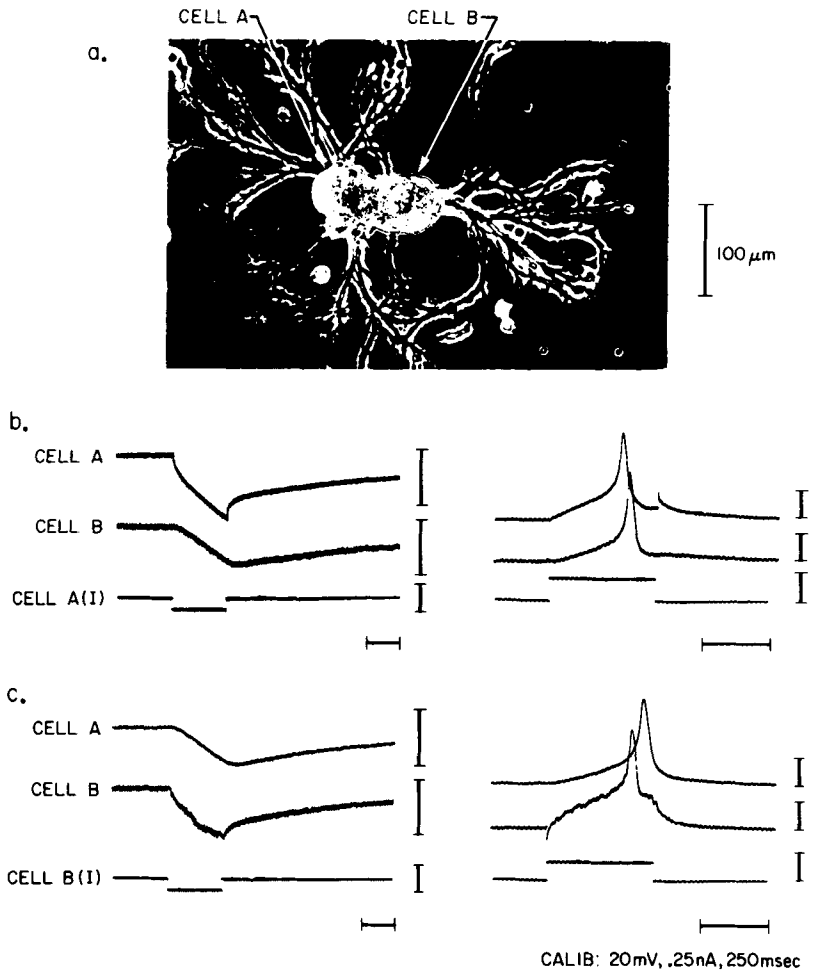

Fig. 3. (a) Two closely-opposed bag cell neurons in cell culture. (b and c) Electrical coupling between the two cells. Traces show the voltage responses to injection of hyperpolarizing or depolarizing current into cell A or B (Kaczmarek et al., 1979).

neurons rather than a time- and voltage-dependent inactivation of potassium currents. Also during the second phase, there are occasional bursts of action potentials. During these short bursts, the conduction velocity is highest for the first spike and decreases for successive spikes (Dudek and Kossatz, 1982). The occurrence of long-duration spikes that conduct slowly down the neurite during the second phase of the afterdischarge would be expected to enhance the amount of hormone released with each action potential.

In vivo, the mean duration of discharges in *Aplysia brasiliana* has been found to be about 21 min (Dudek et al., 1979). It was reported that egg laying may follow afterdischarges that are only 2–3 min in duration, suggesting that sufficient ELH for induction of egg laying may be released during these first few minutes of a discharge. These studies, however, monitored only one of the two clusters of bag cell neurons. Because the duration of discharge in the two clusters may vary considerably, it is possible that a significant amount of ELH could have

been released by a more prolonged second phase in the contralateral cluster.

Propagation of Action Potentials During the Afterdischarge

Because of the tight electrotonic coupling that exists among the bag cell neurons, the afterdischarge can be recorded extracellularly as large compound action potentials that represent simultaneous firing of all of the neurons within a bag cell cluster. Furthermore, extracellular responses of bag cell neurons can be measured in the neurites and distinguished clearly from spikes of other cells on the basis of waveform, location, conduction velocity, and, especially, duration of the action potentials (Dudek and Blankenship, 1977a). Dudek and Blankenship (1976; 1977a,b) used simultaneous intracellular recording at the bag cell somata and extracellular recording from bag cell neurites at various points along the pleuroabdominal nerve to study the ways in which spikes are initiated and propagated during an afterdischarge.

During an afterdischarge, most bag cell spikes are initiated in the distal region of the bag cell processes in one of the pleuroabdominal connectives and propagate into the ipsilateral somata (Dudek and Blankenship, 1977a). Action potentials then propagate toward the contralateral bag cell cluster, where spikes are initiated at a site near the bag cell somata (Haskins and Blankenship, 1979). The spike activity then propagates both into the contralateral cluster and outward toward the head ganglia along the contralateral connective nerve. During such activity, in which one cluster serves as a pacemaker for the other, there is close synchrony in the activity of the two bilateral clusters. The sites of spike initiation often shift spontaneously from the neurite terminals of one cluster to those of the other cluster, with spikes initiated distally and then propagated inward. Occasionally, spike initiation may occur independently at the neurite terminals on both sides, and asynchrony develops between the two clusters.

Spike initiation and a full afterdischarge can occur in neurites that have been severed from the bag cell somata at the junction between the bag cell cluster and the pleuroabdominal connective (Kaczmarek et al., 1978), making it clear that the bag cell somata are not required for the afterdischarge. The somata do, however, allow the propagation of a unilateral afterdischarge to the opposite cluster and serve a pacemaker function, as discussed above.

Finally, action potentials in the bag cell processes frequently fail to invade the somata. Such conduction failure can happen any time during the period of repetitive firing, but occurs most often at the termination of the afterdischarge (Dudek and Blankenship, 1977a; Kaczmarek et al., 1978).

Bag Cell Neurons Enter a Prolonged Inhibited State

Following an afterdischarge, the bag cell neurons enter an inhibited state, during which further stimulation elicits normal action potentials, but either fails to stimulate a second afterdischarge or stimulates an afterdischarge of very short duration (Kupfermann and Kandel, 1970). Recovery from this inhibited state occurs gradually, and a period of 18–20 h is required before full-length afterdischarges can be elicited again. It is likely that the initial part of this inhibited period allows for the completion of the series of egg laying behaviors before initiation of another afterdischarge can occur. It may, perhaps, also permit a new round of oocyte maturation.

The onset of the inhibited period can be associated with the failure of action potentials, which are generated in the neurites, to fully invade the bag cell somata (Dudek and Blankenship, 1977a; Kaczmarek et al., 1978). Nevertheless, it is frequently observed that discharges may persist in the neurites even when the so-

mata are not invaded by action potentials. Therefore, Kaczmarek et al. (1978) defined two independent types of "refractoriness" that contribute to the inhibited period and could be differentiated pharmacologically. Type I refractoriness represents the failure of action potentials generated in the tips of the neurites to invade the somata, whereas Type II refractoriness controls the duration of the discharge itself.

Relationship of Afterdischarge to Behaviors

A great deal of evidence suggests that the bag cell afterdischarge is indeed the trigger that initiates egg laying behavior. Shortly after his finding that bag cell extracts can initiate egg laying behavior, Kupfermann (1970) found that the substance that stimulates egg laying is released during an afterdischarge. The released activity was subsequently shown to be ELH (Stuart et al., 1980). More direct evidence for the involvement of the bag cell discharge in initiating egg laying behavior came from the finding that electrical stimulation of an afterdischarge induces egg laying behavior in freely-behaving animals (Pinsker and Dudek, 1977) and that spontaneous bag cell afterdischarges are always followed by egg laying with a latency of about 30 min (Dudek et al., 1979). Furthermore, surgical removal of bag cell clusters severely diminishes the frequency of egg-laying behaviors (Pinsker and Dudek, 1977). It should be pointed out, however, that although the electrical stimulation of an afterdischarge or the injection of ELH triggers all egg laying behaviors, including rhythmic head movements, in vivo recordings during spontaneous egg laying suggest that such head movements may actually begin before the onset of an afterdischarge (Cobbs and Pinsker, 1982a).

The initiating stimulus that leads to induction of a bag cell afterdischarge and the subsequent egg laying behavior is still unknown. In *Aplysia californica*, a specialized part of the reproductive tract, termed the atrial gland, contains two peptides (peptides A and B) that are capable of stimulating egg laying behavior and a bag cell afterdischarge (Arch et al., 1978; Heller et al., 1980). The atrial gland is found in the wall of the large hermaphroditic duct, and its morphology suggests that it is an exocrine gland that secretes peptides directly into the oviduct rather than into the circulation (Arch et al., 1980; Beard et al., 1982; Painter et al., 1985). There is evidence that some products of the atrial gland may act as pheromes to enhance copulatory behaviors (Susswein and Benny, 1985; Painter et al., 1989) although whether the A and B peptides function in this manner, and the conditions under which they are released, are not yet known. Furthermore, neither the atrial gland nor A and B peptides are present in *A. parvula* (Scheller et al., 1986) although egg laying proceeds normally in this species. Thus, it is unlikely that the presence of atrial gland peptides is necessary for normal initiation of egg laying behavior.

The neural input to the bag cell neurons is not well characterized. Afterdischarges can be evoked by extracellular electrical stimulation of a pathway that leads from the cerebral ganglia, through the pleural ganglia, to the bag cell neurons, via the pleuroabdominal connective nerves, suggesting that the normal neural input arises in, or close to, the cerebral ganglion. This idea is reinforced by the finding that the peptides that trigger bag cell afterdischarges are effective when applied locally to the head ganglia (Heller et al., 1980; Painter et al., 1988, 1989a,b). Stimulation of peripheral nerves afferent to the cerebral ganglia does not, however, trigger afterdischarges. Brown and Mayeri (1987) reported that electrical stimulation of a population of ELH immunoreactive white cells in the right pleural ganglion can initiate a bag cell afterdischarge. In most respects, these cells resemble bag cell neurons that have come to be situated at the pleural, rather than the abdominal, end of the connective nerve. It is possible

that the axons of these cells are the axons responsible for induction of an afterdischarge after stimulation of the pleuroabdominal connective. However, as with the atrial gland, it is unknown whether these cells are involved in the normal initiation of an afterdischarge and egg laying behavior and, if so, what natural conditions result in their activation.

Bag Cell Neurons Are a Multitransmitter System that Secrete ELH and Other Neuroactive Peptides

Purification of ELH

ELH is sensitive to proteases and has a mol wt of about 6000 dalton based on gel filtration (Toevs and Brackenbury, 1969). Subsequently, a polypeptide with a molecular weight consistent with ELH was found to be transported to the axon terminals in the sheath (Arch, 1972b; Loh et al., 1975) and released in a calcium-dependent manner (Arch, 1972a). After a report that ELH is a basic peptide with an isoelectric point of about 9.3 (Arch et al., 1976a), Chiu et al. (1979) used bioassay of induction of egg laying to purify the peptide using cation exchange chromatography, followed by gel filtration. Determination of the primary structure of ELH revealed it to be a 36 amino acid polypeptide with a pI of 9.2 and a calculated mol wt of 4385 dalton.

Processing of Pro-ELH

Based on biochemical studies, it has been appreciated for some time that the bag cell neurons contain a number of peptides that are derived from a single precursor and that ELH and another peptide, termed acidic peptide (AP), are secreted in response to depolarization (Arch, 1972a, 1976a,b; Kupfermann, 1972; Loh et al., 1975; Stuart et al., 1980a; Berry, 1981). Over half of the protein synthesis that occurs in bag cell neurons during the egg laying season is devoted to synthesis of the ELH precursor (Arch et al., 1972b). Thus, it is likely that these polyploid cells contain a proportionately large amount of mRNA coding for this protein. Scheller et al. (1982) isolated recombinant clones encoding a gene that was expressed specifically in the bag cell neurons. These were found to encode a precursor protein that contained the sequence for ELH. Although the nucleotide sequence of independent clones varies, it appears that they all code for the same amino acid sequence (Mahon et al., 1986). Thus, as suggested by biochemical experiments (Berry, 1981), it appears that there may be only one species of ELH precursor protein.

The predicted sequence of the pro-ELH protein contains, at its *N*-terminal, a hydrophobic signal sequence that is characteristic of secretory proteins (Scheller et al., 1983). Also found in the pro-ELH sequence are a number of dibasic residues that have been identified as sites for cleavage by endoproteases. Cleavage at these sites generates ELH, acidic peptide, and several smaller peptides. These include two structurally-related pentapeptides, β-bag cell peptide (β-BCP) and γ-bag cell peptide (γ-BCP), and a nine amino acid peptide (α-bag cell peptide; α-BCP). The relative positions of these peptides in the precursor are shown in Fig. 4. Interestingly, the regions of the pro-ELH gene coding for ELH, α-BCP, and β-BCP are the most highly conserved across different species of *Aplysia*, suggesting that these peptides may have important physiological and behavioral roles in the various species. In biochemical studies, α-, β-, and γ-BCP have each been isolated from bag cell extracts (Rothman et al., 1983a; Sigvardt et al., 1986; Newcomb and Sceller, 1987) and have been shown to be released with ELH and AP during afterdischarge (Rothman et al., 1985). Furthermore, immunocytochemistry confirms that ELH and α-BCP are localized in the same bag cell neurons (Pulst et al., 1986, 1987; New-

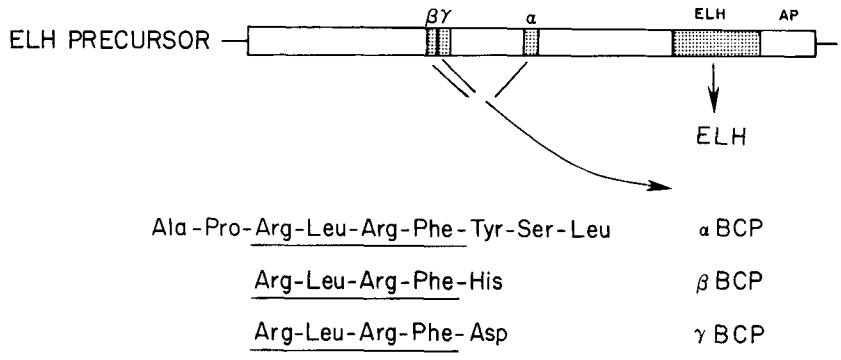

Fig. 4. Diagram showing the relative positions of ELH, AP, and α-, β-, and γ-BCP on the ELH precursor protein. Also shown are the sequences of α-, β-, and γ-BCP.

comb and Scheller, 1987). Based on these data and the physiological data discussed below, it has become clear that the bag cell neurons comprise a multitransmitter system that employs a combination of neuroactive peptides.

Two other peptides derived from the ELH precursor, termed δ-BCP and ε-BCP, have also been found in extracts of bag cell neurons (Nagel et al., 1988b, 1989b). ε-BCP is a 19 amino acid peptide located just before ELH on the precursor. δ-BCP, a 39 residue peptide, is located between γ-BCP and α-BCP. The latter peptide may play a role in stimulating calcium flux into mitochondria of secretory cells in the albumen gland of the reproductive tract.

Packaging of Bag Cell Peptides into Secretory Vesicles

The initial steps of proteolytic processing of the ELH precursor begin in the Golgi apparatus (Yates and Berry, 1984; Fisher et al., 1988). The question of whether a cell separates multiple products before release and, if so, the mechanisms by which the cell achieves such separation is of critical importance for a complete understanding of regulatory mechanisms in peptidergic neurons. If granule products are packaged prior to completion of processing, this would result in simultaneous release of the distinct peptides. However, if the individual peptides derived from a larger precursor are separated before closure of secretory granules, this could allow the products to be routed to release sites and released separately.

Evidence suggests that bag cell peptides may be differentially packaged, transported, and released. For instance, Arch et al. (1986) showed that ELH, and an acidic peptide that may correspond to AP, do not appear to be transported with a fixed stoichiometry. After a pulse incubation with ^{3}H-leucine, ELH and the acidic peptide are synthesized with similar time courses, but labeled AP appears in the neurites before labeled ELH. Furthermore, there is differential release of the two peptides in response to depolarization. Consistent with this, Molloy et al. (1987) found that when vesicle fractions are separated using density-gradient centrifugation, vesicle fractions containing ELH and the acidic peptide do not show strict covariance across the gradient. Taken together, these data suggest that ELH and acidic peptide are in separate secretory granules and are differentially transported and released.

Fischer et al. (1988) showed that ELH and the BCPs located at the amino terminal of the precursor (i.e., α-, β-, and γ-BCPs) are also differ-

entially packaged and transported. Using quantitative immunochemistry with antibodies against the various bag cell products, these workers found that ELH immunoreactivity is localized to the cell body and processes, whereas immunoreactivity to the amino terminal portion of pro-ELH, which contains the BCPs, is primarily localized in the cell body. Furthermore, they found that ELH and amino terminal BCPs are localized in different vesicle classes that have distinct size distributions.

Related Peptides in the Atrial Gland

Genes homologous to the ELH gene are expressed in the atrial gland (Scheller et al., 1982). As mentioned above, two peptides (A and B) that are localized in the atrial gland can initiate bag cell afterdischarges. In addition, the atrial gland contains peptides that are homologous to ELH, some of which are as potent as ELH itself in inducing egg laying when injected into animals (Nagle et al., 1985, 1989b).

The complete nucleotide sequence of two atrial gland peptide genes has been determined (Scheller et al., 1983). One of these encodes a large polyprotein precursor that is homologous to the ELH precursor but, on proteolytic processing, appears to generate only one major active peptide, peptide B (Nagle et al., 1988). Processing of the precursor encoded by the other gene yields peptide A, as well as an ELH-related peptide that is bound by disulfide bridges to another peptide related to AP, which is located adjacent to the ELH-like peptide in the precursor protein (Mahon et al., 1985; Rothman et al., 1986; Nagle et al., 1986, 1989b). These two genes are approximately 90% homologous to the pro-ELH gene (Scheller et al., 1983), but appear to have diverged by single base changes, deletions, and insertions in a way that results in each gene generating a distinct set of nonoverlapping peptides. Biochemical studies have found evidence for additional ELH-related peptides (Nagle et al., 1988), suggesting that other members of the ELH family of genes are also expressed in the atrial gland (Nagle et al., 1986, 1988).

Second Messengers Modulate the Activity of the Bag Cell Neurons

Modulation of Ionic Conductances by Cyclic AMP

The generation of an afterdischarge from the normally silent bag cell neurons represents a long-lasting transformation of the electrical properties of a neuron from one state to another. This form of modulation of neuronal excitability plays an important role in regulating a variety of animal behaviors and is commonly brought about by neurotransmitter-induced generation of second messengers and the subsequent activation of various protein kinases (Kaczmarek and Levitan, 1987). The bag cell neurons provide an excellent model system for studying the molecular mechanisms involved in this type of neuromodulation. A great deal of evidence indicates that cyclic AMP plays a key role in the generation of the bag cell afterdischarge. At the onset of an afterdischarge, there is an increase in cyclic AMP levels (Kaczmarek et al., 1978). Furthermore, bag cell neurons contain cyclic AMP-dependent protein kinase (cAMP-PK) activity (Kaczmarek et al., 1982), and the afterdischarge is accompanied by an increase in the phosphorylation state of at least two endogenous proteins in bag cell neurons, both of which serve as substrates for cAMP-PK in cell-free extracts (Kaczmarek et al., 1982). This suggests that synthesis of cyclic AMP, and subsequent activation of cAMP-PK, may be involved in modulating the excitability of these neurons to give rise to the afterdischarge.

Kaczmarek et al. (1978) demonstrated that cell permeable, phosphodiesterase-resistant

cyclic AMP analogs, such as 8-benzylthio-cyclic AMP and 8-methylthio-cyclic AMP, can initiate an afterdischarge in intact bag cell clusters or isolated bag cell neurites that is similar in all respects to afterdischarges induced by electrical stimulation. Furthermore, the addition of dopamine (which increases cyclic AMP levels in these cells) or methylxanthine phosphodiesterase inhibitors reinitiates afterdischarges when added within 1 min of the termination of an afterdischarge. In contrast to the brief afterdischarges that can sometimes be initiated with electrical stimulation at the end of an afterdischarge, the dopamine and methylxanthine-induced afterdischarges can be longer than the initial afterdischarge.

The role of cyclic AMP and cAMP-PK in modulating the excitability of bag cell neurons was studied further in isolated bag cell neurons grown in primary culture. Bag cell neurons retain many of their morphological and electrical properties when grown in primary culture, and this preparation lends itself well to traditional electrophysiological techniques (Kaczmarek et al., 1979). In common with bag cell neurons in an intact cluster, isolated bag cell neurons respond to the addition of 8-benzylthio-cyclic AMP, with generation of a long-lasting afterdischarge (Kaczmarek and Strumwasser, 1981). The generation of an afterdischarge is preceded by an increase in input resistance, a decrease in the threshold for spikes evoked by depolarizing current pulses, subthreshold membrane potential oscillations, and an increase in the height and width of electrically-evoked action potentials. Similar effects are seen after application of the cyclic AMP analog, adenosine-3',5'-monophosphothioate (Conn et al., 1988a), or the adenylate cyclase activator, forskolin (Kauer and Kaczmarek, 1985; Conn et al., 1988a). Direct evidence that the enhancement of action potentials is mediated by the activation of cAMP-PK comes from the finding that it is mimicked by intracellular injection of the catalytic subunit of cAMP-PK (Kaczmarek et al., 1980) and inhibited by injection of a protein inhibitor of cAMP-PK (Conn et al., 1988a).

Two-microelectrode voltage clamp and whole cell patch clamp techniques indicate that cell permeant cyclic AMP analogs (Kaczmarek and Strumwasser, 1984) and forskolin (Strong 1984; Strong and Kaczmarek, 1986) have little effect on the major net inward current in bag cell neurons. However, two-electrode voltage clamp reveals that 8-benzylthio-cyclic AMP does induce a region of negative slope resistance in the current–voltage relationship of bag cell neurons (Kaczmarek and Strumwasser, 1984). When the membrane potential is clamped for periods as long as 2–6 s, an approximately linear I–V relationship of inward current is observed between –50 and –35 mV. At potentials positive to about –35 mV, net outward currents are activated. Five to ten minutes after the addition of 8-benzylthio-cyclic AMP, a region of negative slope resistance emerges in the I–V relations, giving a large plateau from about –45 to –20 mV (Kaczmarek and Strumwasser, 1984). Such a region of negative slope resistance is characteristic of cells that demonstrate endogenous repetitive firing activity (Benson and Adams, 1987), suggesting that this may be a mechanism by which cyclic AMP may contribute to the onset of discharge in bag cell neurons. The nature of the channel responsible for this change in the current–voltage relationship is not yet known.

Evidence suggests that cyclic AMP also decreases net outward currents in bag cell neurons. The net outward current of these cells includes a transient inactivating potassium current or A current and a delayed potassium current that consists of a calcium-dependent potassium current and two components of delayed rectifier potassium current (Kaczmarek and Strumwasser, 1984; Strong, 1984; Strong and Kaczmarek, 1986). In addition, these cells contain an inward rectifier potassium current (Kauer et al., 1987). Cyclic AMP analogs (Kaczmarek and Strumwasser, 1984) and

forskolin (Strong, 1984; Strong and Kaczmarek, 1986) diminish both the net delayed outward current and the A current in bag cell neurons. Some of these potassium currents are likely to play a role in the repolarization of action potentials, and thus, a decrease in these currents could contribute to spike broadening. An increase in the amplitude of the inwardly rectifying potassium current, in response to cyclic AMP analogs and forskolin, has also been reported (Kauer et al., 1987).

Despite these advances, the exact role of cAMP-PK in initiation and maintenance of the afterdischarge is not known. It is likely that cyclic AMP is required for the expression of spontaneous action potentials, but a direct test of this must await the availability of cell permeable inhibitors of cAMP-PK that can be used to see how inhibition of this enzyme would influence expression of the afterdischarge.

Protein Kinase C Enhances Calcium Action Potentials

Another second-messenger system that has received a great deal of attention is that linked to phosphoinositide hydrolysis, and recent efforts have led to a fairly detailed understanding of this mechanism of signal transduction (For an extensive review, *see* Abdel-Latif, 1986). Stimulation of a phosphoinositide hydrolysis-linked receptor results in activation of a phosphoinositide-specific phosphodiesterase (phospholipase C) and subsequent hydrolysis of membrane phosphoinositides. The primary substrate of receptor-activated phospholipase C is thought to be phosphatidylinositol-1,4,5-trisphosphate (PIP_2). Hydrolysis of PIP_2 results in the liberation of two products, diacylglycerol (DAG) and inositol trisphosphate (IP_3), both of which can serve as second messengers. IP_3 liberates calcium from intracellular stores, whereas DAG activates calcium/phosphatidylserine/DAG-dependent protein kinase (protein kinase C; PKC).

Stimulation of an afterdischarge leads to activation of phosphoinositide hydrolysis, resulting in liberation of IP_3 and, by inference, DAG (Fink et al., 1988). Furthermore, the bag cell neurons are a rich source of PKC and contain a number of PKC substrates (DeReimer et al., 1985a). This enzyme may be activated by synthetic cell-permeable DAGs, such as dioctanoyl glycerol (DOG), or phorbol esters, agents that mimic the action of DAG. Exposure of bag cell neurons grown in primary culture to DOG or the phorbol ester, 12-O-tetradecanoyl-13-phorbol acetate (TPA), results in an enhancement of the height of electrically-evoked action potentials (Fig. 5) (DeReimer et al., 1985b). This effect is mimicked by intracellular injection of purified PKC or exposure to other phorbol esters that activate PKC, but not by phorbol esters or lipids that do not activate this enzyme (DeReimer et al., 1985b; Bley and Kaczmarek, 1986). Futhermore, this effect of TPA is inhibited by two structurally-distinct PKC inhibitors, sphinganine (erythro dihydro-sphingosine) and H-7 (1-[5-isoquinolinesulfonyl]-2-methyl piperazine) (Conn et al., 1988a,b). Taken together, these data strongly suggest that TPA-induced enhancement of bag cell action potentials is mediated by activation of PKC.

Unlike cyclic AMP analogs, activators of PKC have no effect on voltage-dependent potassium currents in bag cell neurons. However, the inward calcium current, measured in cells internally dialyzed with TEA and cesium to block potassium currents, is markedly enhanced by activators of PKC (DeReimer et al., 1985b). Consistent with the enhancement of action potentials, this effect is not mimicked by inactive phorbol esters (DeReimer et al., 1985b) and is prevented by PKC inhibitors (Conn et al., 1988a,b).

Unmasking of a Covert Calcium Channel

In theory, the enhancement of calcium current could be owing to either a change in the

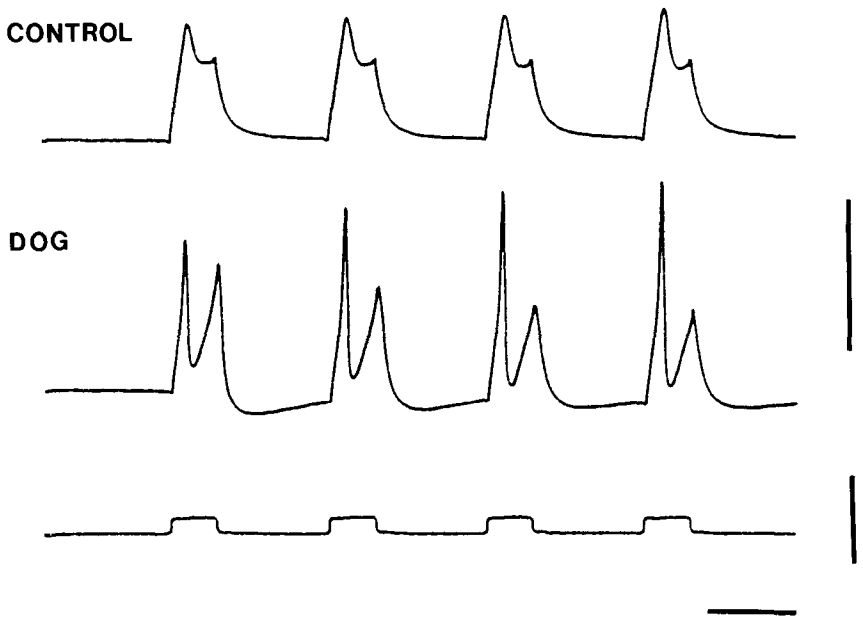

Fig. 5. Enhancement of action potentials in bag cell neurons by dioctanoyl glycerol (DOG). Action potentials in this isolated neuron were evoked by current pulses (lowest trace) (calibration bars: 50 mV, 0.1 mA, 350 ms; Strong et al., 1987b).

properties of calcium channels that are already present in the membrane, or acute recruitment of new calcium channels. These alternatives were examined using cell-attached patch pipets to measure the activity of single calcium channels on the plasma membrane of bag cell neurons (Strong et al., 1987). In cells that have not been treated with PKC activators, a single species of calcium channel is present. Using barium-containing pipets, this channel has a unitary conductance of about 12 ps. It is distributed on the cell membrane in clusters that often contain many channels. After treatment of bag cell neurons with activators of PKC, such as TPA, the 12 ps channel appears to be present in the same density as control cells, and the properties of this channel (i.e., mean open time, and so on) remain unchanged. However, in TPA-treated cells, a new larger conductance (24 ps) channel that is never seen in control cells is also detected (Fig. 6). Unlike the small-conductance channel, this channel is distributed relatively evenly on the cell surface and tends to open in bursts of high frequency. Although the possibility that the large-conductance channel results from interconversion of the small conductance channels cannot be completely ruled out, the fact that the density of the small conductance channel is unchanged following activation of PKC suggests that the large-conductance calcium channel is a novel species of channel that is unmasked in the membrane upon activation of PKC. This hypothesis is further supported by the distinct spatial distributions of the two channels. This acute recruitment of a previously covert species of calcium channel could provide a mechanism for induction of a rapid and long-lasting change in neuronal excitability. An interesting aspect of PKC-induced recruitment of the covert calcium channel is that it does not occur if TPA is added after the cell has been dialyzed. This suggests that cell dialysis disrupts some intracellular process that is important for recruitment of this channel.

The availability of cell-permeable PKC inhibitors allows the direct assessment of the contribution of PKC and the covert calcium channel to the afterdischarge. Incubation of abdominal

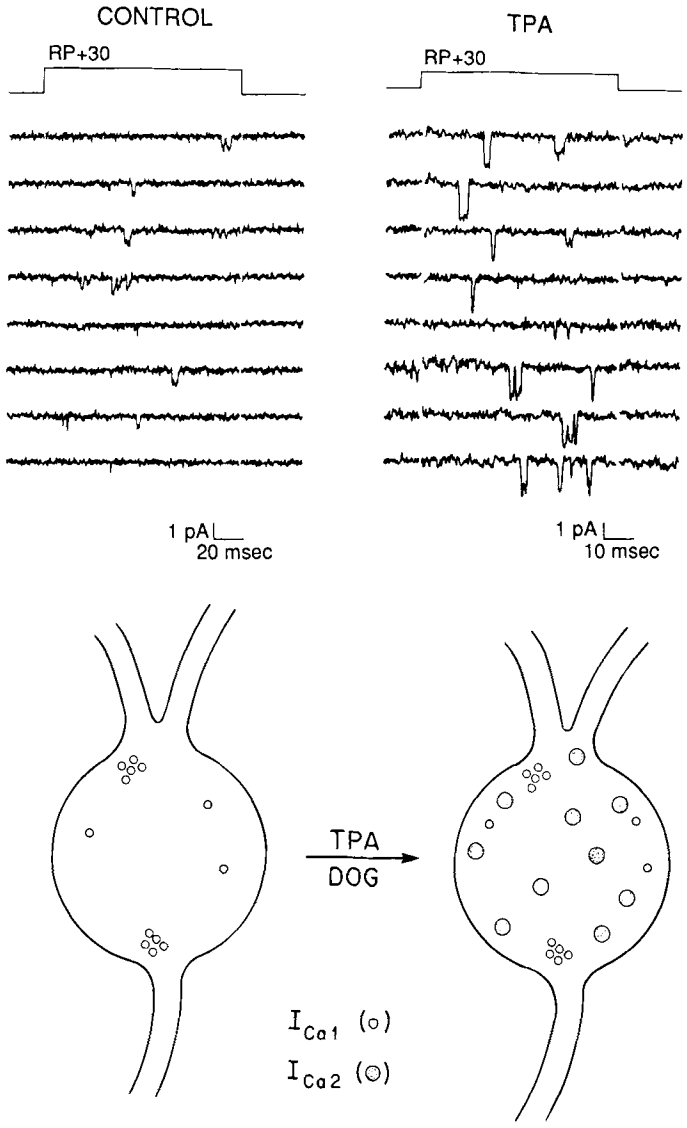

Fig. 6. Unmasking of a new species of calcium channel in bag cell neurons after exposure to an activator of protein kinase C (TPA). In control cells, only one species of channel is detected in cell-attached patch clamp readings. After exposure to TPA, two species of voltage-dependent calcium channels are observed.

ganglia with PKC inhibitors does not prevent initiation or maintenance of an afterdischarge (Conn et al., 1988b), suggesting that PKC and the covert calcium channel are not obligatory for expression of a bag cell afterdischarge. However, as described earlier, intracellular recordings from untreated bag cell neurons during an afterdischarge have shown that bag cell action potentials become markedly enhanced in both height and width in the first 2 min of the afterdischarge and remain enhanced for the duration of the afterdischarge (Kaczmarek et al., 1982). In contrast, in abdominal ganglia that are treated with PKC inhibitors, spike enhancement does not occur during the afterdischarge (Conn et al., 1988b), suggesting that the covert calcium channel may contribute to spike enhancement during the afterdischarge, and perhaps thereby enhance neuropeptide release.

Mechanism of Prolonged Spike Enhancement

The electrical stimulus to the pleuroabdominal nerve that is needed to initiate an afterdischarge is brief, lasting only seconds. However, the enhancement of action potentials persists for the duration of the afterdischarge. Studies in cultured bag cell neurons are beginning to elucidate the mechanism of this prolonged spike enhancement. Although a 15 min preincubation of cultured bag cell neurons with PKC inhibitors prevents TPA-induced spike enhancement, PKC inhibitors cannot reverse the enhancement of action potential height if it is added after the effects of TPA have occurred. This suggests that transient activation of PKC can act as a "trigger" for inducing expression of the covert calcium channel and that, once expressed, the channel activity does not depend on further PKC activity for its maintenance. The mechanism of the persistence of PKC-activated calcium current is not yet known. One possibility is that transient activation of the enzyme results in conversion of PKC, to a proteolytic cleavage product of PKC that is active in the absence of DAG and calcium (Kajikawa et al., 1983). The formation of such an autonomous enzyme that is not sensitive to the PKC inhibitors could explain these results. An equally plausible mechanism is that PKC induces irreversible expression of the covert channel such that the channel remains functional in the absence of ongoing phosphorylation. For example, if in response to the activation of PKC the channel is recruited to the plasma membrane from intracellular vesicles, it may require normal membrane recycling for inactivation.

IP_3-Induced Hyperpolarization of Bag Cell Neurons

Since the afterdischarge is associated with a change in phosphoinositide turnover (Fink et al., 1988), it is possible that the phosphoinositide hydrolysis-derived second messenger, IP_3, also plays a role in modulating bag cell excitability. As has been shown for many nonneuronal cells and also for photoreceptors (Corson and Fein, 1987), microinjection of IP_3 into cultured bag cell neurons elevates intracellular calcium concentrations by liberating calcium from an intracellular store (Fink et al., 1988). Direct measurements of changes in intracellular calcium, using digital imaging of isolated neurons loaded with the fluorescent calcium indicator fura-2, reveal that the IP_3-induced increase in intracellular calcium is primarily localized in the cell body, with little change in the neurites. This is in contrast to the effect of electrically-evoked action potentials that increase intracellular calcium concentrations primarily in the neurites of bag cell neurons (Fink et al., 1988).

The electrophysiological effects of microinjections of IP_3 are consistent with those that would be expected to occur with an increase in intracellular calcium. Injection of IP_3 induces a transient hyperpolarization of the membrane and diminishes the height of electrically-evoked action potentials. Intracellular injection of calcium mimicks the effect of IP_3. Furthermore, this effect is accompanied by an increase in conductance and is abolished by the addition of the potassium channel blocking agent, tetraethylammonium ions (TEA), suggesting that it may be related to an increase in a potassium current. The net delayed potassium current in bag cell neurons includes a calcium-dependent potassium current (Kaczmarek and Strumwasser, 1984), and the electrophysiological effects of IP_3 suggest that IP_3-induced increases in intracellular calcium may activate this current. This is supported by the finding that injection of IP_3 increases the activity of a 40 ps channel that is believed to be a calcium-activated potassium channel. The increase in the activity of this channel may occur through a shift in its voltage-dependence to more negative potentials (Fink et al., 1988).

Intracellular recordings from bag cell neurons during an afterdischarge reveal that bag cell neurons undergo a transient hyperpolari-

zation immediately following electrical stimulation of the pleuroabdominal connective. This hyperpolarization precedes the onset of spontaneous firing. It is possible that IP_3 is responsible for this hyperpolarization. IP_3-induced increases in calcium concentration may also have other functions in regulating activity of bag cell neurons during the afterdischarge, such as contributing to calcium-dependent neurotransmitter release or activating PKC or calcium/calmodulin-dependent protein kinase (Ca/CaM-PK)(DeReimer et al., 1984).

Modulation of Peptide Synthesis and Granule Transport

Synthesis of sufficient ELH and other peptides for secretion during repeated afterdischarges in the reproductive season may place a major demand on the peptide synthetic machinery of the bag cell neurons. The synthesis of ELH undergoes seasonal variation and is approximately twofold higher in the late summer egg laying season than in midwinter (Berry, 1982). This suggests that regulatory mechanisms exist that replenish ELH stores during periods of high secretory demand. Evidence suggests that generation of an afterdischarge may also regulate pro-ELH synthesis. Depolarization of bag cell neurons with a high potassium medium enhances their synthesis of pro-ELH (Berry and Arch, 1981). This effect does not occur if the neurites are removed from the bag cell somata or the experiment is done in a low calcium medium.

Since stimulation leading to an afterdischarge results in activation of phosphoinositide hydrolysis and elevation of cyclic AMP, Bruel and Berry (1985) began to test the hypothesis that these second messenger systems regulate pro-ELH synthesis during an afterdischarge. They found that a variety of agents that increase cyclic AMP levels in these cells stimulate ELH synthesis. These include a cell permeable cyclic AMP analog, forskolin, a phosphodiesterase inhibitor, dopamine, and serotonin. Although some of these treatments can stimulate an afterdischarge, serotonin elevates cyclic AMP levels in these cells without activating an afterdischarge, suggesting that this effect is not secondary to a cyclic AMP-induced afterdischarge. Furthermore, Berry (1988) has found that application of a peptide that decreases cyclic AMP production in bag cell neurons (α-BCP) decreases the synthesis of pro-ELH.

In addition to stimulating synthesis of ELH, cyclic AMP may also stimulate transport of secretory granules to the terminal regions of bag cell neurites. Forscher et al. (1988) used video-enhanced microscopy and digital image analysis techniques to show that forskolin induces a rapid increase in directed organelle transport into the terminal regions of growth cones from bag cell neurons grown in primary culture. The majority of the organelles appear to be the 200 nm dense core granules that contain ELH. This effect is potentiated by the addition of phosphodiesterase inhibitors and mimicked by cell-permeable cyclic AMP analogs, suggesting that it is mediated by cyclic AMP. If such an effect of cyclic AMP on granule movement also occurs in intact abdominal ganglia, this could increase the availability of ELH and other secretory products for exocytotic release.

Elevation of intracellular calcium and the activation of PKC may oppose the actions of cyclic AMP on pro-ELH synthesis (Bruehl and Berry, 1985; Berry, 1986). Biosynthesis of ELH is increased in zero calcium/EGTA medium and decreased by the addition of a calcium ionophore (Bruehl and Berry, 1985). The effect of the ionophore does not occur in calcium-free medium, suggesting that it is not a calcium-independent effect of the drug (Berry, 1986). Calmidazolium, which inhibits both PKC and calmodulin-dependent enzymes, increases ELH synthesis, providing tentative evidence that the effect of calcium could be mediated by Ca/CaM-PK or PKC (Bruehl and Berry, 1985). More direct evidence for a role of PKC came

from the finding that TPA (a PKC-activating phorbol ester), but not a phorbol ester that fails to activate PKC, mimicks the effect of the calcium ionophore. It is possible that Ca/CaM-PK is also involved in the regulation of pro-ELH synthesis, but this remains to be tested directly.

Termination of the Afterdischarge

Because of evidence that cyclic AMP plays a role in the generation of an afterdischarge, Kauer and Kaczmarek (1985) tested the hypothesis that the termination of the afterdischarge is owing to either a decrease in the synthesis of cyclic AMP, or a decrease in the responsiveness of bag cell neurons to cyclic AMP. Cyclic AMP analogs and phosphodiesterase inhibitors cause a prolongation of the duration of afterdischarges. Discharges can also be restarted if cyclic AMP levels are elevated pharmacologically within a few minutes of the normal termination of a discharge, suggesting that termination is unlikely to be associated with a major decrease in the sensitivity of bag cell neurons to cyclic AMP (Kaczmarek et al., 1978; Kauer and Kaczmarek, 1985).

In contrast, the ability of the cells to elevate cyclic AMP levels may be modified near the termination of a discharge. Kauer and Kaczmarek (1985) examined the ability of forskolin and theophylline to increase cyclic AMP levels in unstimulated bag cell clusters, compared with clusters that had been stimulated to afterdischarge. They found that the ability of forskolin/theophylline to increase cyclic AMP levels is greatly diminished at the time when an afterdischarge terminates. This presumably represents either a decrease in cyclic AMP synthesis, or an increase in cyclic AMP breakdown and could play an important role in termination of the afterdischarge.

Consistent with this hypothesis, Kauer et al. (1987) found that one of the bag cell peptides, α-BCP, can decrease cyclic AMP levels in stimulated bag cell clusters and decreases the ability of forskolin and theophylline to elevate cyclic AMP levels. Evidence indicates that this peptide acts directly at an autoreceptor on the bag cell neurons. The application of α-BCP to bag cell neurons during an afterdischarge causes premature termination of the discharge (Fig. 7). Moreover, this inhibitory effect can be antagonized by prior treatment with a cyclic AMP analog or by forskolin and theophylline.

Establishment of the Prolonged Inhibited State

As discussed above, after termination of an afterdischarge, the bag cell neurons enter a prolonged inhibited state during which further long-lasting afterdischarges cannot be initiated. Relatively little is known about the molecular mechanisms involved in the development of this inhibited state. One mechanism that appears to be involved in establishment of the inhibited state involves a decrease in the response of bag cell neurons to cyclic AMP (Kauer and Kaczmarek, 1985). In contrast to the effect of forskolin/theophylline immediately after termination of the afterdischarge, the addition of forskolin/theophylline, or stimulation of the pleuroabdominal connective after bag cell clusters have entered the inhibited state (1 h after termination of the afterdischarge), results in an increase in cyclic AMP levels similar to that seen in previously unstimulated bag cell clusters. However, the electrophysiological responses of bag cell neurons to agents that increase cyclic AMP are greatly attenuated 1 h after termination of an afterdischarge. In particular, cyclic AMP analogs or agents that elevate cyclic AMP either fail to trigger or prolong discharges once the cells have entered the inhibited state.

There is also evidence to suggest that the elevation of intracellular calcium ions during an afterdischarge may contribute to the onset of the inhibited period. Afterdischarges can be stimulated in a sodium-free medium provided potassium channel blockers, such as TEA, are

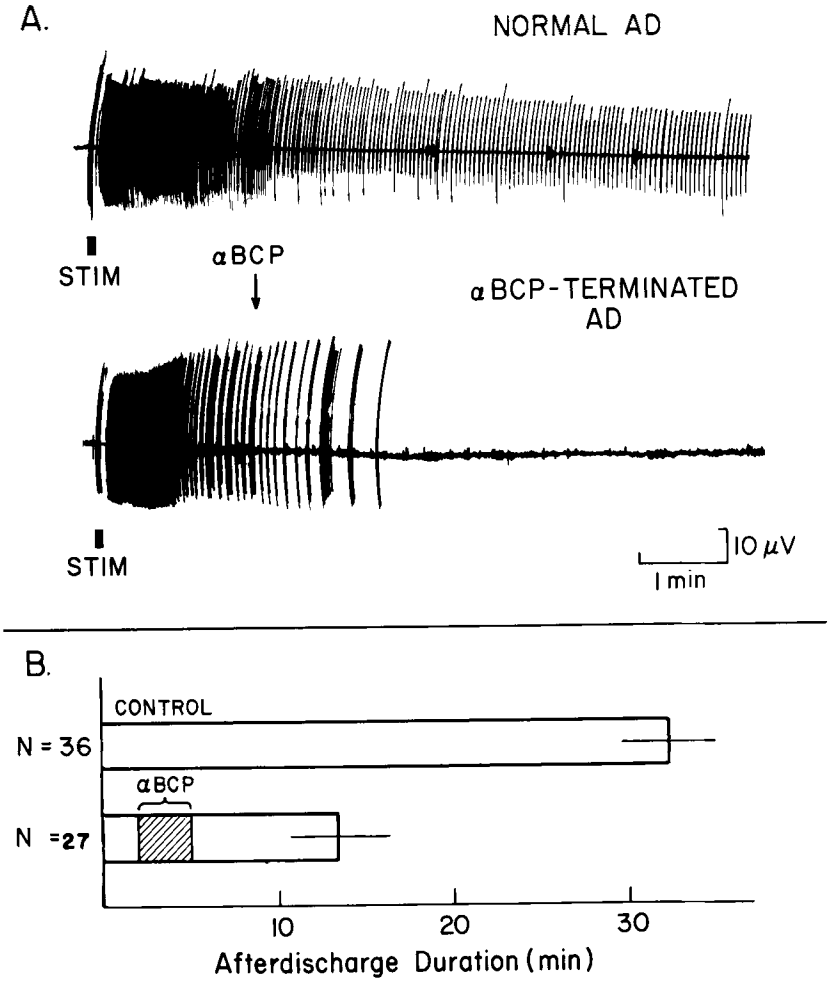

Fig. 7. (A) Inhibition of an ongoing discharge in bag cell neurons by α-BCP (1–7). (B) Mean durations of discharges in control ganglia and those exposed to α-BCP within 2–5 min of the onset of discharge (Kauer et al., 1987).

included. The omission of sodium abolishes the rapid-firing phase of an afterdischarge, but leaves the longer slow-firing phase intact. Such afterdischarges are followed by a normal inhibited state. In contrast, afterdischarges that are triggered in calcium-free media express only the rapid-firing phase of the afterdischarge. These discharges, however, can be stimulated repeatedly and do not give rise to a normal inhibited state (Kaczmarek et al., 1982), suggesting that calcium entry may be required for initiation of the inhibited state. The hypothesis that intracellular calcium is involved in the development of this prolonged refractory state was also tested using the cationophore, X537A. Incubation of intact bag cell clusters in X537A in a calcium-containing medium induces a state that resembles the natural inhibited state that follows an afterdischarge (Kaczmarek and Kauer, 1983). In common with the normal inhibited state, there is no change in the resting potential of the cells or their ability to generate action potentials, but stimulation of the afferent connective fails to generate an afterdischarge or generates only an afterdischarge of short duration. Furthermore,

there is a gradual recovery from exposure to X537A that has a very similar timecourse to that which follows electrical stimulation of an afterdischarge. Although further experiments with other ionophores and other techniques for elevating intracellular calcium are needed, these data suggest that calcium may play a role in the expression of the inhibited state. However, it is not known whether these effects are owing to a direct effect of calcium on an intracellular enzyme, such as Ca/CaM-PK or a calcium-dependent protease, or whether this effect of calcium is secondary to calcium-dependent release of neurotransmitters from the bag cell neurons or a presynaptic cell.

Inhibitory Control of Bag Cell Neurons

Because of the duration of the behaviors associated with egg laying, it is likely that in vivo bag cell afterdischarges are only triggered under appropriate environmental conditions. Thus, in addition to a mechanism for generating an afterdischarge and the associated egg laying behaviors, mechanisms must exist for inhibitory regulation of the bag cell neurons.

One neurotransmitter that can inhibit bag cell activity is serotonin. Serotonin (10 μmol) prevents atrial gland peptide-induced stimulation of egg laying behavior when injected into the body cavity of *Aplysia*, but does not inhibit the response to ELH. Furthermore, serotonin (0.1–1 μ*M*) has been found to inhibit bag cell afterdischarges that have been initiated either by electrical stimulation or application of atrial peptide B (Jennings et al., 1981). Nanomolar or micromolar concentrations of serotonin typically do not prevent initiation of an afterdischarge, but terminate an afterdischarge within 30 s of addition if added during the second, slow-firing phase. This suggests that serotonin selectively inhibits the second phase of the afterdischarge. This effect is mimicked by two serotonin agonists, tryptamine and bufotenine, and inhibited by a serotonin antagonist, butaclamol. The question of whether serotonin normally serves an inhibitory function on bag cell neurons is not known at present, nor is the mechanism by which serotonin inhibits bag cell afterdischarges. Somewhat paradoxically, serotonin stimulates accumulation of cyclic AMP in bag cell neurons (Kaczmarek et al., 1978; Bernier et al., 1982). Based on the data discussed above, this would be predicted to have an excitatory rather than inhibitory effect on these cells.

The source, if any, of serotonergic input to the bag cell neurons has not been determined. A histofluorescence study has suggested that the bag cell neurons themselves may contain serotonin (Tritt et al., 1983). In contrast, studies with antibodies to serotonin suggest that cells with serotonin immunoreactivity have processes that are extensively branched in the sheath surrounding the bag cell cluster, but that the bag cell neurons themselves are devoid of serotonin immunoreactivity (Hopkins et al., 1982).

Autoreceptor-Mediated Effects of Bag Cell Peptides on the Bag Cell Neurons

Evidence suggests that the bag cell peptides themselves may play an important role in modulating the excitability of the bag cell neurons (Rothman et al., 1983; Brown and Mayeri, 1986, 1989; Rock et al., 1986; Sigvardt et al., 1986; Kauer et al., 1987; Loechner and Kaczmarek, 1987; Loechner et al., 1988). Application of α-, β-, or γ-BCP to bag cell neurons in the intact nervous system is able to produce a depolarization (Brown and Mayeri, 1986, 1989). During a discharge such a depolarization produced by newly-released peptides may contribute to maintaining the bag cell neurons in a depolarized state. The actions of these peptides, however, are multifaceted. For instance, Kauer et al. (1987) found that α-BCP acts directly on the bag cell neurons to decrease basal and afterdischarge- or forskolin-induced increases in cyclic

AMP levels. Furthermore, addition of α-BCP to abdominal ganglia shortly after stimulation of an afterdischarge, results in premature termination of the ongoing afterdischarge. This effect could be prevented or reversed by pharmacological elevation of cyclic AMP, suggesting that α-BCP has an autoinhibitory effect on bag cell neurons that may, at least in part, be mediated by a decrease in cyclic AMP levels. Consistent with this, α-BCP increases delayed potassium currents (Loechner and Kaczmarek, 1987). Furthermore, α-BCP antagonizes the effects of the adenylate cyclase activator, forskolin, on voltage-dependent potassium currents in isolated bag cell neurons (Kauer et al., 1987). During an afterdischarge, there is a progressive increase in the rate of release of peptides from bag cell neurons (Loechner et al., 1988). It is possible that the bag cell neurons are self-regulating and employ α-BCP to terminate the afterdischarge. Owing to the progressive increase in the release of peptides during the afterdischarge, concentrations of α-BCP that are sufficient for inhibiting the discharge may not be achieved at early time points during the afterdischarge. This hypothesis is consistent with the finding that forskolin-induced increases in cyclic AMP levels are diminished at the end of an afterdischarge (Kauer and Kaczmarek, 1985).

The role of α-BCP in regulating the excitability of bag cell neurons is more complicated than a simple autoinhibitory role mediated by decreasing cyclic AMP levels. For instance, α-BCP also enhances an inwardly rectifying potassium current in cultured bag cell neurons (Kauer et al., 1987). This effect is independent of the decrease in cyclic AMP levels, and the role of this effect in the regulation of repetitive firing of bag cell neurons is unclear. As noted above, α-BCP can also depolarize the bag cell neurons, and occasionally, this depolarization initiates an afterdischarge in intact abdominal ganglia (Rothman et al., 1983; Rock et al., 1986). Thus, it is possible that α-BCP has multiple functions in modulating bag cell excitability.

The effects of other bag cell peptides on bag cell neurons have not been characterized as intensively as those of α-BCP. However, it is becoming clear that the other bag cell peptides may also have autoregulatory effects on the bag cell neurons. Both β-BCP and γ-BCP can also depolarize bag cell neurons (Brown and Mayeri, 1988, 1989), suggesting that depolarization occurs by a mechanism that is likely to be unrelated to changes in cyclic AMP levels. In common with α-BCP, γ-BCP decreases cyclic AMP levels in intact bag cell clusters and increases delayed potassium currents in isolated bag cell neurons (Loechner and Kaczmarek, 1987). β-BCP increases cyclic-AMP levels, decreases delayed potassium currents, and enhances action potentials in these cells (Loechner and Kaczmarek, 1987), suggesting that this peptide may play a purely autoexcitatory role. Finally, it is likely that changes in cyclic AMP levels, induced by bag cell peptides, also regulate the synthesis and transport of neuropeptides during the afterdischarge. For instance, as mentioned above, exogenously applied α-BCP has been shown to reduce pro-ELH synthesis in bag cell neurons (Berry, 1988).

Bag Cell Afterdischarge Controls Egg Laying Behavior by Modulating the Physiology of a Number of Target Cells

Egg laying is a complex behavior involving changes in the physiology of nonneuronal cells in the reproductive system, as well as alterations in the excitability of many neuronal circuits, including those controlling feeding, locomotion, and head movements. Although the actions of ELH and some of the effects of other bag cell peptides on specific neuronal and nonneuronal targets have been investigated, a com-

plete picture of how egg laying behaviors are orchestrated is not yet available. The following is a brief account of work in this developing area.

ELH Acts Directly on the Oveotestis to Evoke Egg Release

Soon after the discovery that an extract of bag cell neurons is capable of stimulating egg laying behavior (Kupfermann, 1967), Coggeshall (1970) proposed that a hormone secreted from these cells stimulates egg release by acting directly on the muscle cells in the ovotestis. Stimulation of contraction of the small muscle cells that surround the follicles was hypothesized to induce egg expulsion from the follicles. Direct support for this hypothesis came with the finding that an extract taken from bag cell clusters, but not other areas of the *Aplysia* nervous system, stimulates egg release from isolated ovotestis fragments in vitro (Dudek and Tobe, 1978). The egg-releasing activity was found to be secreted from bag cell clusters upon depolarization (Dudek et al., 1980) and subsequently was purified and identified as ELH (Rothman et al., 1983b). Whether this effect of ELH is mediated by stimulation of the muscle cells surrounding the follicles is not yet known, but these data are consistent with the hypothesis that ELH has a hormonal action on the ovotestis to stimulate egg extrusion.

Inhibition of Feeding

There are two general mechanisms by which bag cell afterdischarge could trigger behaviors associated with egg laying. The primary action of ELH could be on the ovotestis to induce egg release. The external appearance of eggs, which occurs after a bag cell afterdischarge, could then serve as a stimulus for induction of the associated behaviors. Alternatively, ELH or other bag cell products could act directly on the nervous system to alter behavior. Evidence suggests that appearance of the eggs may serve as a stimulus for at least some behaviors associated with egg laying, such as head weaving (Ferguson et al., 1986). Moreover, there is evidence that the movement of eggs through the reproductive tract may play a role in the maintenance of some egg laying associated behaviors, such as rhythmic head movements and inhibition of feeding (Cobbs and Pinsker, 1982b). It has been shown, however, that cessation of feeding, following injection of a bag cell extract, is not dependent on egg extrusion (Stuart and Strumwasser, 1980), suggesting that this effect is mediated by a direct action of the bag cell products on the neuronal circuitry involved in feeding.

The buccal musculature in *Aplysia* is innervated by nerve B4 (for nomenclature, *see* Kandel, 1979). Axons of motor neurons course through this nerve from the buccal ganglion to control the musculature involved in feeding. The addition of partially-purified ELH to the head ganglia in vitro induces repetitive firing in a bilaterally-situated pair of neurons that are located on the surface of the right and left buccal ganglia between the identified buccal neurons, B3 and B4, that send their axons into the B4 nerves (Stuart and Strumwasser, 1980). These cells have subsequently been identified as the right and left cholinergic neurons, B16 (Ram, 1983). B16 cells make excitatory connections with the radula retractor muscle, I5 (also known as the accessory radula closer muscle) (Cohen et al., 1978; Weiss et al., 1978), and stimulation of B16 results in contraction of this muscle (Ram, 1982). Contraction of I5 closes and retracts the radula (Cohen et al., 1978), the organ used to grasp food. Thus, ELH-induced contraction of muscle I5 is consistent with inhibition of feeding. Furthermore, after addition of ELH, there is a 5–7 min delay before the firing starts, and the peak rate is after 10–25 min. This time course matches the cessation of feeding that occurs after the injection of ELH into intact animals, suggesting that the two could be related.

Kirk and Scheller (1986) have investigated the ionic currents in neuron B16 that are modulated by ELH and found that ELH has no effect on any of the known potassium currents in this cell, but increases a slow inward current that is carried by sodium. The sodium current is particularly enhanced in the region from –40 to –25 mV, where a negative slope resistance is observed in the current–voltage relationship. Based on the long duration and long latency of the response, Kirk and Scheller (1986) suggested that the ELH-induced inward current may be mediated by generation of a second messenger. Ram et al. (1986) found that the effect of ELH is mimicked by 8-bromo-cyclic AMP, phosphodiesterase inhibitors, and forskolin and is inhibited by tolbutamide, a putative inhibitor of cAMP-PK. Based on this, Ram et al. (1986) suggested that the effect of ELH on these neurons is mediated by formation of cyclic AMP and subsequent activation of cAMP-PK. This is consistent with the finding that ELH increases cyclic AMP levels in other *Aplysia* neurons (Levitan et al., 1987).

Effects similar to those of ELH can be produced on application of serotonin to neuron B16 (Ram et al., 1986; Sossin et al., 1987). Furthermore, the effect of serotonin is apparently also mediated by activation of adenylate cyclase (Ram et al., 1986). These results are surprising in that serotonin is believed to mediate arousal of feeding in *Aplysia*. Ram et al. (1986) suggested that the behavioral responses to ELH and serotonin may depend on effects on different populations of neurons, both of which include B16. Consistent with this notion, Sossin et al. (1987) found that serotonin, in addition to stimulating B16, also excites neuron B15, premotor neurons that synapse onto B15 and B16, neurons that send excitatory input to these premotor neurons, and muscle I5.

In addition to its effects on neuron B16, ELH also increases the rate of firing of another pair of neurons in the buccal ganglia, each of which sends an axon into a cerebrobuccal connective nerve (Stuart and Strumwasser, 1980). Furthermore, ELH influences synaptic input onto B15, B16, and premotor neurons in the buccal ganglion (Sossin et al., 1987). The exact ways in which these various effects of ELH upon the circuitry of the buccal ganglion modulate feeding behavior are not known at present.

Inhibition of Locomotion

Prior to egg laying, *Aplysia* stop locomotion. *Aplysia* locomotion is a centrally-programmed, fixed action pattern that can be modulated under a variety of different conditions (Mackey and Carew, 1983). Evidence suggests that serotonin plays a primary role in stimulating locomotion, and it has been found that injection of a bag cell extract (but not extracts of other areas of the *Aplysia* nervous system) reversibly suppresses 5HT-triggered locomotion. This effect is likely to be owing to effects of the extract on cells in the pleural-pedal ganglia (Mackey and Carew, 1983). Inhibition of 5HT-induced locomotion is abolished by incubation of the extract with pronase, suggesting that the activity is likely to be one of the peptides released from the bag cell neurons. To date, the only secretory product of bag cell neurons examined, with relation to the circuitry involved in locomotion, is ELH. When ELH is added to head ganglia in vitro, it induces large increases in the activity of units in the pedal nerves (Stuart and Strumwasser, 1980), which travel from the pedal ganglia to innervate the foot. The relevant pedal neurons remain to be identified, but it is possible that increased activity of these cells contributes to the regulation of locomotion.

Modulation of the Respiratory Motor Program

Another behavioral change induced by initiation of an afterdischarge is facilitation of respiratory pumping of the gill and siphon (Schaefer and Brownell, 1986). Respiratory pumping is a spontaneous rhythmic behavior that is timed and coordinated by the interactions of a group

of interneurons and two groups of motor neurons that innervate the gill and siphon (Kupfermann et al., 1974; Perlman, 1979; Byrne, 1983). Stimulation of a bag cell discharge causes relaxation of the siphon, increased frequency of respiratory pumping, and increased amplitude of gill and siphon contractions during respiratory pumping. Immediately after initiation of an afterdischarge, the gill motor neuron (LD_{G1}) is transiently inhibited (1–2 min) and then weakly excited for a period of 10–30 min, whereas the siphon motor neuron (LD_{S1}) undergoes a prolonged period (30–60 min) of excitation. The amount of increase in activity of these two neurons varies from animal to animal but correlates well with an increase in the amplitude of gill and siphon contractions, respectively (Schaefer and Brownell, 1986). The effects of individual of bag cell peptides on these neurons have not been characterized.

Redistribution of Circulation to Motor Areas Involved in Egg Laying Behavior

The dramatic change in metabolic activity of various organ systems during egg laying behavior causes a decrease in the circulatory demand of certain tissues, while increasing others. To accommodate this, the bag cell afterdischarge exerts actions on the circulatory system of *Aplysia* (Ligman and Brownell, 1985). In a semi-intact preparation of visceral organ systems innervated by the abdominal ganglion, stimulation of a bag cell afterdischarge induces vasoconstriction of two of the three major arteries conducting blood from the heart of *Aplysia* (Ligman and Brownell, 1985). Vasoconstriction occurs in the anterior and gastroesophageal arteries. This is likely to result in decreased blood flow through these arteries, while increasing blood flow through the abdominal artery. Rerouting blood flow in this manner may increase blood supply to tissues that are highly metabolically active during egg laying, such as the ovotestis and oviduct, while decreasing blood flow to relatively inactive tissues, such as those involved in digestion and locomotion. Evidence suggests that this effect is owing to a direct hormonal action of secretory products of the bag cell neurons on the *Aplysia* vasculature.

Local Hormonal Action of ELH on Neuron R15 and Other Abdominal Ganglion Neurons

In addition to the effects described above, ELH has a variety of effects on neurons within the abdominal ganglion (Fig. 8). One of the most thoroughly characterized actions of a bag cell peptide on an abdominal ganglion neuron is the effect of ELH on identified neuron R15. R15 is a neurosecretory cell that is thought to be involved in the regulation of water balance in *Aplysia* (Kupfermann and Weiss, 1976). Normally, R15 is spontaneously active and fires with an endogenously-generated rhythmic bursting pattern in which bursts of about 8 to 12 action potentials are separated by brief periods of hyperpolarization. Stimulation of a bag cell afterdischarge in an excised abdominal ganglion, or application of purified ELH, augments the bursting activity of neuron R15 by enhancing both the hyperpolarizing and depolarizing phases of the burst cycle (Branton et al., 1978a,b; Mayeri et al., 1979b, 1985). ELH application results in an increase in the number and frequency of spikes during R15 bursts and an increase in the amplitude of the interburst hyperpolarization. This is likely to increase neurosecretion by R15 and, thereby, alter water balance. It appears that ELH acts directly on R15 and not via an intermediate neuron (Mayeri et al., 1985; Levitan et al., 1987;). The effect of ELH on R15 excitability is long-lasting and persists long after ELH has been removed. Thus, the ELH released during a 30 min afterdischarge could influence water balance for the entire period during which the egg laying associated behaviors occur.

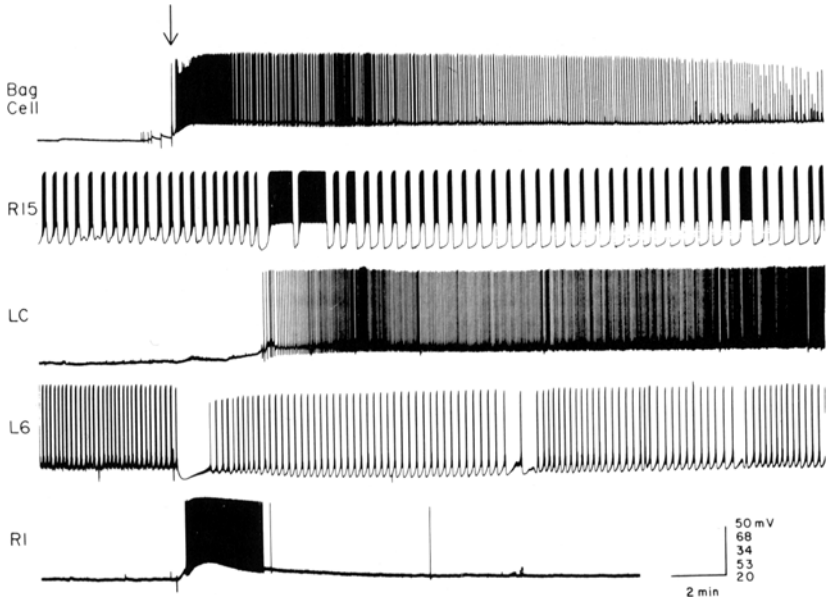

Fig. 8. Composite figure showing the changes that occur in the electrical activity of different identified abdominal ganglion neurons following stimulation of a discharge in the bag cell neurons (top trace) (Mayeri and Rothman, 1981).

The persistent nature of ELH-mediated effects on neuron R15 suggests that ELH exerts its effects by activation of an intracellular second messenger system that then alters the properties of specific ionic conductances. Consistent with this, ELH has been found to increase cyclic AMP levels in neuron R15 (Levitan et al., 1987). Furthermore, cyclic AMP analogs, phosphodiesterase inhibitors, the adenylate cyclase activator forskolin, and other neurotransmitters that activate adenylate cyclase in R15 mimick the effect of ELH on this neuron (Levitan et al., 1987; Treistman and Levitan, 1976). Levitan et al. (1987) used voltage clamp techniques to determine which ionic conductances are altered by ELH to give rise to the augmentation of bursting activity. Activation of an afterdischarge, or exogenous application of synthetic ELH on R15 in an intact abdominal ganglion, results in an increase in an inwardly rectifying potassium current and a voltage-gated calcium current.

Stimulation of an afterdischarge (Mayeri et al., 1979a) or application of ELH to the abdominal ganglion (Mayeri et al., 1985) also alters the excitability of other, less well-characterized, neurons. In particular, cells in two identified clusters of neurons (LB and LC) in the left lower quadrant (LLQ) of the abdominal ganglion respond to activation of an afterdischarge or application of ELH with a prolonged excitation that greatly outlasts the afterdischarge or the period of exposure to exogenously applied ELH. In addition, a number of unidentified LLQ cells undergo prolonged excitation in response to stimulation of an afterdischarge (Mayeri et al., 1979a). These excitatory responses occur within 2–4 min of exposure to exogenous or endogenous ELH and may persist for as long as 2 h.

Voltage clamp experiments suggest that the excitation of LC and LB cells is mediated by an ELH-induced increase in at least three separate currents (Jansen and Mayeri, 1988). The currents that are evoked by ELH include a slow

voltage-dependent inward current that is calcium sensitive and sodium insensitive, an inward rectifier potassium current, and a slow voltage-independent inward current that is likely to be carried by sodium. The precise roles, if any, of these cells in contributing to egg laying behavior are unknown. However, the LC and LB clusters contain a variety of motorneurons (Koester and Kandel, 1977) that could potentially be involved in the regulation of the various behaviors associated with egg laying.

It is likely that responses to ELH are mediated by processes in the neuropil. Unlike conventional synaptic responses, the responses to ELH have a slow onset, are smoothly graded, and have a prolonged response duration. In addition, serial perfusion of an abdominal ganglion with the medium from a second abdominal ganglion, in which the bag cell clusters are discharging, results in modulation of abdominal ganglion neurons in a manner identical to that seen during an afterdischarge (Mayeri et al., 1985). This suggests that ELH is secreted in sufficient quantities to reach target cells at concentrations that would have a biological effect by diffusing through the extracellular space.

Effects of Other Bag Cell Peptides on Neurons in the Abdominal Ganglion

In addition to the prolonged excitatory effects on R15 and LC and LB cells, bag cell afterdischarges induce at least two other types of responses in abdominal ganglion neurons. Perhaps the most prominent effect of an afterdischarge on abdominal ganglion cells is a prolonged inhibition that occurs in at least 21 identified neurons and other unidentified neurons (Brownell and Mayeri, 1978; Mayeri et al., 1979a,b, 1985). The identified cells include the ink-gland motoneurons (L14A, B, and C), left upper quadrant (LUQ) neurons L2–L6, the giant cell R2, the right upper quadrant white cells R3–R13, and white cell R14. This inhibition is seen as a slow hyperpolarization that occurs within seconds of stimulation of an afterdischarge and may last as long as 3 h after initiation of the afterdischarge. Another response to bag cell afterdischarges is a transient excitation that occurs in two symmetrically-located, silent cells, R1 and L1. Stimulation of bag cell activity results in vigorous repetitive spike activity in these cells that persists for 3–7 min after initiation of the afterdischarge (Mayeri et al., 1979a).

In common with augmentation of R15 bursting and prolonged excitation of LB and LC cells, evidence suggests that the above effects are owing to a local hormonal action rather than synaptic input. However, unlike the effects on R15 and LB and LC neurons, the effects of an afterdischarge on L2–L4, L6, L1, and R1 are not mimicked by ELH (Mayeri et al., 1985), suggesting that these effects may be mediated by one of the other peptides that are secreted by the bag cell neurons. The effect of ELH on the other neurons that respond to an afterdischarge with slow inhibition were not determined. However, β-BCP was found to mimick bag cell-induced excitation of neuron L1 (Mayeri et al., 1985), and α-BCP were found to mimick the bag cell-induced inhibition of LUQ neurons (Rothman et al., 1983; Rock et al., 1986; Sigvardt et al., 1986) and neuron R2 (Rock et al., 1986). The acidic peptide AP has not yet been found to have any effect on target neurons. It is unclear at this point what role α-BCP, β-BCP, and γ-BCP play in mediating egg laying behavior, but these data do suggest that the bag cell neurons serve as a multitransmitter system in which different neuropeptides are released to act on different targets to bring about the final neuronal response.

Summary

In conclusion, egg laying behavior in *Aplysia* is a tightly regulated, all or none event that is under the control of the bag cell neurons in the abdominal ganglion. The properties of the bag

cell neurons are particularly suited for regulating such an important behavior. They synthesize and secrete a number of neuropeptides, each of which has specific effects on target cells that are involved in egg laying behavior. The electrical properties of the bag cell neurons allow them to act as a "switch" in that no neuropeptides are released under normal conditions, during which these neurons are silent, but maximal efficiency of neuropeptide release is achieved in response to an appropriate stimulus. This maximized efficiency of neurotransmitter release is brought about by the combined actions of various second messengers. These second messengers may alter ionic conductances to induce repetitive firing of spikes with high amplitude and width, increase neuropeptide synthesis to replenish depleted stores of neuropeptides, and enhance vesicle transport in a manner that could supply the terminals with neurosecretory products.

Once an afterdischarge is initiated, the morphological properties of the bag cell neurons allow them to secrete their neuropeptide products into the neuropil to act on neurons in the abdominal ganglion, and into the vascular system so that they can reach distal targets through the general circulation in a hormonal fashion. The neuropeptides can then act on peripheral target organs, such as the ovotestis and the vascular system, as well as neuronal targets that are involved in altering animal behavior, to bring about the egg laying response. In addition, some of the neuropeptides also serve an autoregulatory function and act directly on the bag cell neurons themselves to alter the duration and properties of the after-discharge. The unique properties of the bag cell neurons have made them an attractive model system for studying the cellular and molecular mechanisms involved in the control of a long-lasting sequence of behaviors. This system has already provided valuable insights into a variety of aspects of the molecular and cellular biology of peptidergic neuronal systems, some of which are likely to be applied to the more complex mammalian neuropeptidergic systems.

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References

Abdel-Latif A. A. (1986) Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacological Reviews* **38,** 227–270.

Acosta-Urquidi J. and Dudek F. E. (1981) Soma spike of neuroendocrine bag cells of *Aplysia californica*. *J. Neurobiol.* **12,** 367–378.

Arch S. (1972a) Polypeptide secretion from the isolated parietovisceral ganglion of *Aplysia californica*. *J. Gen. Physiol.* **59,** 47-59.

Arch S. (1972b) Biosynthesis of the egg-laying hormone (ELH) in the bag cell neurons of *Aplysia californica*. *J. Gen. Physiol.* **60,** 102–119.

Arch S. (1976) Neuroendocrine regulation of egg laying in *Aplysia californica*. *Am. Zool.* **16,** 167–175.

Arch S., Earley P., and Smock T. (1976a) Biochemical isolation and physiological identification of the egg-laying hormone in *Aplysia californica*. *J. Gen. Physiol.* **68,** 197–210.

Arch S., Linstedt A., Whitney G., Teal P., and Smock T. (1986) Neuropeptide routing in the bag cells: kinetic differences in the appearance of newly labeled peptides in transport and secretion. *J. Neurosci.* **6,** 1545–1552

Arch S., Lupatkin J., Smock T., and Beard M. (1980) Evidence for an exocrine function of the *Aplysia* atrial gland. *J. Comp. Physiol.* **141,** 131–137.

Arch S. and Smock T. (1977) Egg-laying in *Aplysia californica*. *Behav. Biol.* **19,** 45–54.

Arch S., Smock T., and Earley P. (1976b) Precursor and product processing in the bag cell neurons of *Aplysia californica*. *J. Gen. Physiol.* **68,** 211–225.

Arch S., Smock T., Gurvis R., and McCarthy C. (1978) Atrial gland induction of the egg-laying response in *Aplysia californica*. *J. Comp. Physiol.* **128,** 67–70.

Audesirk T. E. (1977) Chemoreception in *Aplysia californica*. III. Evidence for pheromones influencing reproductive behavior. *Behav. Biol.* **20,** 235–245.

Audesirk T. E. (1979) A field study of growth and reproduction in *Aplysia californica*. *The Biological Bulletin* **157,** 407–421.

Beard M., Millechia L., Masuoka C., and Arch S. (1982) Ultrastructure of secretion in the atrial gland of a mollusc (*Aplysia*). *Tissue and Cell* **14,** 297–308.

Benson J. A. and Adams W. B. (1987) The control of rhythmic neuronal firing, *Neuromodulation*, Kaczmarek L. K. and Levitan I. B., eds., Oxford University Press, New York, pp. 100–118.

Bernier L., Castellucci V. F., Kandel E. R., and Schwartz J. H. (1982) Facilitatory transmitter causes a selective and prolonged increased in adenosine 3':5'-monophosphate in sensory neurons mediating the gill and siphon withdrawal reflex in *Aplysia*. *J. Neurosci.* **2,** 1682–1691.

Berry R. W. (1981) Proteolytic processing of the neurosecretory egg-laying hormone in *Aplysia*. 1. Precursors, Intermediates, and products. *Biochemistry* **20,** 6200–6205.

Berry R. W. (1982) Seasonal modulation of synthesis of the neurosecretory egg-laying hormone of *Aplysia*. *J. Neurobiol.* **13,** 327–335.

Berry R. W. (1986) Calcium and protein kinase C inhibit biosynthesis of *Aplysia* egg-laying hormone. *Mol. Brain Res.* **1,** 185–187.

Berry R. W. (1988) α bag cell peptide reduces stimulated cAMP levels and proELH synthesis in bag cells. *Mol. Brain Res.* **4,** 267–271.

Berry R. W. and Arch S. (1981) Activation of neurosecretory cells enhances their synthesis of secretory protein. *Brain Res.* **215,** 115–123.

Berry R. W. and Schwartz A. W. (1977) Axonal transport and axonal processing of low molecular weight proteins from the abdominal ganglion of *Aplysia*. *Brain Res.* **129,** 75–90.

Berry R. W. and Yates M. E. (1986) Neuropeptide processing activity in *Aplysia* bag cell homogenates. *Peptides* **7,** 637–643.

Blankenship J. E. and Haskins T. (1979) Electrotonic coupling amoung neuroendocrine cells in *Aplysia*. *J. Neurophysiol.* **42,** 347–355.

Blankenship J. E., Rock M. K., Robbins L. C., Livingston C. A., and Lehman H. K. (1983) Aspects of copulatory behavior and peptide control of egg laying in *Aplysia*. *Fed. Proc.* **42,** 96–100.

Bley K. R. and Kaczmarek L. K. (1986) Modulation of *Aplysia* bag cell neuron excitability by synthetic diacylglycerols. *Soc. Neurosci. Ab.* **12,** 1194.

Branton W. D., Arch S., Smock T., and Mayeri E. (1978a) Evidence for mediation of a neuronal interaction by a behaviorally active peptide. *Proc. Natl. Acad. Sci. USA* **75,** 5732–5736.

Branton W. D., Mayeri E., Brownell P., and Simon S. B. (1978b) Evidence for local hormonal communication between neurones in *Aplysia*. *Nature* **274,** 70–72.

Brown R. O. and Mayeri E. (1986) Evidence for excitatory autotransmission in *Aplysia* bag cell neurons mediated by alpha-, beta-, and gamma-bag cell peptides. *Soc. Neurosci. Ab.* **12,** 946.

Brown R. O. and Mayeri E. (1987) Activation of the bag cells by ELH. BCP-immunoreactive neurons in the right pleural ganglion of *Aplysia californica*. *Soc. Neurosci. Ab.* **13,** 39.

Brown R. O. and Mayeri E. (1989) Positive feedback by autoexcitatory neuropeptides in neuroendocrine bag cells of *Aplysia*. *J. Neurosci.* **9,** 1443–1451.

Brownell P. and Mayeri E. (1979) Prolonged inhibition of neurons by neuroendocrine cells in *Aplysia*. *Science* **204,** 417–420.

Brownstein M. J., Russel J. T., and Gainer H. (1980) Synthesis, transport, and release of posterior pituitary hormones. *Science* **207,** 373–378.

Bruehl C. L. and Berry R. W. (1985) Regulation of synthesis of the neurosecretory egg-laying hormone of *Aplysia*: antagonistic roles of calcium and cyclic adenosine 3':5'- monophosphate. *J. Neurosci.* **5,** 1233–1238.

Buma P., Roubos E. W., and Brunekreef K. (1986) Role of cAMP in electrical and secretory activity of the neuroendocrine caudo-dorsal cells of *Lymnaea stagnalis*. *Brain Res.* **380,** 26–33.

Byrne J. H. (1983) Identification and initial characterization of a cluster of command and pattern-generating neurons underlying respiratory pumping in *Aplysia californica*. *J. Neurophysiol.* **37,** 996–1019.

Carefoot T. H. (1967) Growth and nutrition of *Aplysia punctata* feeding on a variety of marine algae. *J. Mar. Biol. Assoc. UK* **47,** 565–589.

Castellucci V. F., Frost W. N., Goelet P., Montarolo P. G., Schacher S., Morgan J. A., Blumenfeld H., and Kandel E. R. (1986) Cell and molecular analy-

sis of long-term sensitization in *Aplysia*. *J. Physiol.* (Paris) **81**, 349–357.

Chiu A. Y., Hunkapiller M., Heller E., Stuart D. K., Hood L. E., and Strumwasser F. (1979) Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* **76**, 6656–6660.

Cobbs J. S. and Pinsker H. M. (1982a) Role of bag cells in egg deposition of *Aplysia brasiliana*. I. Comparison of normal and elicited behaviors. *J. Comp. Physiol.* **147**, 523–535.

Cobbs J. S. and Pinsker H. M. (1982b) Role of bag cells in egg deposition of *Aplysia brasiliana*. II. Contribution of egg movement to elicited behaviors. *J. Comp. Physiol.* **147**, 537–546.

Coggeshall R. E. (1967) A light and electron microscope study of the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**, 1263–1287.

Coggeshall R. E. (1970) A cytologic analysis of the bag cell control of egg laying in *Aplysia*. *J. Morphol.* **132**, 461–486.

Cohen S. L., Weiss K. R., and Kupfermann I. (1978) Motor control of buccal muscles in *Aplysia*. *J. Neurophysiol.* **41**, 157–179.

Conn P. J., Strong J. S., Azhderian E. M., Nairn A. C., Greengard P., and Kaczmarek L. K. (1988a) Protein kinase inhibitors selectively block phorbol ester- or forskolin-induced changes in excitability of *Aplysia* neurons. *J. Neurosci.* **9**, 473–479.

Conn P. J., Strong J. A., and Kaczmarek L. K. (1988b) Inhibitors of protein kinase C prevent enhancement of calcium current and action potentials in peptidergic neurons of *Aplysia*. *J. Neurosci.* **9**, 480–487.

Coombs J. and Thompson S. (1987) Forskolin's effect on transient K current in nudibranch neurons is not reproduced by cAMP. *J. Neurosci.* **7**, 443–452.

DeReimer S. A., Greengard P., and Kaczmarek L. K. (1985a) Calcium/phosphatidylserine/diacylglycerol-dependent protein phosphorylation in the *Aplysia* nervous system. *J. Neurosci.* **5**, 2672–2676.

DeReimer S. A., Kaczmarek L. K., Lai Y., McGuinness T. L., and Greengard P. (1984) Calcium/calmodulin-dependent protein phosphorylation in the nervous system of *Aplysia*. *J. Neurosci.* **4**, 1618–1625.

DeReimer S. A., Strong J. A., Albert K. A., Greengard P., and Kaczmarek L. K. (1985b) Enhancement of calcium current in *Aplysia* neurons by phorbol ester and protein kinase C. *Nature* **313**, 313–316.

Dudek F. E. and Blankenship J. E. (1976) Neuroendocrine (bag) cells of *Aplysia:* Spike blockade and a mechanism for potentiation. *Science* **192**, 1009, 1010.

Dudek F. E. and Blankenship J. E. (1977a) Neuroendocrine cells of *Aplysia brasillana*. I. Bag cell action potentials and afterdischarge. *J. Neurophysiol.* **40**, 1301–1311.

Dudek F. E. and Blankenship J. E. (1977b) Neuroendocrine cells of *Aplysia brasiliana*.. II. Bag cell prepotentials and potentiation. *J. Neurophysiol.* **40**, 1312–1324.

Dudek F. E., Cobbs J. S., and Pinsker H. M. (1979) Bag cell electrical activity underlying spontaneous egg laying in freely behaving *Aplysia brasiliana*. *J. Neurophysiol.* **42**, 804–817.

Dudek F. E. and Kossatz A. (1982) Conduction velocity and spike duration during afterdischarge in neuroendocrine bag cells of *Aplysia*. *J. Neurobiol.* **13**, 319–326.

Dudek F. E. and Tobe S. S. (1978) Bag cell peptide acts directly on ovotestis of *Aplysia californica:* Basis for an *in vitro* bioassay. *Gen. Comp. Endocrinol.* **36**, 618–627.

Dudek F. E., Weir G., Acosta-Urquidi J., and Tobe S. S. (1980) A secretion from neuroendocrine bag cells evokes release *in vitro* from ovotestis of *Aplysia californica*. *Gen. Comp. Endocrinol.* **40**, 241–244.

Eales N. B. (1921) *Aplysia*. Liverpool Marine Biology Committee, Memoir #24, Proc. and Trans., *Liverpool Biol. Soc.* **35**, 183–266.

Ferguson G. P., Parsons D. W., Maat A., and Pinsker H. M. (1986) Spontaneous and elicited bag cell discharges in gonadectomized *Aplysia*. *J. Exp. Biol.* **123**, 159–173.

Fink L. A., Connor J. A., and Kaczmarek L. K. (1988) Inositol Trisphosphate releases intracellularly stored calcium and modulates ion channels in moluscan neurons. *J. Neurosci.* **8**, 2544–2555.

Fisher J. M., Sossin W., Newcomb R., and Scheller R. H. (1988) Multiple neuropeptides derived from a common precursor are differentially packaged and transported. *Cell* **54**, 813–822.

Forscher P., Kaczmarek L. K., Buchanan J. A., and Smith S. J. (1987) Cyclic AMP induces changes in distribution and transport of organelles within

growth cones of *Aplysia* bag cell neurons. *J. Neurosci.* **7,** 3600–3611.

Frazier W. T., Kandel E. R., Kupfermann I., Waziri R., and Coggeshall E. (1967) Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica. J. Neurophysiol.* **30,** 1288–1351.

Gainer H., Loh Y. P., and Neale E. A. (1982) The organization of post-translational precursor processing in peptidergic neurosecretory cells, *Proteins in the Nervous System: Structure and Function,* Haber B., Perez-Polo J. R., and Coulter J. D., eds., Liss, New York, pp. 131–145.

Geraerts W. P. M. and Hogenes Th. M. (1985) Heterogeneity of peptides released by electrically active neuroendocrine caudodorsal cells of *Lymnaea Stagnalis. Brain Res.* **331,** 51–61.

Gev S., Achituv Y., and Susswein A. J. (1984) Seasonal determinants of the life cycle in two species of *Aplysia* found in shallow waters along the Mediterranean coast of Israel. *J. Exp. Mar. Biol. Ecol.* **74,** 67–83.

Gumbiner B. and Kelly R. B. (1981) Secretory granules of an anterior pituitary cell line, AtT-20, contain only mature forms of corticotropin and β-lipotropin. *Proc. Natl. Acad. Sci. USA* **78,** 318–322.

Haskins J. T. and Blankenship J. E. (1979) Interactions between bilateral clusters of neuroendocrine cells in *Aplysia. J. Neurophysiol.* **42,** 356–367.

Haskins J. T., Price C. H., and Blankenship J. E. (1981) A light and electron microscopic investigation of the neurosecretory bag cells of *Aplysia. J. Neurocytology* **10,** 729–747.

Heller E., Kaczmarek L. K., Hunkapiller M. W., Hood L. E., and Strumwasser F. (1980) Purification and primary structure of two neuroactive peptides that cause bag cell afterdischarge and egg-laying in *Aplysia. Proc. Natl. Acad. Sci. USA* **77,** 2328–2332.

Hoshi T., Garber S. S, and Aldrich R. W. (1988) Effect of forskolin on voltage-gated K+ channels is independent of adenylate cyclase activation. *Science* **240,** 1652–1655.

Jamieson J. D. and Palade G. E. (1977) Production of secretory proteins in animal cells, *International Cell Biology,* Brinkley B. R. and Porter K. R., eds., Rockefeller University Press, New York, pp. 308–317.

Jansen R. F. and Mayeri E. (1988) The neuropeptide egg-laying hormone modulates multiple ionic currents in single target neurons of the abdominal ganglion of *Aplysia. J. Neurosci.* **8,** 3074–3084.

Jennings K. R., Host J. J., Kaczmarek L. K., and Strumwasser F. (1981) Serotonergic inhibition of afterdischarge in peptidergic bag cells. *J. Neurobiol.* **12,** 579–590.

Kaczmarek L. K., Finbow M., Revel J. P., and Strumwasser F. (1979) The morphology and coupling of *Aplysia* bag cells within the abdominal ganglion and in cell culture. *J. Neurobiol.* **10,** 535–550.

Kaczmarek L. K., Jennings K., and Strumwasser F. (1978) Neurotransmitter modulation, phosphodiesterase inhibitor effects, and cyclic AMP correlates of afterdischarge in peptidergic neurites. *Proc. Natl. Acad. Sci. USA* **75,** 5200–5204.

Kaczmarek L. K., Jennings K. R., and Strumwasser F. (1982) An early sodium and late calcium phase in the afterdischarge of peptide secreting neurons of *Aplysia. Brain Res.* **238,** 105–115.

Kaczmarek L. K., Jennings K. R., Strumwasser F., Nairn A. C., Walter U., Wilson F. D., and Greengard P. (1980) Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture. *Proc. Natl. Acad. Sci. USA* **77,** 7487–7491.

Kaczmarek L. K. and Kauer J. A. (1983) Calcium entry causes a prolonged refractory period in peptidergic neurons of *Aplysia. J. Neurosci.* **3,** 2230–2239.

Kaczmarek L. K. and Levitan I. B., eds. (1986) *Neuromodulation: The Biochemical Control,* Oxford University Press, New York.

Kaczmarek L. K. and Strumwasser F. (1981) The expression of long lasting afterdischarge by isolated *Aplysia* bag cell neurons. *J. Neurosci.* **1,** 626–634.

Kaczmarek L. K. and Strumwasser F. (1984) A voltage-clamp analysis of currents underlying cyclic AMP-induced membrane modulation in isolated peptidergic neurons of *Aplysia. J. Neurophysiol.* **52,** 340–349.

Kajikawa N., Kishimoto A., Shiota M., and Nishizuka Y. (1983) Ca^{2+}-Dependent neutral protease and proteolytic activation of Ca^{2+}-activated, phospholipid-dependent protein kinase. *Meth. Enzymol.* **102,** 279–290.

Kandel E. R. (1979) *Behavioral Biology of Aplysia: A Contribution to the Comparitive Study of Opistho-*

branch Molluscs, W. H. Freeman, San Francisco, CA.

Kandel E. R. and Schwartz J. H. (1982) Molecular biology of learning: Modulation of neurotransmitter release. *Science* **218**, 433–443.

Kauer J. A., Fisher T. E., and Kaczmarek L. K. (1987) α bag cell peptide directly modulates the excitability of the neurons that release it. *J. Neurosci.* **7**, 3623–3632.

Kauer J. A. and Kaczmarek L. K. (1985) Peptidergic neurons of *Aplysia* lose their response to cyclic adenosine 3':5'-monophosphate during a prolonged refractory period. *J. Neurosci.* **5**, 1339–1345.

Kirk M. D. and Scheller R. H. (1986) Egg-laying hormone of *Aplyisa* induces a voltage-dependent slow inward current carried by Na^+ in an identified motoneuron. *Proc. Natl. Acad. Sci. USA* **83**, 3017–3021.

Koester J. and Kandel E. R. (1977) Further identification of neurons in the abdominal ganglion of *Aplysia* using behavioral criteria. *Brain Res.* **121**, 1–20.

Kreiner T., Sossin W., and Scheller R. H. (1986) Localization of *Aplysia* neurosecretory peptides to multiple populations of dense core vesicles. *J. Cell Biol.* **102**, 769–782.

Kriegstein A. R. (1977) Development of the nervous system of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* **74**, 375–378.

Kriegstein A. R., Castellucci V., and Kandel E. R. (1974) Metamorphosis of *Aplysia californica* in laboratory culture. *Proc. Natl. Acad. Sci. USA* **71**, 3654–3658.

Kupfermann I. (1967) Stimulation of egg laying: possible neuroendocring function of bag cells of abdominal ganglion of *Aplysia californica*. *Nature* **216**, 814, 815.

Kupfermann I. (1970) Stimulation of egg laying by extracts of neuroendocrine cells (bag cells) of abdominal ganglion of *Aplysia*. *J. Neurophysiol.* **33**, 865–876.

Kupfermann I. (1972) Studies on the neurosecretory control of egg laying in *Aplysia*. *Am. Zool.* **12**, 513–519.

Kupfermann I., Carew T. J., and Kandel E. R. (1974) Local, reflex, and central commands controlling gill and siphon movements in *Aplysia*. *J. Neurophysiol.* **33**, 865–876.

Kupfermann I. and Kandel E. R. (1970) Electrophysiological properties and functional interconnections of two symetrical neurosecretory clusters (bag cells) in abdominal ganglion of *Aplysia*. *J. Neurophysiol.* **33**, 865–876.

Kupfermann I. and Weiss K. R. (1976) Water regulation by a presumptive hormone contained in identified neurosecretory cell R15 of *Aplysia*. *J. Gen. Physiol.* **67**, 113–123.

Levitan E. S., Kramer R. H., and Levitan I. B. (1987) Augmentation of bursting pacemaker activity by egg-laying hormone in *Aplysia* neuron R15 is mediated by a cyclic AMP-dependent increase in Ca^{2+} and K+ currents. *Proc. Natl. Acad. Sci. USA* **84**, 6307–6311.

Lewis R. S. and Cahalan M. D. (1988) The plasticity of ion channels: parallels between the nervous and immune systems. *Trends in Neurosci.* **11**, 214–218.

Ligman S. H. and Brownell P. H. (1985) Differential hormonal action of the bag cell neurons on the arterial system of *Aplysia*. *J. Comp. Physiol.* **157**, 31–37.

Loechner K. J., Azhderian E., Dreyer R., and Kaczmarek L. K. (1988) Progressive release of peptides from bag cell neurons of *Aplysia* during a discharge. *Soc. Neurosci. Ab.* **14**, 176.

Loechner K. J. and Kaczmarek L. K. (1987) Regulation of isolated bag cell neurons of *Aplysia* by α, β, and γ-bag cell peptides. *Soc. Neurosci. Ab.* **13**, 1071.

Leonard J. L. and Lukowiak K. (1986) The behavior of *Aplysia californica Cooper* (Gastropoda; Opisthobranchia): I. Ethogram. *Behavior* **98**, 320–360.

Loh Y. P., Sarne Y., and Gainer H. (1975) Heterogeneity of proteins synthesized, stored, and released by the bag cells of *Aplysia californica*. *J. Comp. Physiol.* **100**, 283–295.

MacGinnitie G. E. (1934) The egg-laying activities of the sea hare, *Tethys californicus* (Cooper). *Biol. Bull.* **67**, 300–303.

Mackey S. and Carew T. J. (1983) Locomotion in *Aplysia:* triggering by serotonin and modulation by bag cell extract. *J. Neurosci.* **3**, 1469–1477.

Mahon A. C., Nambu J. R., Taussig R., Shyamala M., Roach A., and Scheller R. H. (1985) Structure and expression of the egg-laying hormone gene family in *Aplysia*. *J. Neurosci.* **5**, 1872–1880.

Mayeri E., Brownell P., and Branton W. D. (1979a) Multiple, prolonged actions of neuroendocrine bag cells in *Aplysia*. II. Effects on beating pace-

maker and silent neurons. *J. Neurophysiol.* **42,** 1185–1197.

Mayeri E., Brownell P., Branton W. D., and Simon S. B. (1979b) Multiple, prolonged actions of neuroendocrine bag cells on neurons in *Aplysia.* I. Effects on bursting pacemaker neurons. *J. Neurophysiol.* **42,** 1165–1184.

Mayeri E. and Rothman B. S. (1981) Nonsynaptic peptidergic neurotransmission in the abdominal ganglion of *Aplysia, Neurosecretion—Molecules, Cells, Systems,* Farner P. S. and Lederis K. eds., Plenum, New York, pp. 305–316.

Mayeri E. and Rothman B. S. (1985) Neuropeptides and the control of egg-laying behavior in *Aplysia, Model Neural Networks and Behavior,* Selverston A. I., ed., Plenum, New York, pp. 285–301.

Mayeri E., Rothman B. S., Brownell P. H., Branton W. D., and Padgett L. (1985) Nonsynaptic characteristics of neurotransmission mediated by egg-laying hormone in the abdominal ganglion of *Aplysia. J. Neurosci.* **5,** 2060–2077.

McAllister L. B., Scheller R. H., Kandel E. R., and Axel R. (1983) *In situ* hybridization to study the origin and fate of identified neurons. *Science* **222,** 800–808.

Molloy S., Bruns C., and Arch S. (1987) Dissimilar associations of two secretory peptides with a neurosecretory granule-enriched fraction from the bag cells. *Peptides* **8,** 829–836.

Nagle G. T., de Jong-Brink M., Painter S. D., Blankenship J. E., and Kurosky A. (1988b) Purification and structure of the delta and epsilon bag cell peptides of *Aplysia. Soc. Neurosci. Ab.* **14,** 176.

Nagle G. T., Painter S. D., and Blankenship J. E. (1989a) Post-translational processing in model neuroendocrine systems: The precursors and products that coordinate reproductive activity in *Aplysia* and *Lymnaea. J. Neurosci. Res.* **23,** 359–370.

Nagle, G.T., Painter, S. D., and Blankenship, J. E. (1989b) The egg-laying hormone family: Precursors, products, and functions. *Biol. Bull.,* **177.**

Nagle G. T., Painter S. D., Blankenship J. E., Dixon J. D., and Kurosky A. (1986) Evidence for the expression of three genes encoding homologous atrial gland peptides that cause egg laying in *Aplysia. J. Biol. Chem.* **261,** 7853–7859.

Nagle G. T., Painter S. D., Blankenship J. E., and Kurosky A. (1988a) Proteolytic processing of egg-laying hormone-related precursors in *Aplysia.* Identification of peptide regions critical for biological activity. *J. Biol. Chem.* **263,** 9223–9237.

Nagle G. T., Painter S. D., Kelner K. L., and Blankenship J. E. (1985) Atrial gland cells synthesize a family of peptides that can induce egg laying in *Aplysia. J. Comp. Physiol. B* **156,** 43–55.

Nambu J. R. and Scheller R. H. (1986) Egg-laying hormone genes of *Aplysia:* Evolution of the ELH gene family. *J. Neurosci.* **6,** 2026–2036.

Newcomb R. and Scheller R. H. (1987) Proteolytic processing of the *Aplysia* egg-laying hormone and R3-14 neuropeptide precursors. *J. Neurosci.* **7,** 854–863.

Painter S. D., Gustavson A. R., Kalman V. K., Nagle G. T., and Blankenship J. E. (1989a) Induction of copulatory behavior in *Aplysia:* Atrial gland factors mimic the excitatory effects of freshly deposited egg cordons. *Behav. Neural Biol.* **51,** 222–236.

Painter S. D., Gustavson A. R., Nagle G. T., and Blankenship J. E. (1988) Induction of mating behavior in *Aplysia* by freshly deposited egg cordons and atrial gland factors. *Symposia Biologica Hungarica* **36,** 287–296.

Painter S. D., Kalman V. K., Nagel G. T., and Blankenship J. E. (1989b) Localization of immunoreactive alpha-bag-cell peptide in the central nervous system of *Aplysia:* Implications for the physiological induction of bag-cell activity. *J. Comp. Neurology,* **287,** 515–530.

Painter S. D., Kalman V. K., Nagle G. T., Zuckerman R. A., and Blankenship J. E. (1985) The anatomy and functional morphology of the large hermaphroditic duct of three species of *Aplysia,* with special reference to the atrial gland. *J. Morphol.* **186,** 167–194.

Painter S. D., Rock M. K., Nagle G. T., and Blankenship J. E. (1988) Peptide B induction of bag-cell activity in *Aplysia:* Localization of sites of action to the cerebral and pleural ganglia. *J. Neurobiol.* **19,** 695–706.

Perlman, A. J. (1979) Central and peripheral control of siphon-withdrawal reflex in *Aplysia californica. J. Neurophysiol.* **42,** 510–529.

Pinsker, H. M. and Dudek, F. E. (1977) Bag cell control of egg-laying in freely behaving *Aplysia. Science* **197,** 490–493.

Pinsker H. M. and Parsons D. W. (1985) Temperature dependence of egg laying in *Aplysia brasiliana* and *A. californica*. *J. Comp. Physiol. B* **156,** 21–27.

Pulst S.-M., Gusman D., Rothman B. S., and Mayeri E. (1986) Coexistence of egg-laying hormone and α-bag cell peptide in bag cell neurons of *Aplysia* indicates that they are a peptidergic multitransmitter system. *Neurosci. Lett.* **70,** 40–45.

Pulst S. M., Rothman B. S., and Mayeri E. (1987) Presence of immunoreactive α-bag cell peptide[1–8] in bag cell neurons of *Aplysia* suggests novel carboxypeptidase processing of neuropeptides. *Neuropeptides* **10,** 249–259.

Ram J. L. (1982) *Aplysia* egg-laying hormone increases excitatory input into a retractor muscle of the buccal mass. *Brain Res.* **236,** 505–510.

Ram J. L. (1983) Neuropeptide activation of an identifiable buccal ganglion motoneuron in *Aplysia*. *Brain Res.* **288,** 177–186.

Ram J. L., Haller K. A., and Levran Z. (1986) Sensitivity of a peptide-activated neuron in *Aplysia* to serotonin and cyclic AMP-relevant agents. *Comp. Biochem. Physiol.* **83C,** 279–283.

Rock M. K., Shope S. B., Blankenship J. E., and Schlesinger D. H. (1986) Effects of synthetic bag cell and atrial gland peptides on identified nerve cells in *Aplysia*. *J. Neurobiol.* **17,** 273–290.

Rothman B. S., Hawke D. H., Brown R. O., Lee T. D., Dehghan A. A., Shively J. E., and Mayeri E. (1986) Isolation and primary structure of the califins, three biologically active egg-laying hormone-like peptides from the atrial gland of *Aplysia californica*. *J. Biol. Chem.* **261,** 1616–1623.

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

Rothman B. S., Sigvardt K. A., and Mayeri E. (1985) Co-release of five peptides, ELH, AP, α–, β– and γ-BCP, derived from a common precursor protein of the bag cells of *Aplysia*. *Soc. Neurosci. Ab.* **11,** 482.

Rothman B. S., Weir G., and Dudek F. E. (1983b) Egg-laying hormone: Direct action on the ovotestis of *Aplysia*. *Gen. Comp. Endocrinol.* **52,** 134–141.

Schaefer M. and Brownell P. H. (1986) Modulation of a respiratory motor program by peptide-secreting neurons in *Aplysia*. *J. Neurobiol.* **17,** 121–126.

Scheller R. H., Jackson J. F., McAllister L., Rothman B. S., Mayeri E., and Axel R. (1983) A single gene encodes multiple neuropeptides mediating a stereotyped behavior. *Cell* **32,** 7–22.

Scheller R. H., Jackson J. F., McAllister L. B., Schwartz J. H., Kandel E. R., and Axel R. (1982) A family of genes that codes for ELH, a neuropeptide eliciting a stereotyped pattern of behavior in *Aplysia*. *Cell* **28,** 707–719.

Schlesinger D. H., Babirak S. P., and Blankenship J. E. (1981) Primary structure of an egg laying peptide from the atrial gland of *Aplysia californica, Symposium on Neurohypophyseal Peptide Hormones and Other Biologically Active Peptides,* Schlesinger D. H., ed., Elsevier/North Holland, New York, pp. 137–150.

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

Sossin W. S., Kirk M. D., and Scheller R. H. (1987) Peptidergic modulation of neuronal circuitry controlling feeding in *Aplysia*. *J. Neurosci.* **7,** 671–681.

Strong J. A. (1984) Modulation of potassium current kinetics in bag cell neurons of *Aplysia* by an activator of adenylate cyclase. *J. Neurosci.* **4,** 2772–2783.

Strong J., Fink L., Fox A., and Kaczmarek L. K. (1987b) Multiple roles for calcium and calcium-dependent enzymes in the activation of peptidergic neurons of *Aplysia, Cell Calcium and the Control of Membrane Transport,* Eaton D. and Mandel L., eds., Rockefeller University Press, New York, pp. 187–199.

Strong J. A., Fox A. P., Tsien R. W., and Kaczmarek L. K. (1987) Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature* **325,** 714–717.

Strong J. A. and Kaczmarek L. K. (1986) Multiple components of delayed potassium current in peptidergic neurons of *Aplysia:* Modulation by an activator of adenylate cyclase. *J. Neurosci.* **6,** 814–822.

Strumwasser F., Kaczmarek L. K., Chiu A. Y., Heller E., Jennings K. R., and Viele D. P. (1980) Peptides

controlling behavior in *Aplysia, Peptides: Integrators of Cell and Tissue Function*, Bloom F. E., ed., Raven, New York, pp. 197–217.

Stuart D. K., Chiu A. Y., and Strumwasser F. (1980) Neurosecretion of egg-laying hormone and other peptides from electrically active bag cell neurons of *Aplysia*. *J. Neurophysiol.* **43**, 488–498.

Stuart D. K. and Strumwasser F. (1980) Neuronal sites of action of a neurosecretory peptide, egg-laying hormone in *Aplysia californica*. *J. Neurophysiol.* **43**, 499–519.

Susswein A. (1984) Effects of food deprivation upon behavioral patterns and time budgeting of *Aplysia fasciata*. *Beh. Neural Biol.* **42**, 127–133.

Susswein A. J. and Benny M. (1985) Sexual behavior in *Aplysia fasciata* induced by homogenates of the distal large hermaphroditic duct. *Neurosci. Lett.* **59**, 325–330.

Susswein A. J., Gev S., Achituv Y., and Markovich S. (1984) Behavioral patterns of *Aplysia fasciata* along the mediterranean coast of Israel. *Behav. Neural Biol.* **41**, 7–22.

Switzer-Dunlap M., Meyers-Schulte K., and Gardner E. A. (1984) The effect of size, age, and recent egg laying on copulatory choice of the hermaphroditic mollusc *Aplysia juliana*. *Int. J. Invert. Reprod. Develop.* **7**, 217–225.

terMaat A., Geraerts W. P. M., Jansen R. F., and Bos N. P. A. (1988) Chemically mediated positive feedback generates long-lasting afterdischarge in a molluscan neuroendocrine system. *Brain Res.* **438**, 77–82.

Toevs L. A. and Brackenbury R. W. (1969) Bag cell-specific proteins and the humoral control of egg laying in *Aplysia californica*. *Comp. Biochem. Physiol.* **29**, 207–216.

Treistman S. N. and Levitan I. B. (1976) Alteration of electrical activity in molluscan neurones by cyclic neucleotides and peptide factors. *Nature* **261**, 62–64.

Tritt S. H., Lowe I. P., and Byrne J. H. (1983) A modification of the glyoxylic acid-induced histofluorescence technique for demonstration of catecholamines and serotonin in tissues of *Aplysia californica*. *Brain Res.* **259**, 159–162.

Weiss K. R., Cohen J. L., and Kupfermann I. (1978) Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia*. *J. Neurophysiol.* **41**, 181–203.

Woolum J. C. and Strumwasser F. (1988) Calcium changes in isolated peptidergic neurons during activation by a cyclic AMP analog. *Brain Res.* **444**, 1–9.

Yates M. E. and Berry R. W. (1984) Subcellular sites of processing of precursors to neurosecretory peptides in the bag cells of *Aplysia*: Inferences from the effects of monensin, FCCP, and chloroquine. *J. Neurobiol.* **15**, 141–155.