Title
Increased activity in the bursting neuron R15 following activation of the neurosecretory 'bag' cells of Aplysia

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INCREASED ACTIVITY IN THE BURSTING NEURON R15 FOLLOWING ACTIVATION OF THE NEUROSECRETORY 'BAG' CELLS OF *APLYSIA*

by

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THESIS

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I. INTRODUCTION

In recent years, many investigators have been looking at information processing in biology on a cellular level. Cell - cell communication is essential in all tissues so that growth and differentiation can occur, whole organ function can be coordinated and overall homeostasis of the organism can be maintained. The two professional communications systems in the body, the nervous and endocrine systems have received particular attention since it is their full time job to perceive, integrate and dispatch information governing behavior. Detailed studies of nervous and endocrine actions are beginning to define the cellular mechanisms that form the vocabularies of these complex information systems.

In the nervous system, for example, spatial and temporal summation of synaptic potentials are the simplest levels of information integration. Reverberating circuits, synaptic decrement and facilitation are further elements that have been described (30) and slow excitatory and inhibitory synaptic potentials have been added to the mechanisms of integration (23, 37). While these examples all describe elements of communication confined to the nervous system itself, there are also intercommunications between the nervous and endocrine systems. In fact, the two systems are not really distinct entities but contain overlapping elements: catacholamines act as both hormones and synaptic transmitters, and neurosecretory cells are of neural origin but secrete endocrine products. Neural input can alter hormone release and humoral presence of hormones can modify nerve cell behavior. The mechanisms of these neuroendocrine interactions, then, have also received much attention.

Recently, there have been increasing numbers of studies of hormone effects on individual neurons or groups of neurons in vertebrate systems (15, 25, 32). Peptide hormones in particular have been shown to have excitatory effects on some types of neurons (15, 32). For example,

the peptide hormones LRF, TRF and oxytocin were found to excite nerve cells in the rat hypothalamus (15) and the small peptide called substance P (actually a putative neurotransmitter) excites frog and rat spinal motoneurones (32).

More detailed analyses of hormone effects on nerve cells and the relation of these effects to behavior have been done using simple invertebrate systems (5,8,9,19,24,40,46,58). One well known study in the area of hormonal control of invertebrate behavior is the work by Truman and others (58) which describes the behavioral effects of eclosion hormone of silk moths. The eclosion hormone is released during the transition from pupal to adult behavior as these insects emerge from their inactive state to become fully developed moths. The transition involves activation of a whole new pattern of motor activity, a sudden 'turning on' of preexisting motor circuits which have been inactive prior to release of eclosion hormone. The 'turning on' mechanism has not been determined, but could conceivably involve direct hormonal activation of the silk moth neurons.

In another invertebrate system, Barker and Gainer (5) have shown that the peptide hormones vasopressin and oxytocin can excite an identified neuron in the land snail. This cell is silent when the snail is hibernating and active when the snail is active. Hormonal activation of individual nerve cells, then, might play a role in the snails hibernation behavior.

In our laboratory we have been studying the egg laying hormone ('bag cell hormone') of Aplysia Californica to determine it's effects on the Aplysia nervous system. Bag cell hormone is a substance found in a group of neurosecretory cells which is released when the bag cells are electrically stimulated, and which induces egg laying when injected into another Aplysia (34). Injections of bag cell hormone lead not only to

expulsion of eggs, but to a whole pattern of stereotyped behavior associated with egg laying, such as attachment to a vertical surface and characteristic head motions. While one primary action of bag cell hormone is probably to directly cause contraction of smooth muscles surrounding egg follicles, and so expel the Aplysia oocytes (11), it must also have at least an indirect effect in initiating the accompanying behavioral changes. There is, in fact, some evidence that bag cell hormone could affect cardiovascular function in Aplysia by increasing the size of synaptic potentials in nerve cells of the heart circuitry (Mayeri and Simon, unpublished). While investigating this action of bag cells on heart circuitry, we found that electrical activation of the neurosecretory bag cells also causes striking changes in the nerve cell called R15 by Frazier et al. (20). This cell normally fires in regular bursts of action potentials separated by silent periods (see figure 6). Within minutes after the bag cells have been activated, this bursting behavior becomes markedly exaggerated and the cell occasionally slips into periods of sustained continuous spiking. This change in firing pattern takes on added significance because while R15 retains properties similar to other Aplysia neurons, it is also thought to be a neurosecretory cell (34), releasing a hormone involved in water retention (36). This phenomenon, then, affords the opportunity to examine an action of one neurosecretory cell on another under close to physiologic conditions. We have not yet established that the bag cell effect on R15 is mediated by the bag cell hormone, but evidence in favor of this hypothesis will be presented here.

If the changes in R15 are in fact produced by a hormone, the mechanisms of the bag cell - R15 system might have even wider application since the system would illustrate (1) a physiologic case of a hormone turning on a neural element, (2) probably a case of a peptide

exciting a neural element, a subject currently being investigated (14, 15,32,44) (see later section for evidence that bag cell hormone is a peptide) and (3) probably a case of a hormone activating a neurosecretory cell, thus accelerating release of a second hormone for at least an hour following activation.

The system is an appealing one to use to study these phenomena since the cells involved are large and accessible, are located in the same ganglion and can be observed under close to physiologic conditions. The bag cells can be made to secrete physiologic doses of hormone at the physiologic site of release under fairly constant experimental conditions. And, the behavioral significance of the system is approachable because Aplysia nerve cells have been widely studied to determine individual neuron functions.

II. THE APLYSIA PREPARATION

The nervous system of the Aplysia Californica is a relatively small network of interconnected ganglia (figure 1). Each ganglion has a single outer layer of large nerve cell bodies which send processes to the core, or neuropil of the ganglion (9,54), and thence to the periphery or to the neuropil of other ganglia via the connecting nerves. Synaptic contacts are restricted to the neuropil; no synapses have been found on cell bodies. The whole ganglion is wrapped in a vascular connective tissue sheath, which for some ganglia can be easily penetrated with microelectrodes.

Because the Aplysia nerve cells are large, often pigmented and lie in a fairly constant position from one animal to another, much work has been done to map these neurons and establish their individual electrical properties, interconnections and behavioral functions (20,22,27,28,29,33,39,43, 54). Only the abdominal ganglion was used for the present studies, and some of the circuitry in this ganglion has already been established (see for example 39).

DISSECTED APLYSIA

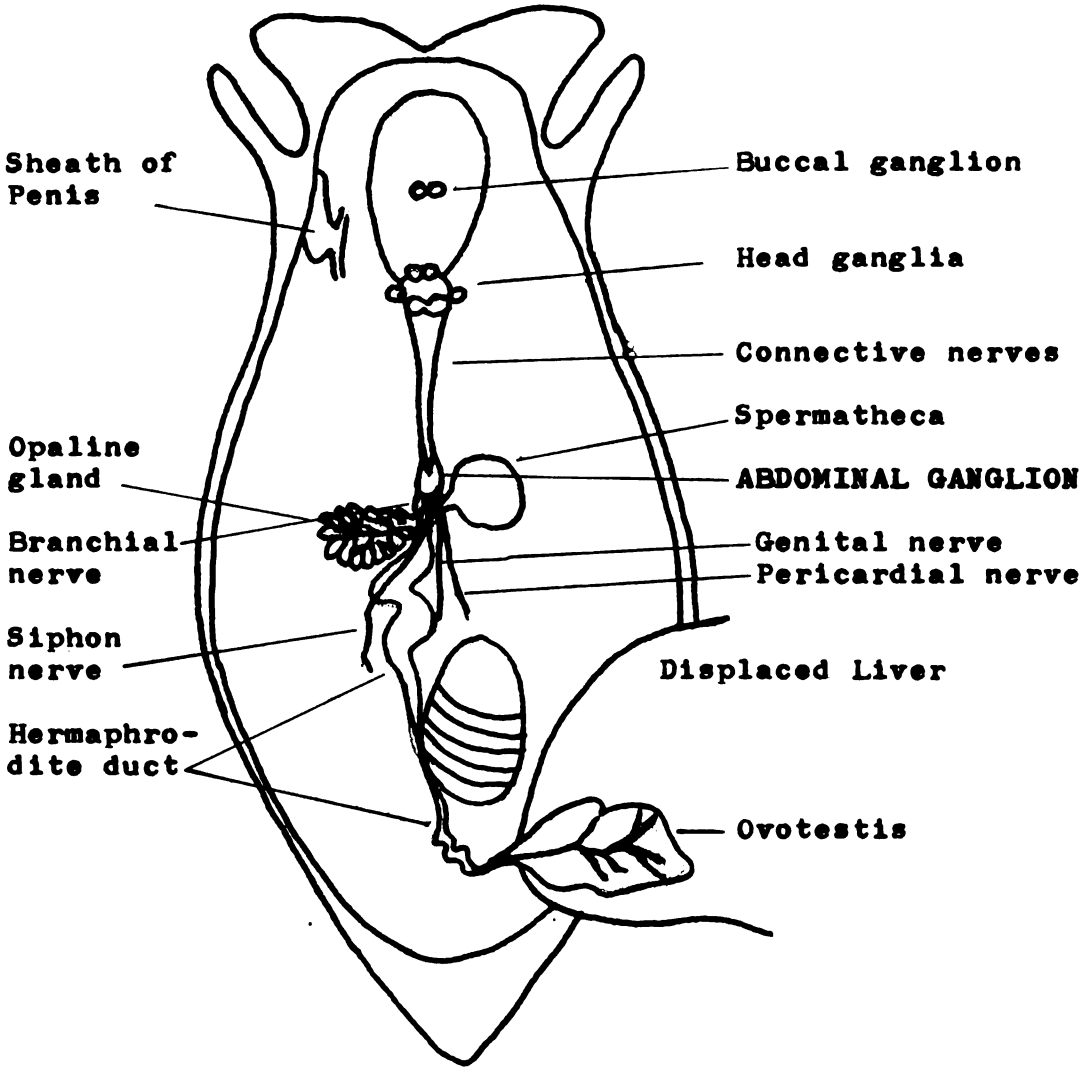


FIGURE 1 Ventral view of a disected Aplysia californica showing some relevant anatomy of the reproductive and nervous systems. Adapted from Toevs (57).

The abdominal ganglion (figures 2 and 3) is made up of three functionally separate cell groups: the pigmented cells, the white cells, and the bag cells (20). The pigmented cells make up the motor and sensory pathways, the white cells are fairly large white cells that contain dense core secretory granules, and the bag cells are two very distinct clusters of smaller neurosecretory (also white) cells which secrete a hormone that induces egg laying (34) (see figure 3). This work describes interactions between the bag cells and one particular white cell called R15 (figure 3).

A. BAG CELLS

1. Anatomy

The bag cells lie in two interconnected clusters located at the rostral end of the abdominal ganglion at the point where the connectives from the head ganglia enter (figure 2). Most of the cell bodies lie on the ventral surface, but a variable number of bag cells spread to the dorsal side. Some processes arising from the bag cells wrap around the axons in the connectives forming a sort of cuff, and many turn into the sheath where they end bordering the vascular system and do not re-enter the ganglion. Bag cell processes ending in the sheath are distributed over most of the ganglion surface. Apparently, no processes are sent into the neuropil and there is no evidence of synapses with other cells (20).

The bag cells vary in size and number according to the age and weight of the animal. In the sexually mature animal (usually greater than 200 grams) there are about 400 cells in each cluster, each cell measuring fifty or less microns in diameter (20). Bag cells are very white, owing to their secretory granules (about 1000 angstrom moderately dense core granules); the degree of whiteness is presumed to correlate with the amount of hormone in the cell (20).

Physiological evidence suggests that all of the bag cells in both

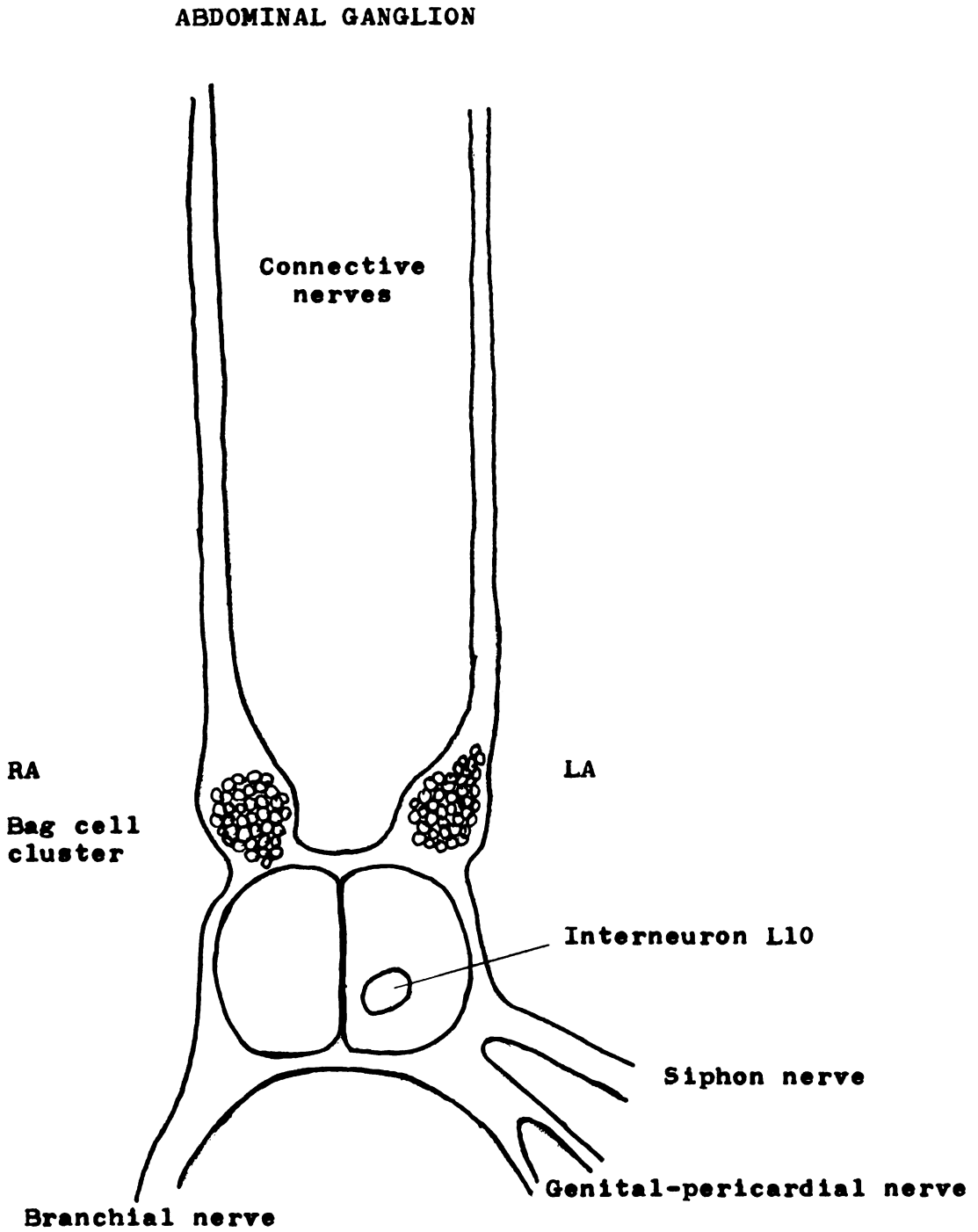


FIGURE 2 Ventral surface of Aplysia abdominal ganglion showing the location of the RA and LA bag cell clusters surrounding the entering connective nerves. (Schematic)

ABDOMINAL GANGLION

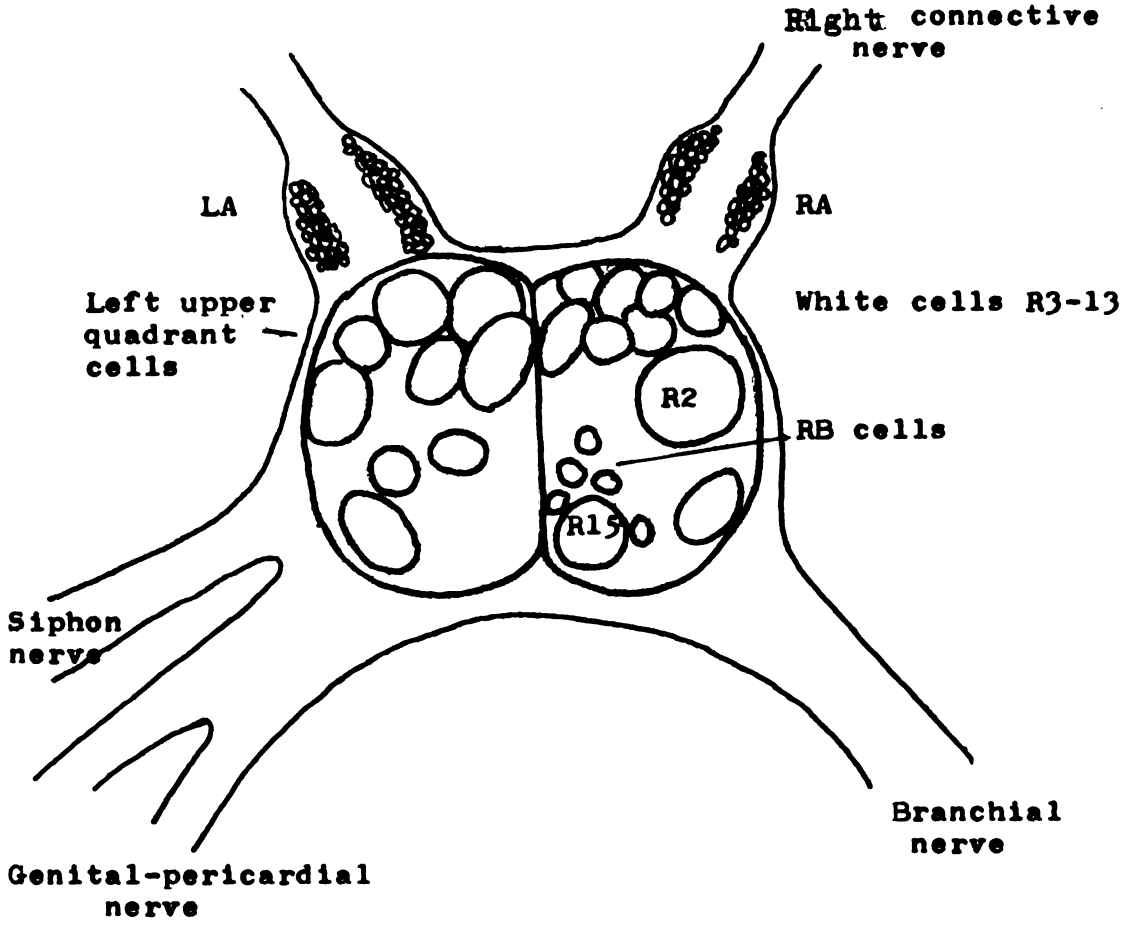


FIGURE 3 Dorsal surface of the abdominal ganglion showing the location of R15, the bag cells and other identified neurons. Redrawn from Kandel, et. al. (28).

clusters are electrically coupled (33), but no anatomical evidence for this coupling or for any kind of bag cell synapse has been found yet. No cell has been found to receive synaptic input from the bag cells and the best evidence for synaptic input to the bag cells is the observation that bag cells spike in response to electrical stimulation of the connectives.

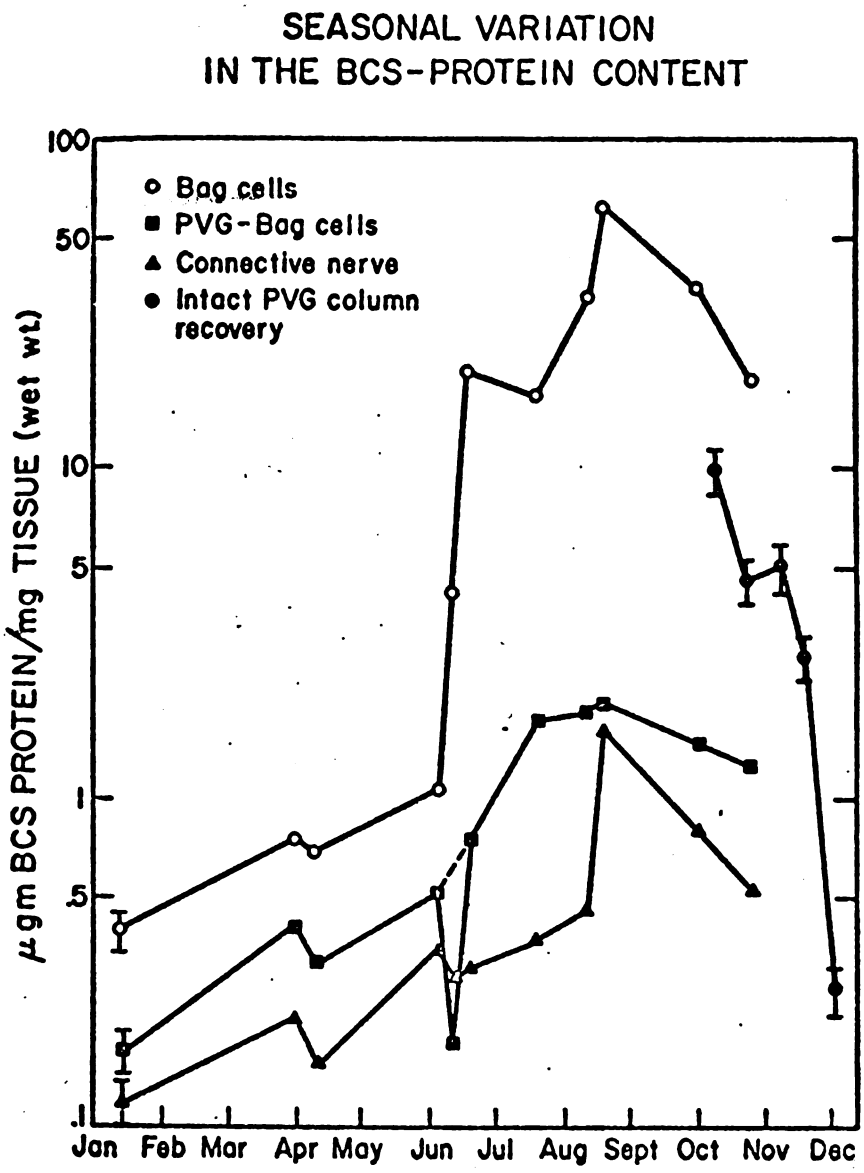
Anatomical evidence, then, suggests that the bag cells are an autonomous unit, neurally isolated from the rest of the cells in the ganglion.

Blood flows into the abdominal ganglion sheath via a small artery from the heart, and presumably picks up the bag cell hormone and carries it to the ovotestes via the hemocoel to cause oocyte expulsion in egg laying.

It is worth emphasizing that the bag cells are located in the rostral abdominal ganglion, far from the ovotestis target organ, far from the head ganglia where the presence of other neurohormones is suspected, and surrounding cells to which they are anatomically unrelated. From an anatomical point of view, then, it is reasonable to propose a role for bag cell hormone in controlling some egg laying behavior by acting on the abdominal ganglion neurons.

2. Stimulation of egg laying

Because the bag cells are anatomically separable from the rest of the abdominal ganglion, they can be harvested and ground up. Behavioral effects and biochemical nature of this 'bag cell extract' can then be studied. When bag cell extract is injected into another Aplysia, that animal will usually lay eggs within one or two hours (34,35,53). This response to bag cell extract varies according to the season (figure 5). The greatest number of positive responses occur during June and July when the animal normally has its egg laying season (34,38,53). Egg laying can also be induced by injecting an animal with the bathing medium of bag cells which have been electrically stimulated to fire action poten-



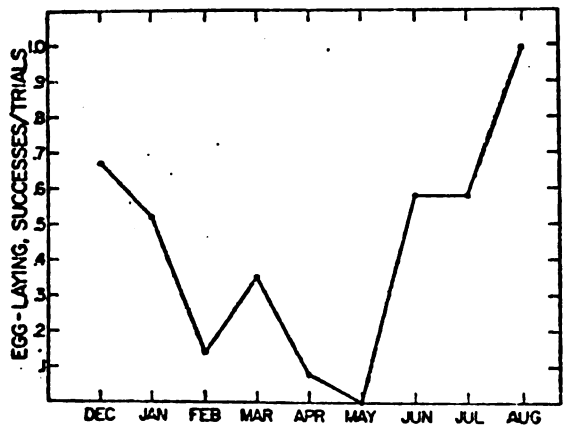


FIGURE 4 (above) Open dot curve shows seasonal variation in the amount of peptide found in the bag cells (presumably the hormone). BCS stands for bag cell specific, PVG for parieto-visceral ganglion. From Toevs (57).

FIGURE 5 (left) Seasonal variation in ability of bag cell extract to stimulate egg laying. From Strumwasser (53).

Both graphs reflect the summer/fall egg laying season, low values occuring in the months February - May.

tials, and so to release their hormone (34).

Anatomical evidence for a role of bag cell hormone in egg laying was found by studying the ovotestis which nurtures, stores and expels oocytes (11,12). Application of bag cell extract causes the ovotestis to expel eggs within one minute following contact (11). Ovotestis muscles are not innervated, so no neural stimulus regulates their contraction (12). Electron micrographs taken before and after application of bag cell extract show evidence of contraction of the tiny smooth muscles surrounding the ovotestis egg follicles. Coggeshall suggests, then, that bag cell hormone directly affects these muscle cells much as oxytocin causes contraction of smooth muscles in the human mammary gland (12).

3. Biochemistry of bag cell hormone

The active substance secreted by the bag cells has not been isolated, but is thought to be a small polypeptide with a molecular weight in the 6000 dalton range (2,21,56,57). More recent unpublished work (and see 21) suggests the hormone may be an even smaller fragment of this 6000 dalton peptide. The active agent in bag cell extract is destroyed by proteolytic enzymes and high temperature (34,56,60). SDS gels show a protein peak in the 6000 dalton range for the bag cells (21,56,57), and a change in the protein composition of the bathing medium is seen following bag cell stimulation, suggesting that polypeptide is released (2). The amount of this 6000 dalton polypeptide found in Aplysia bag cells varies according to the animal's sexual maturity and according to the season of the year (34,56,57). Figure 4 shows the seasonal variation in the 6000 molecular weight substance. It should be noted, however, that the study shown in figure 4 did not include animal weights, and could therefore reflect the seasonal ability to obtain sexually mature animals; large animals are not found as easily in the winter months.

4. Seasonal responsiveness

In laboratory Aplysia both spontaneous egg laying and egg laying induced by bag cell extract occur much more frequently during the Summer and early Fall. As yet, there is no single explanation for this seasonality. There is seasonal variation in the amount of hormone synthesized (57); there is comparable variation in the number of oocytes produced by an animal (11); and we have seen seasonal variability in the electrical excitability of the bag cells. In addition, variation in receptor responsiveness is suspected. It is likely that other hormones are exerting influence on the system.

Our attempts to alter spontaneous egg laying behavior by varying feeding schedule, light/dark schedule and water temperature have not been successful.

5. Electrophysiology

The electrophysiology of the bag cells has been thoroughly investigated by Kupfermann (33,35). The bag cells are usually silent with a resting potential of 20 to 60 mv and no sign of spontaneous synaptic input. Electrical stimulation of the connectives or direct depolarization of the bag cell cluster using a focal electrode will cause all of the bag cells of both clusters to begin firing in synchrony. Once activated, the bag cells will spike repetitively for about one to twenty minutes--even up to an hour at times. The burst is characterized by spikes of very long duration, even for a snail (ranging from 30-150 msecs or about 5 times the duration of spikes in pigmented cells (33)) and the spike heights decrease as the burst continues (maximum spike height about 80 mv). The rate of spiking is initially high, speeds up, then slows again to a stop. The action potentials of cells within a cluster are tightly synchronized, but cells in opposite clusters may fall out of synchrony by the end of a burst. Once activated, the bag cells are refractory to further bursting for at least an hour (33).

Our observations of bag cell properties generally agree with Kupfermann's (33,35). We only monitored bag cells with resting potentials greater than 30 mvs and found some bag cells with resting potentials of over 70 mv. We found it more difficult, however, to evoke more than one bag cell discharge in one day.

B. IDENTIFIED CELL R15

1. Anatomy

The single identified cell named R15 by Frazier et al. (20) is located in the right caudal quadrant of the dorsal surface of the abdominal ganglion (figure 3). It is a large (about 300 μ) milky white cell which, like other white cells, contains secretory granules in its cell body and throughout the length of its processes. This white coloring is more apparent in large adult animals. Unlike other neurosecretory cells, however, R15 does not send processes into the vascular sheath surrounding the abdominal ganglion (27). It does send large axons out the branchial nerve (figure 3), the pericardial nerve and probably the spermathecal nerve (20,50, Mayeri, unpublished). The process in the branchial nerve carries information from the osphradium, a chemoreceptive and perhaps osmoreceptive organ; the pericardial nerve axon may innervate the kidney or the heart; the spermathecal branch has been reported to innervate the spermatheca (50), a balloon-like structure that stores and digests extraneous sperm following copulation (55).

R15 is surrounded by pigmented cells, so is not a part of the cluster of white cells R3-R13 (see figure 3) which also receive input from osphradial stimulation, and are believed to be involved in food detection (27). R15 receives input from abdominal ganglion pigmented cells, notably from L10 and Interneuron II, the interneurons of the cardiovascular/respiratory system (39).

2. Biochemistry

Like the other white cells in the ganglion, R15 contains dense core granules and is thought to secrete a small polypeptide. The white cells in general have been shown to synthesize unusually large amounts of small polypeptides (21). Recent investigations suggest an R15 specific polypeptide (around 1500 daltons) which is synthesized in the cell body and transported out the axons (Gainer and Poh, personal communication). This would support the theory that R15 secretes a small protein hormone. Although hormone secretion by R15 has not been demonstrated, its characteristic secretory granules and the ability of R15 homogenate to cause water retention in Aplysia (36) indicate that R15 is probably neurosecretory.

3. Role in water retention

Evidence has been collected to show that R15 is involved in Aplysia water retention (3,26,27,36,45,50). The presence of a hypotonic solution around the osphradium (an external organ located in the mantle cavity near the gill) will cause long lasting inhibition of the tonically active R15 cell (27,50). Recently, R15 cells were dissected out of the abdominal ganglion and injected into other Aplysia. The injections caused a 5-10% weight gain in the recipient animals (36).

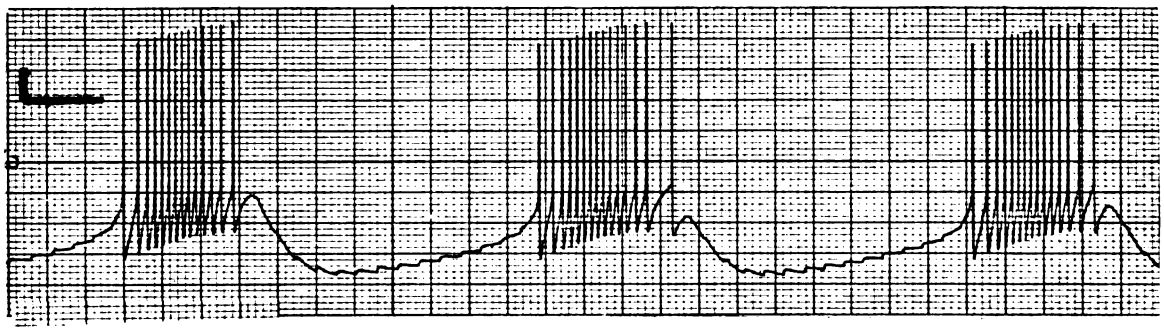
The full story of water retention by a neurohormone secreted by R15 is under investigation, but presently has some puzzling aspects. First, although the paths of R15 axons have not been fully worked out, the currently known anatomy does not necessarily fit the proposed role for R15, There is no current evidence that R15 processes end in the vascular system, unless perhaps the pericardial axon releases secretory material into the heart. The function of the spermathecal branch remains unexplained. Electrophysiologically, R15 is an autogenic burster which follows a diurnal rhythm and will be shown to be excited by bag cells.

The benefits of varying tonicity according to time of day and during egg laying are not evident. Therefore, it is quite possible that other roles for R15 have yet to be discovered. The spermathecal axon links R15 to the reproductive system, so perhaps R15 has a local effect there, such as changing the permeability of the spermatheca. Or perhaps this hormone, like vasopressin, has effects on the vascular system in addition to its ability to cause water retention. Changes in vascular function might be more consistent with egg laying behavior than changes in water load. Further studies will be required to clarify the function of R15.

4. Electrophysiology

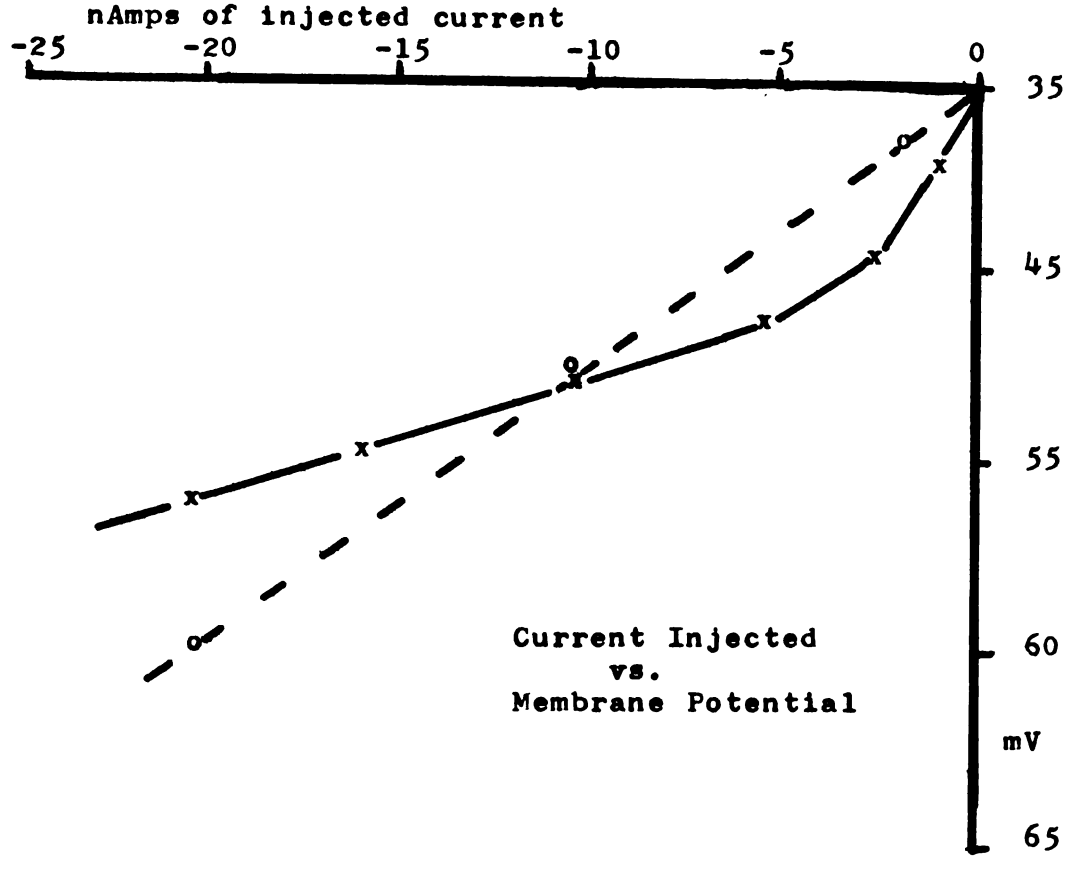
Because it is a large, accessible, identifiable cell, R15 has been extensively studied as an example of a bursting pacemaker neuron (1,6,7, 10,26,31,36,41,42,48). There are several other bursting neurons in Aplysia ganglia and bursters exist in other animals as well. Bursting neurons are of more general interest, also, because neural burst mechanisms may apply to bursting in pacemaker muscle such as the smooth muscle of heart or gastrointestinal tract, and it is conceivable that bursting neurosecretory cells might be responsible for pulsatile release of neurohormones in vertebrate systems.

BURSTING NEURONS: For conceptual convenience, nerve cells can be divided into three categories: driven, beating and bursting. A driven cell is the classically taught nerve cell which is silent until activated by sufficient excitatory input. The beating cell fires spikes at regular or irregular intervals independent of excitatory input. And, the bursting cell fires rhythmic bursts of spikes time by a slowly oscillating membrane potential (figure 6). Recently investigators have shown that some cells may have the capacity to behave in any of these three modes depending on their temperature and humoral environment (4,5,13,31).



(Calibration is 10 mv, 2 seconds)

FIGURE 6 Some basic characteristics of a bursting cell. Above is an example of R15 burst activity demonstrating the rhythmic bursts of spikes seperated by silent periods. The small EPSP seen during the silent phases is from L10. The graph below (from Barker (4)) shows a burster cell current-voltage plot. The dashed line is a usual non-burster linear relation, while the burster curve becomes nonlinear. This nonlinearity is termed anomalous rectification and is typical of the bursting neurons.



The general characteristics of a bursting cell are most easily described by a picture (figure 6), but include: (A) a slowly oscillating membrane potential (sometimes called the bursting pacemaker potential (BPP), or the slow potential). This potential continues even when the burst spikes are blocked by tetrodotoxin (52). (B) a characteristic burst of spikes occurring at the crest of the slow potential. The spikes first accelerate and then slow by the end of the burst (thus the name 'parabolic burster', spiking being a parabolic function of time). (C) a characteristic nonlinear current-voltage relationship (figure 6) indicating unstable membrane resistance (anomalous rectification).

Burster cells are modulated by, but not usually driven by synaptic input. At least part of the burst generating mechanism lies in the cell body (1).

MODEL FOR BURST MECHANISM: The mechanisms that initiate and control bursting behavior in R15 and other cells have been explored using a variety of techniques (1,5,6,7,10,13,17,26,31,48,51,52). Smith et al.'s voltage clamp analysis during bursting (48) suggests bursting cells are leaky to sodium, and that this constant depolarizing sodium current is counterbalanced by a voltage dependent, slow acting potassium current. The sodium current has both a voltage dependent and voltage independent component. According to this study of burst control (48) the following ionic changes describe a burst cycle*. At the start of a burst of spikes, there is no slow potassium current. Because the membrane has been depolarizing, though, the voltage threshold for the potassium current has been exceeded and eventually the current turns on, slowing the spiking to a stop and then driving the membrane potential down to its lowest

*Other models for burst control have been suggested and it is possible that an inward Ca^{++} current is involved.

point, during the silent phase. The hyperpolarization turns off the potassium current and unmasks the ever-present sodium current. This depolarizing current drives the membrane potential up again, turning on the voltage dependent sodium current as well, initiating and accelerating spiking again until the potassium current begins to exert its influence again, and so the cycle continues.

Physiological factors which can modulate bursting patterns include temperature, divalent cation concentrations (4,7), synaptic input (41, 42), peptide hormones (5,26) and time of day (51). All of these will be discussed in later sections.

In the isolated abdominal ganglion, R15 receives very little spontaneous synaptic input. In some preparations, bursting is periodically shut off for several minutes by inhibitory input, but in general, there is little synaptic modulation of the inherent bursting. In semi-isolated preparations, there are two strong influences on R15 bursting. Long periods of inhibition are transmitted via the branchial nerve (50), and there is periodic spontaneous excitation from the head ganglia via the right connective nerve (Interneuron XIII). Higher threshold inhibitory input can be electrically stimulated in either connective, but this may or may not be physiologic.

As mentioned earlier, the activity of R15 follows a diurnal rhythm, with peak activity (greatest average spiking frequency) occurring at dawn (51). Under our experimental conditions these diurnal changes occur slowly and would not interfere with the faster changes in R15 presented in this work.

III. METHODS

Aplysia Californica were obtained from Pacific Biomarine Co., Venice, California. The animals were kept in artificial sea water aquaria (Instant Ocean Systems, Inc., Wickliffe, Ohio) on a 24 hour light cycle

of 14 hours light, 10 hours dark. Animals ranged in weight between 200 and 600 grams. Experiments for this study were carried out between August, 1974 and April, 1975. All experiments were performed during the day, dissection usually occurring around 10:30 a.m. with preparations lasting through the evening.

In order to reduce the likelihood of causing the bag cells to discharge in response to dissection, the inside of the animal was doused with cold (below 6 degrees centigrade) sea water at the time of the first incision. The abdominal ganglion was then kept in cold sea water until all cells were impaled and the bag cells could be monitored. In one of the preparations (8/31/74) room temperature sea water with a high magnesium concentration was used instead of cold water to anesthetize the animal and suppress bag cell spikes (33). Results were equivalent with this procedure.

The dissected abdominal ganglion was pinned dorsal side up in a wax bottom dish perfused with artificial sea water containing 1 gm/liter of glucose and buffered to 7.6 pH with 10 mM Tris HCl. Glass micropipettes prepared from pyrex 7740 glass tubing, 1 mm outer diameter, filled with two molar potassium citrate (5-20 megohms resistance) were used for intracellular recording. Simultaneous recordings could be made from up to four cells at a time. R15 is easily impaled visually with the aid of a dissecting microscope. Although individual bag cells are not usually visible, recordings from bag cells were obtained by inserting the electrode into a discernible bag cell cluster. Since the bag cell clusters are homogeneous, this blind impalement is usually safe. Positive identification was achieved by observing the cell's electrical properties, particularly the evoked burst of spikes. In cases where bursts could not be stimulated, the cell was identified by eliciting a spike or subthreshold response via a focal electrode on the surface of the ganglion over

the cell body.

Cut nerve endings were wrapped around current passing electrode posts in the dish. Current could be injected into cells through the micropipettes via a Wheatstone bridge circuit. A focal stimulating electrode that could be placed on the surface of the ganglion was generally used for electrical activation of the bag cells.

Except for cold perfusion during pinning and impalement, experiments were carried out at room temperature (21-24 degrees centigrade). Temperature changes during the course of R15 responses were less than one degree centigrade. At initial impalement during cold perfusion there was little or no spike activity in any of the cells. Bag cells were never seen spiking during cold perfusion. After the preparation had warmed to room temperature, an hour or more of waiting allowed the cells to stabilize to normal room temperature firing patterns.

The ganglion was not desheathed for any of the experiments reported here. Electrodes were tapped through the sheath and then into the cells.

All experiments were recorded in entirety on paper chart output (Brush 440 chart recorder), and at least in part on FM magnetic tape as well (Hewlett Packard 3960 tape recorder). Except for figures 7, 15 and 16, figures showing electrical activity were made from tape replays with the replay tape speed at 1/8 the recorded speed onto the chart recorder. The effective bandwidth of the chart recorder was 400 Hz at full scale, well above the fundamental frequency of the recorded spikes (100 Hz or less).

The basic procedure for each experiment was: (A) to impale all cells under cold perfusion (cells always included R15 and a bag cell) (B) to stop perfusion and allow the bathing medium to come to room temperature either by letting it stand or recirculating the medium using an air pump (no differences between these two methods have been observed)

(C) to wait for stable behavior in all cells monitored (D) to stimulate a bag cell discharge by delivering pulses via a focal stimulating electrode placed over one of the two bag cell clusters (stimulus type based on Kupfermann's description (33)) (E) to observe changes in R15 over the next one to five hours.

CALCULATIONS AND GRAPHS

Values for charts and graphs presented in the next section are calculated from tape replay samples of typical R15 burst activity. Samples were deliberately chosen during periods of time when no unusual synaptic activity was influencing the burst characteristics. Whenever possible, figures given are based on average values over a minimum of five complete consecutive burst cycles, usually covering a time span of one to two minutes. Under such limited conditions, variability in burst characteristics is remarkably small--less than 5% total variation from average for most samples of most variables. Measurements of slow potential amplitude were slightly more variable, and the largest deviation from average was seen in measurements of the time span of the interburst interval. Averages over five bursts seemed to give good representative estimates, though even for these two variables. Because the variability was so small compared to the effects seen and in order to preserve clarity, no variability measurements are graphed.

Spike velocity was calculated as the number of spikes in a burst divided by the length of time spent in the depolarized phase of the burst cycle. This time interval was measured from the threshold of the first spike to the beginning of the long hyperpolarizing excursion.

Average spikes per minute was calculated as the total spikes in a five burst sample divided by the time required to complete the five burst cycles.

Figure 10 shows the interval measured to reflect the amplitude of

the slow potential. It should be noted that this variable is not a true measurement of the whole slow potential excursion; the peak of that excursion is obscured by the spiking activity. The most depolarized point preceding the hyperpolarizing phase was chosen as a reference point because this measure is more convenient and reflects changes accurately as judged by remeasuring the slow potential using spike threshold and peak spike overshoot as alternative reference points for the top of the burst cycle.

'Peak' values for response characteristics were measured at a given point in time when the response as a whole was judged to be a maximum based on the slow potential amplitude and the number of spikes occurring in each burst. Not all burst characteristics peaked at this chosen time (as is evident in figures 10 and 11). Control values are from samples just preceding or overlapping the start of bag cell discharges.

No samples were taken during periods of sustained spiking, so that variables such as spike velocity and spikes per burst reflect the actual burst behavior rather than overall cell behavior. Time spent in prolonged spiking is accounted for separately in the chart of table I.

IV. RESULTS AND DISCUSSION

A. Character of response

Under our experimental conditions, R15 shows very stable electrical behavior through the day. R15 is silent or beating during cold perfusion and begins bursting as the temperature is raised. At room temperature the burst pattern is quite regular with about 10-15 spikes in a burst, a small (10-20 mv) slow potential amplitude, and fairly constant interburst intervals (typically 5-15 seconds). In some preparations, interburst intervals are occasionally prolonged for up to a few minutes by inhibitory input. We have not seen healthy control R15 cells slip into periods of sustained spiking or undergo large increases in slow potential amplitude

even over hours of monitoring R15 activity*. Typically, very slow changes in R15 do take place over the day, so that small increases in the number of spikes in each burst and variability in burst timing is typical. In contrast to this very stable control behavior, striking changes are seen in R15 within minutes after a bag cell discharge.

Five to fifteen minutes after the bag cells start bursting, marked alterations in R15 burst characteristics become apparent. The two most noticeable features of this response are 1. a large increase in the amplitude of the slow potential, and 2. a tendency for R15 to slip into periods of constant spiking without the usual silent phase appearing. Two dramatic examples of these response features are given in figure 7, where slow paper speed records of the half hour during and following two bag cell discharges are shown. Control periods are not shown in this figure but can be seen in other figures to be comparatively stable, with no visible changes in slow potential amplitudes and no intervals of sustained spiking at all.

On closer inspection, two more features of the response become evident: 3. an increase in the number of spikes occurring in each burst develops and 4. an increase in the velocity of spiking during each burst accompanies the increase in spike number. (See table I for quantitative increases). These two characteristics combined tend to mask each other when looking at paper output, for the length of time spent in the spiking phase of the burst cycle may increase, decrease, or remain the same during the response--an increased velocity would disguise the increased number of

*It should be noted, however, that most of our observations were in winter months. We did see one preparation where R15 had an unusually large slow potential even at the start of the day.

TABLE I: **Summary of R15 responses. See next page for explanations.**

DATE	ANIMAL WEIGHT (gms)	STIMULUS SITE	TIME OF BAG CELL BURST	BURST LENGTH (mins)	LATENCY (mins)	PEAK LATENCY (mins)	AVE SPIKE RATE	TIME MONITORED (hrs)
8/31/74	420	-	1:13	6	7	30	I 74%	2:12**
10/29/74	500	-	1:45	4.5	7	20	I 19%	3:10**
12/6/74	510	RA	5:42	5.5	6	60	I 79%	3:00
2/12/75	400	LA	4:10	1.5	15	40	I 75%	4:20
2/17/75	310	LA	2:35	*	6	50	I 30%	6:00**
3/25/75	335	LA	2:24	4	15	60	I150%	5:30

DATE	BURST LENGTH (mins)	INTER BURST LENGTH	SPIKES PER BURST	SPIKE VELO-CITY	SIZE SLOW POTENTL	SLOPE HP PHASE	EPISODES LONG SPIKING	MINUTES IN LONG SPIKING
8/31/74	I 45%	I 20%	I 50%	I 25%	I 96%	I 100%	3	14.5
10/29/74	I 24%	I 86%	I 82%	I 19%	I 74%	I 60%	7	18.5
12/6/74	I 7%	D 26%	I 48%	I 36%	I 79%	I 130%	8	7
2/12/75***	I 16%	D 29%	I 52%	I 33%	I 13%	I 40%	0	
2/17/75	D 11%	I 48%	I 51%	I 71%	I 120%	I 150%	10	2
3/25/75	D 5%	0%	I 81%	I 101%	I 52%	I 140%	0	

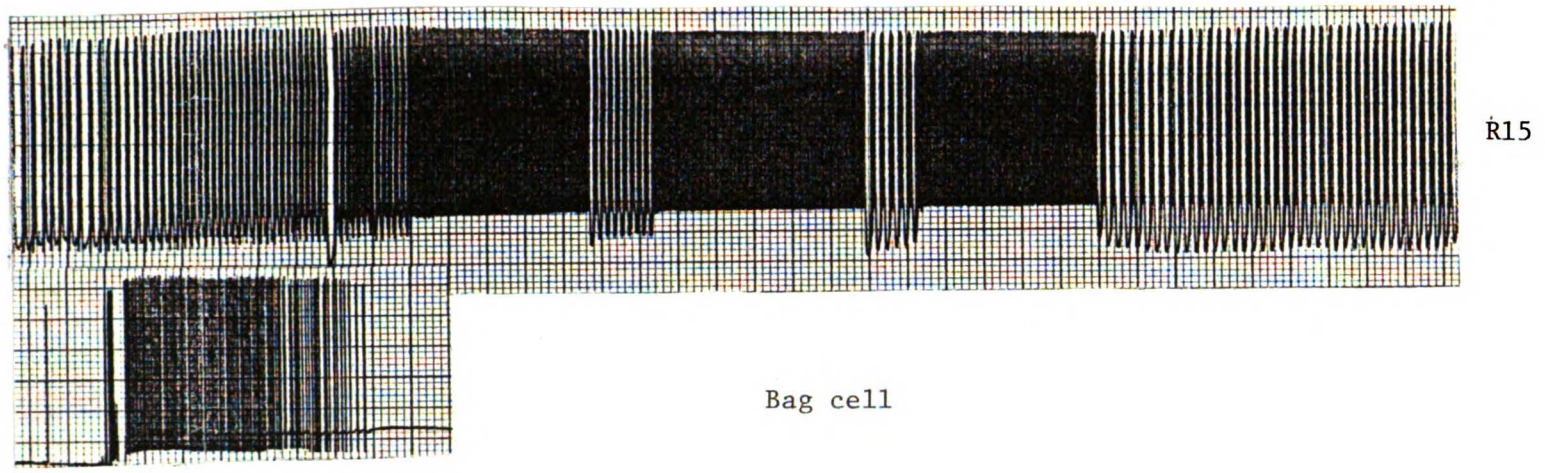
TABLE I CONTINUED (see previous page) Charts summarizing experiments where R15 responded to bag cell discharges. The first chart gives information about the experimental conditions. Stimulus site tells whether the focal electrode was placed over the right (RA) or left (LA) bag cell cluster. Bag cell burst length is the number of minutes from first to last bag cell spike. Latency is a subjective measure indicating the number of minutes before the response was noticeable to the observer. Peak latency is the time to maximal slow potential increase. Average spike rate is calculated as the number of spikes in five consecutive typical bursts, divided by the total length of time needed to complete the five burst cycles. Sustained spiking episodes were not used for this calculation.

The bottom chart shows percentage changes from control level to peak response for the characteristics of R15 burst cycles, (I for increases, D for decreases). The last two columns reflect influence of sustained spiking. Time of sustained spiking is the total number of minutes R15 spent in these phases.

*No bag cell monitor on 2/17/75. Discharge was assumed because of subsequent development of the typical bag cell discharge response.

**These 3 responses were back toward control levels (especially the slow potential) before three hours after the bag cell burst. The other three responses did not wear off during the time monitored. See figures 10 and 11 for examples.

***Percentage changes for 2/12/75 are not calculated to peak but underestimate the peak values. Experimental procedures during peak prevented adequate sampling of maximal response.



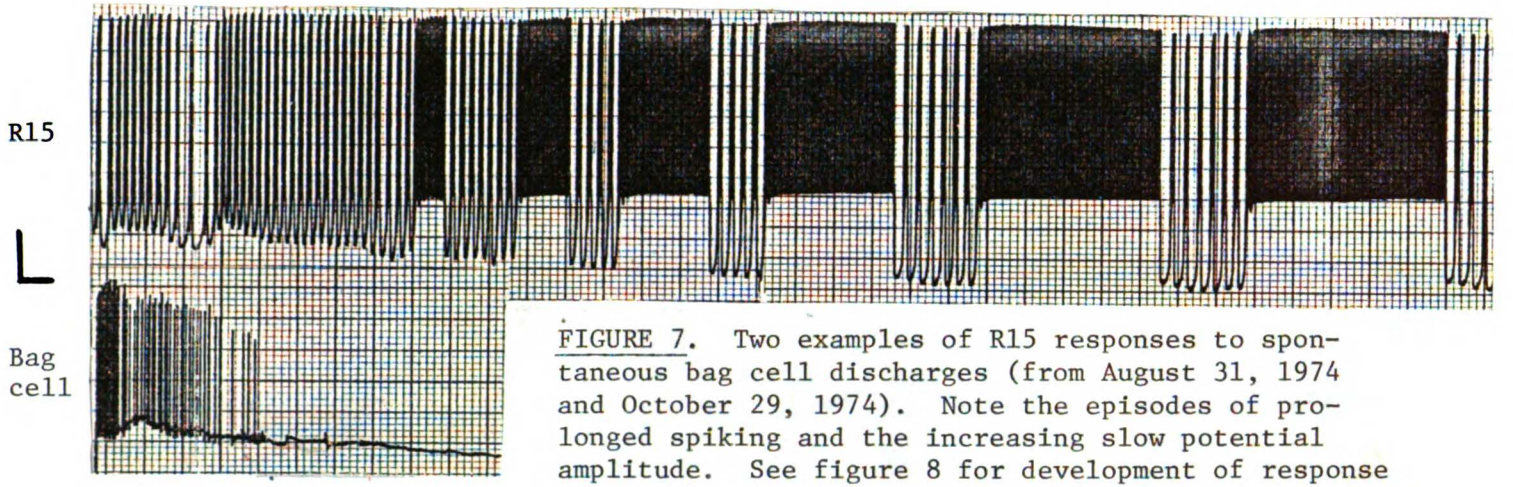
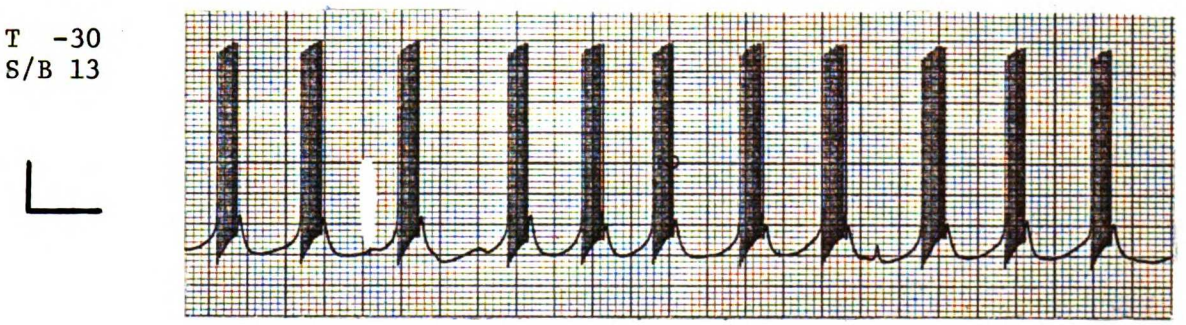
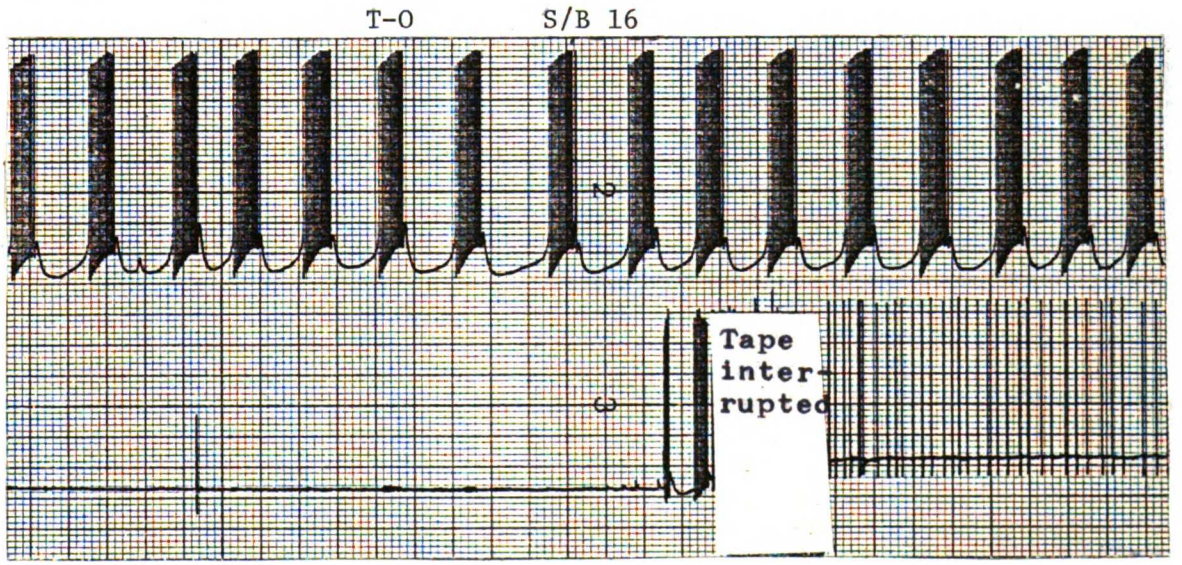


FIGURE 7. Two examples of R15 responses to spontaneous bag cell discharges (from August 31, 1974 and October 29, 1974). Note the episodes of prolonged spiking and the increasing slow potential amplitude. See figure 8 for development of response at faster paper speed. Calibration is 20 mv, 1 minute.

FIGURE 8 (two pages) Development of the bag cell response of August 31, 1974. Bag cells began firing spontaneously. For double records, the top recording is R15 and the bottom a bag cell. Numbers accompanying readouts give time from bag cell discharge in minutes and number of spikes per burst for the sample. Calibration is 20 mv, 15 second.





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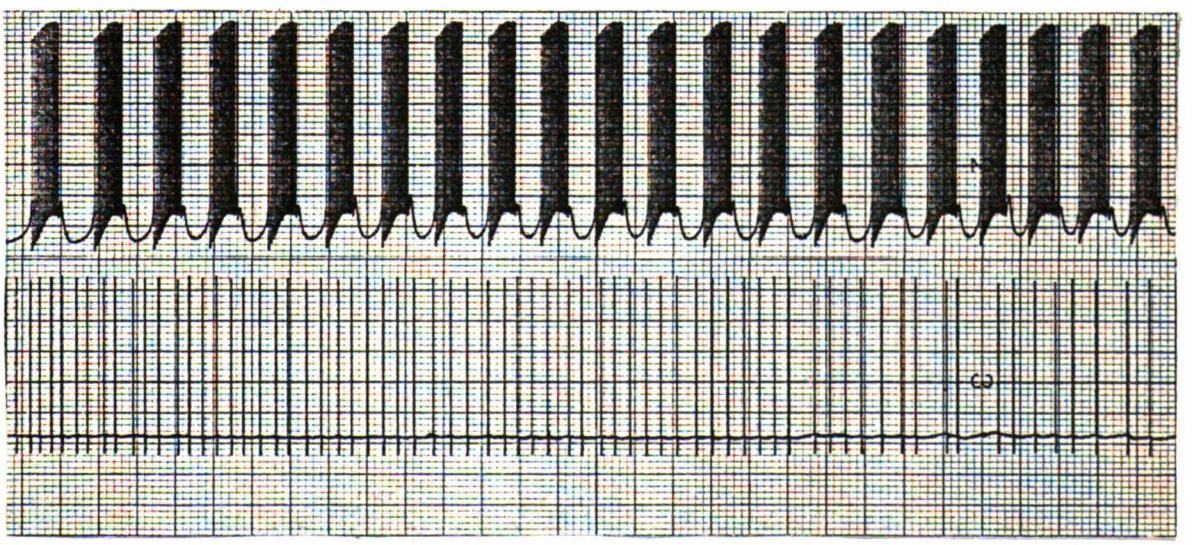

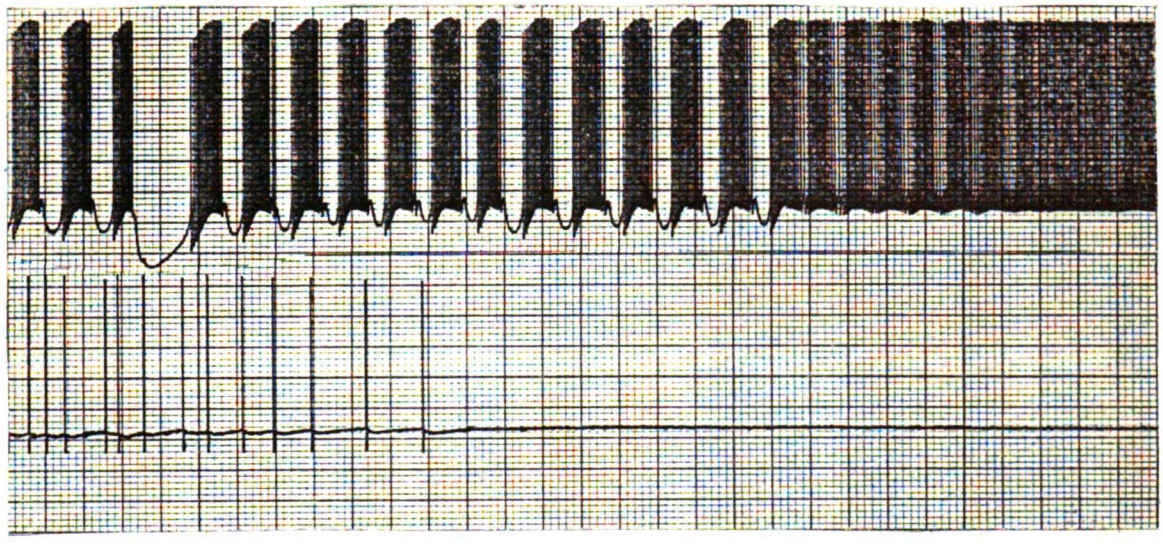

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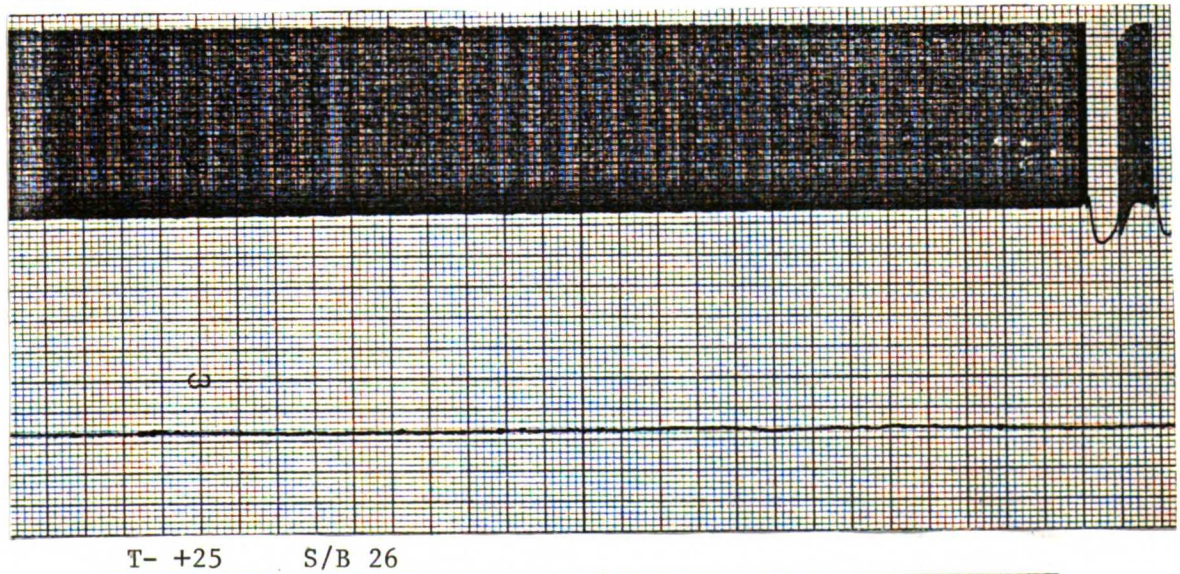

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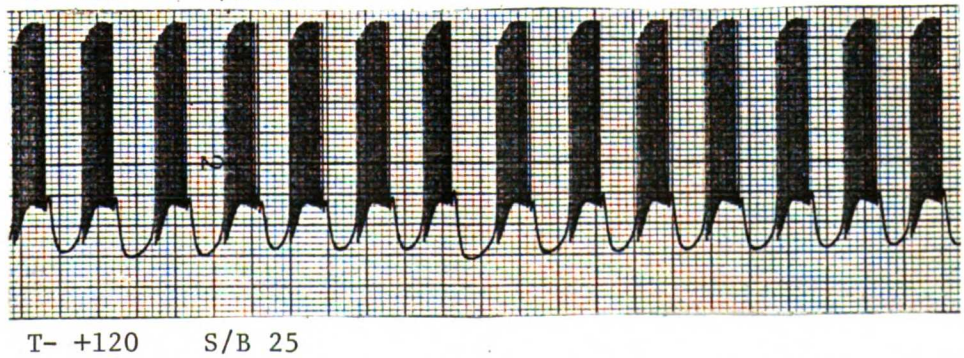

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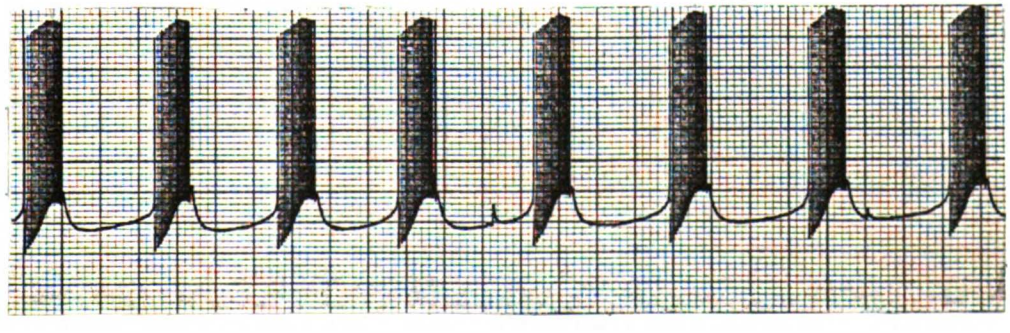


spikes. For this reason, the number of spikes per burst is listed along with most samples of R15 bursts in the figures of this section.

These four changes in R15--increased slow potential amplitude, periods of sustained spiking, increased number of spikes in a burst and increased spike rate during the burst, then are the major characteristics of the bag cell response. Together, they produce an overall increase in total R15 spike activity for at least an hour after the bag cell discharge.

Figure 8 shows the development of one particular response expanded on a faster paper speed. The development of the large hyperpolarizing excursion, the episodes of sustained spiking and the increase in spikes per burst and spiking rate are apparent. Compare this progression to the samples of figure 9. Figure 9 shows a typical control day when we were unable to evoke a bag cell discharge. R15 behavior in this figure is very constant.

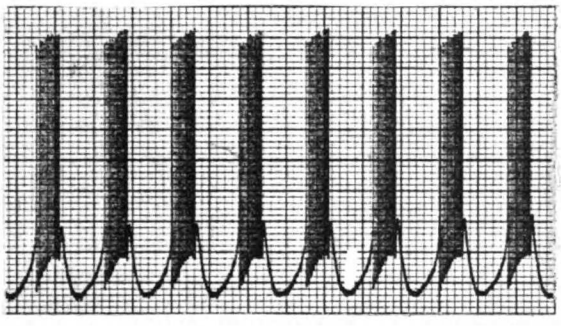
We have been able to follow six R15 responses to bag cell discharges during the months August, 1974 to April, 1975. Table I summarizes the results of these six responses. The responses took place at different times of day and under different methods of bag cell activation. For three preparations the left bag cell cluster was stimulated, for one preparation the right cluster was stimulated, and for two preparations the bag cells began firing on their own with no experimental intervention. Qualitatively, the responses are very similar; quantitatively, they differ somewhat (Table I). In the six responses presented the average spiking activity went up an average of 71% (range 19 - 150%), slow potential amplitude increased 72% (13 - 120%), the slope of the hyperpolarizing phase of the slow potential increased 103% (40 - 140%), spikes in each burst increased 61% (50 - 82%) and spike rate during the burst increased 47% (19 - 101%). The average latency to the start of the response was

9 minutes (range - 6 - 15 minutes) and latency to peak averaged about 45 minutes (20 - 60 minutes). The strength of a response was judged as the amount of increased spiking in R15 which is reflected by the amount of time R15 spent in sustained spiking mode and by the amount of increase in average spiking while in burst mode. The increase in the slow potential amplitude is also a measure of the strength of the response because this probably reflects the changes in the mechanisms underlying the increased spike activity. The two strongest responses came from spontaneous bag cell discharges illustrated in figures 7 and 8. Samples from the other responses will be shown in subsequent figures.

The important burst characteristic which does not change consistently in the six responses is the timing of the slow potential. In some cases the length of time between bursts increases and in others it decreases during the response (Table I). This is important to the possible functional role of the bag cell response because the burst timing will help determine the average number of spikes occurring during a given time interval, and this would presumably reflect the amount of hormone R15 secretes during that time. Table I includes a column for average spikes per minute, and it is evident that in all responses there was an increase in spike activity despite the direction of change in the timing mechanism.

B. Specificity of response

As mentioned earlier, we have never seen R15 undergo large spontaneous changes in burst behavior. Sustained spiking and large slow potential amplitudes are definitely not characteristic under our conditions (and during the months when our observations were made). Siphon nerve and connective stimulations which do not evoke bag cell discharges have not produced characteristics similar to the bag cell response (see figure 9). Responses occurred following bag cell discharges regardless of



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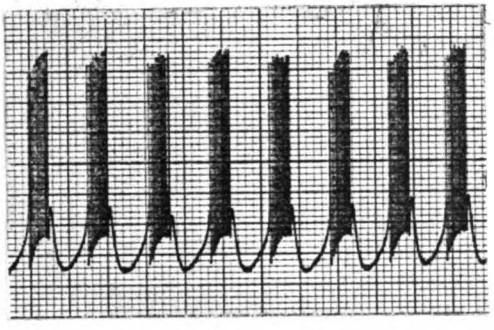

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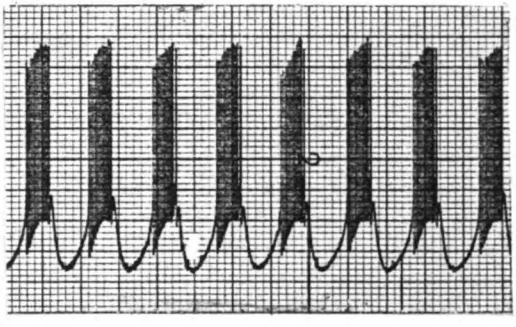

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FIGURE 9 Sample records of R15 activity on a typical control day (February 26, 1975). LA bag cells were electrically stimulated with a bipolar focal electrode at time zero, but could not be made to fire even with relatively extreme stimulus intensities. Time from zero time (in minutes) and number of spikes in a typical burst are printed below each sample. No striking changes in R15 burst activity were seen. The decrease in spikes per burst at +20 minutes is probably due to prolonged heavy stimulation of connectives while trying to activate the bag cells. Strong stimuli to the connectives evoke very effective inhibitory input to R15. Calibration is 20 mv, 15 seconds.

whether the discharges were spontaneous or the result of electrical stimulation (table I). And the responses that followed stimulated discharges were similar regardless of whether LA or RA cells were stimulated, and regardless of stimulus intensity (Intensities ranged from 2.8 to 13 mAMPs, with 5 msec pulses, variable numbers of pulses being required to activate the cells). Mild temperature changes and artificial hyperpolarization or depolarization of R15 do not mimic the effects (figure 16). The similarity of response latencies shown in table I and the specificity of occurrence of the response make it clear that the bag cell discharge is prerequisite to the R15 responses we have seen.

C. Time course of response

In all six responses monitored, the four main characteristics described in part A were developed to the point of easy recognition within fifteen minutes of the bag cell discharge. Note the similar time courses of the two responses shown in figure 7. The latency to the peak of the response varied between twenty minutes and an hour, averaging 45 minutes over the six responses.

The total duration of the response was more variable. For three of the responses, the slow potential was back to control level within three hours after the bag cell discharge and for three others the response never washed out while R15 was being monitored (maximum six hours). For all six responses, however, the possibly functionally important characteristics of increased average spikes per minute and sustained spiking episodes were decreased within two hours. Even when the slow potential increase lasted for many hours, episodes of sustained spiking tended to take place only during the first hour. And, we have never seen large declines in any of the described response characteristics in less than an hour. Despite the variability of the duration of slow potential changes, then, we can say that effects on R15 last for at least an hour, and that the possible functionally significant changes probably last one to two hours.

Figures 10 and 11 show the time course of two rather different responses.

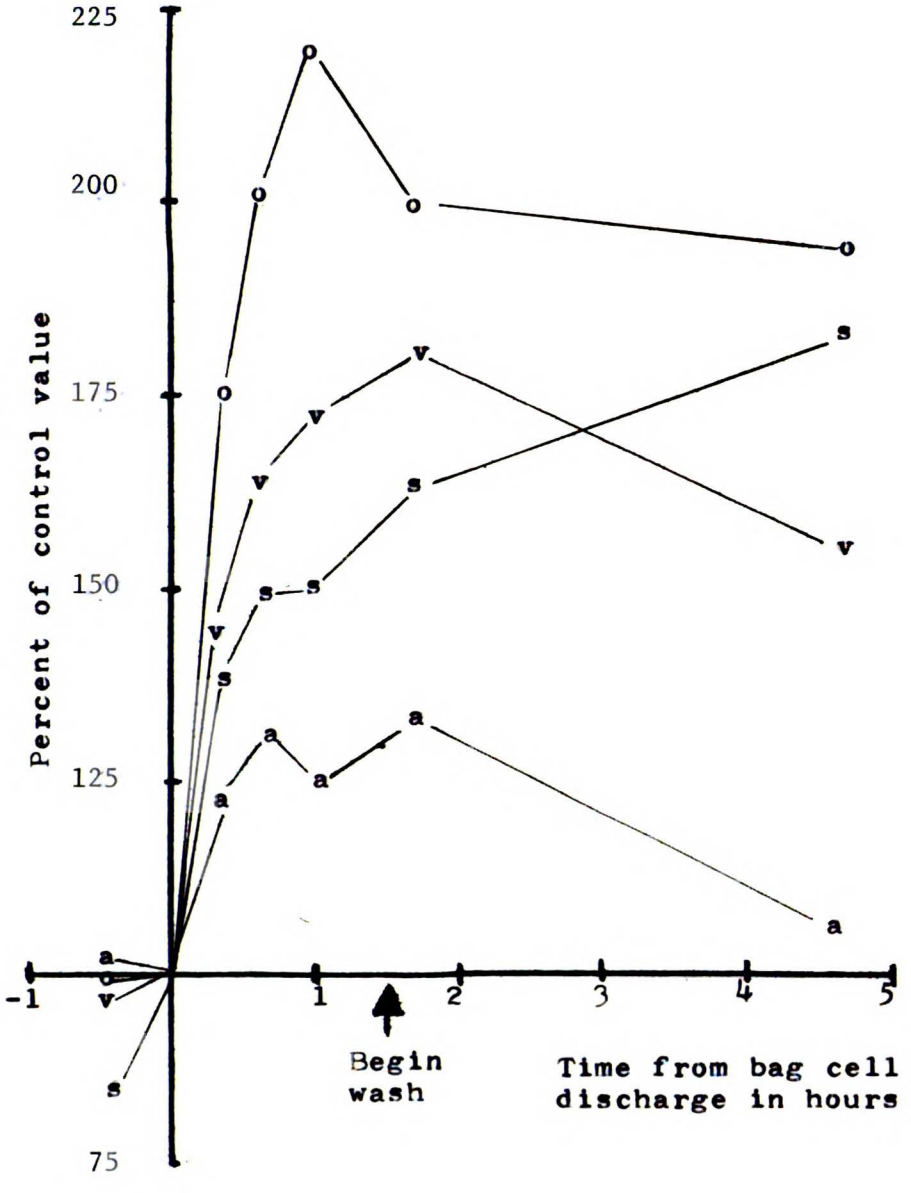
The February 17 example is a response that did not wear off even after several hours of washing and the August 31 example shows a response that declined in two hours with no washing. Further experience may reveal a clearer pattern for the time course of the decline of the slow potential increase. In these experiments the strongest responses do not last the longest times.

Figures 10 and 11 also demonstrate the variable course of the individual characteristics of a response. Time course of development is similar but not identical for all characteristics. The variability in the average spikes per minute is at least in part dependent upon the amount of spontaneous inhibitory input for the particular preparation, and so its time course would be expected to be more variable than time courses for other characteristics.

D. Attempts to reverse or enhance the response

In addition to trying to wash out the responses, we have used active means to try to reverse them. A variety of strengths of electrical stimuli to the connectives or to the siphon nerve have been tried and found to have at most transient effects. Suppression of all spike activity with eight minutes of strong hyperpolarization, shown in figure 12, likewise has no effect. Changing illumination and reimpaling the cell cause no changes.

We have also found the R15 changes to be refractory to enhancement. Under our experimental conditions, we have had difficulty stimulating more than one bag cell discharge in the same day especially during the summer months (see also 33). For two of the reported responses, however,we were able to get second discharges late in the day. In neither case did we see enhancement of the R15 response to the first discharge. Figure 13 shows the results of one of these experiments. In this example, the second discharge took place two hours after the first. The first discharge lasted for five minutes and the second lasted seven. See table I for details of the first response.



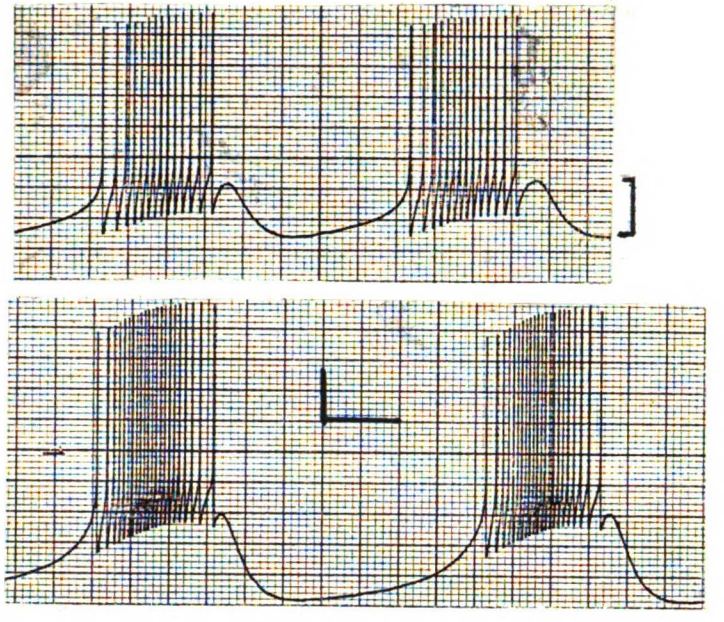


FIGURE 10 Response of February 17, 1975. Above are readouts of R15 burst activity before and during the bag cell response. Calibration is 10 mv, 3 sec. At left is a graph following four burst characteristics over time. Symbols are: o-slow potential oscillation amplitude (sample measurement shown at right of top burst readout above), s-number of spikes per burst, v-velocity of spiking during burst, a-average number of spikes per minute. All values are expressed as percentages of their control value at time zero. Time zero is the time electrical stimulation of the bag cells occurred. See text for discussion.

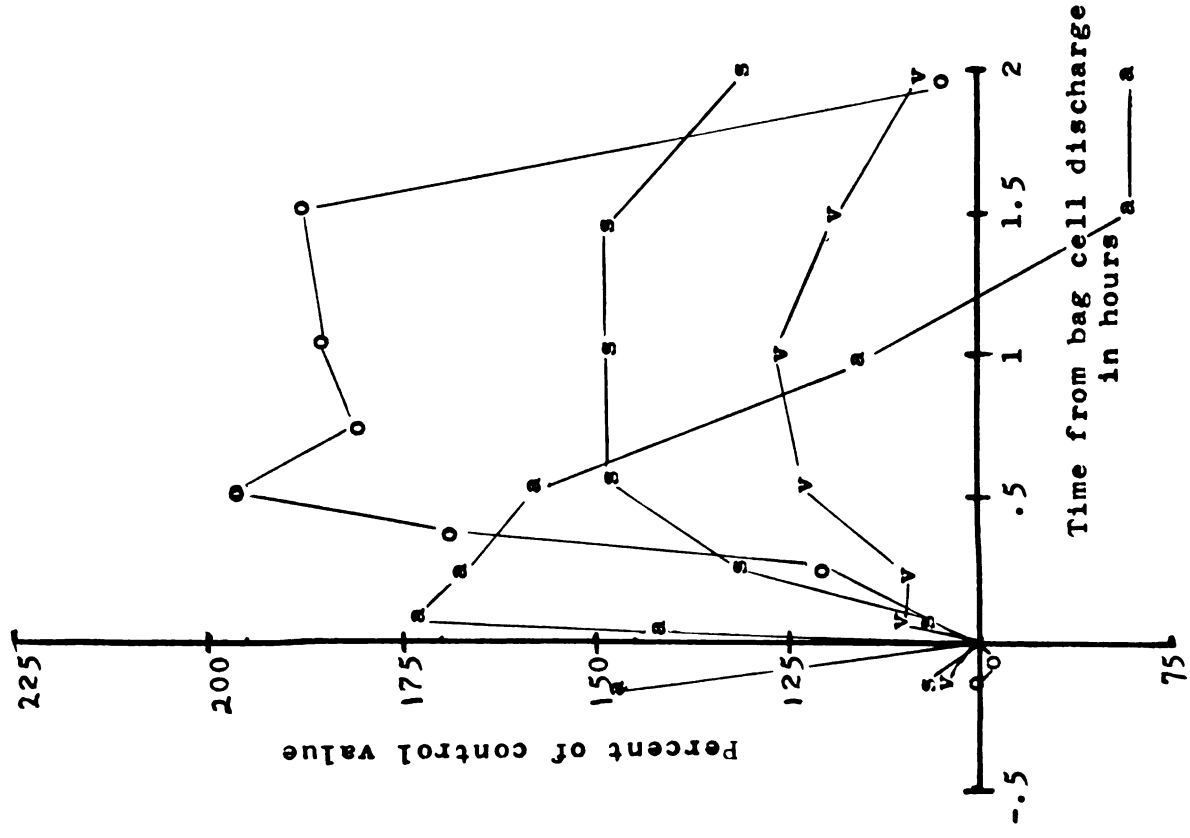
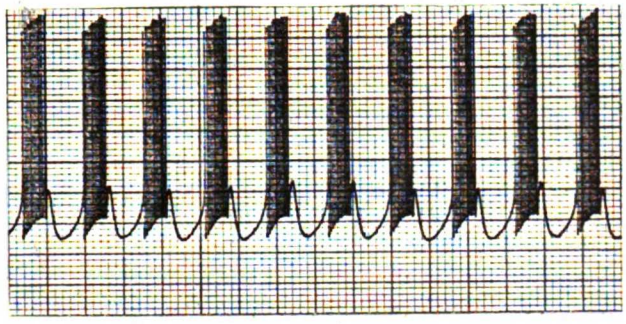
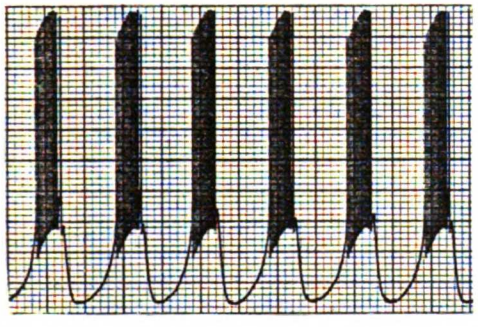


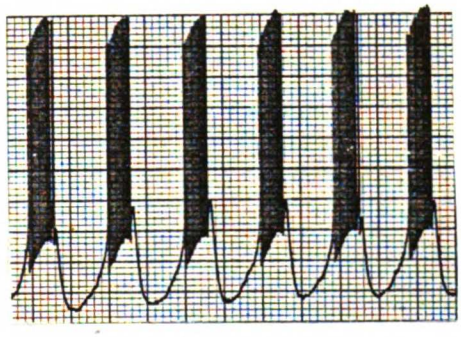
FIGURE 11 Response of August 31, 1974. As in previous figure, four burst properties are followed over time. In comparing the two graphs, note expanded time scale in this figure. As before, symbols are o-amplitude of slow oscillating potential, v-velocity of spiking during a burst, s-number of spikes in a burst and a-average spikes per minute. All values are given as percentages of their control value at time zero. Time zero in this case is the first spike of a spontaneous bag cell discharge. During this response there were several periods of sustained spiking (see figure 7) which are not shown as part of the spikes per burst (s) curve. And, during these episodes of spiking, the average spiking velocity was decreased by about fifty percent. In order to preserve the trend of increased velocity during actual burst conditions, these decreases are not included in the spike velocity (v) curve. There was no wash period this day.



Control S/B 14



Before HP S/B 22



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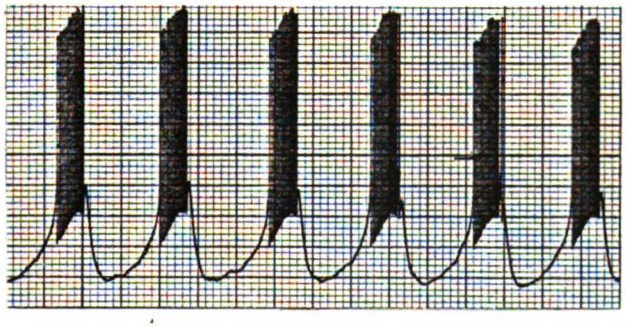
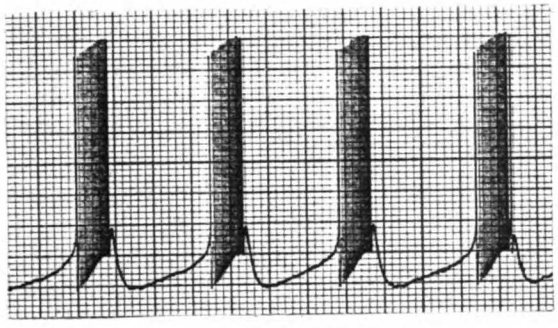


5 hours after bag cell discharge
S/B 24

FIGURE 12 Hyperpolarizing R15 will not reverse the bag cell response characteristics. Top line shows control R15 activity. Middle samples show R15 during bag cell response before and after eight minutes of 20 nAMP hyperpolarization. Spikes per burst are included under each record. Bottom line is R15 five hours after the bag cell discharge, illustrating that the response did not wear off this day (from February 17, 1975). Calibration is 20 mv, 15 seconds.



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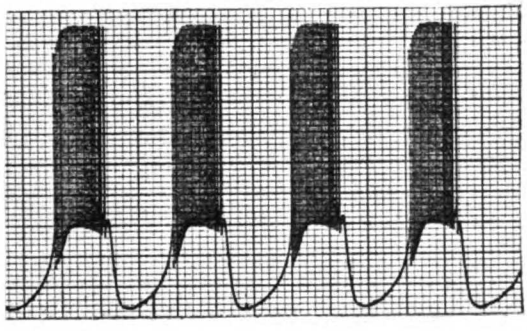
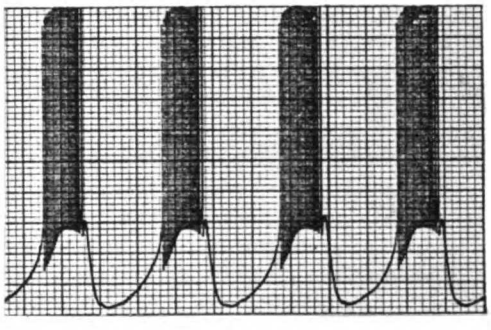


20 minutes post first bag cell discharge S/B 33



20 minutes post second bag cell discharge S/B 32

FIGURE 13 Failure of second bag cell discharge to enhance response. Top line shows R15 control sample. Second line shows R15 during response to a bag cell discharge and then one hour later after a second bag cell discharge was stimulated. No enhancement is apparent. (From December 6, 1974) Calibration is 20 mv, 15 seconds.

E. Variability in responses

While the six responses have much in common, there are, of course, differences to be accounted for. Some of these differences have already been discussed, others are apparent from table I.

As mentioned earlier, the timing of the burst oscillations does not change consistently in response to bag cell discharge. Perhaps timing is simply not affected by bag cell hormone, or it is possible that the inconsistency is introduced by experimental method. Although we try to use low voltage stimuli to activate the bag cells, we often excite the axons of the underlying connective and generate input to R15. Possibly the different stimulus amplitudes affect R15 burst timing by way of this input (41, 42). Note that in the two cases of spontaneous bag cell discharges, the burst timing changes were at least in the same direction (burst rate decreased). More experience with spontaneous discharges might confirm the trend.

Since we suspect that the bag cell effect on R15 is mediated by the bag cell hormone, it would be reasonable to expect that a dose-response relationship would account for much of the quantitative variability in the responses. Using table I to compare response strength with the length of time the bag cells fired does not reveal any relationship. The length of time of the discharge, however, is not necessarily a good indication of how much hormone is released during the burst. Some bag cell discharges have a greater spiking speed than others, so that the length of the burst may not reflect the number of spikes that were generated. And more importantly, there is evidence that the amount of hormone in the bag cells may vary by season (figure 5) and animal size (20, 57). Since we are working with a seasonal system, one might expect to see seasonal variation in the R15 response also (whether due to dose of hormone or ability to respond). In fact, the data collected so far would support this theory in that the

two best responses took place during the egg laying season, and responses have been more difficult since that time. Perhaps a dose-response relationship would be evident in a number of responses looked at in the same month of the year. Or, another way to approach the dose-response relation would be to add bag cell hormone in measured doses to the bathing medium. Since the hormone has not been isolated, bag cell extract (again, during a restricted time of year) would have to be used.

The past history of R15 might also be an important factor in determining response amplitude. For example, it is possible that the response becomes relatively refractory for many hours following egg laying.

F. Negative results

We have not seen the R15 response described above in every experiment we have tried. A seventh positive experiment which is consistent with the data reported here was not included because experimental manipulation preceding the bag cell discharge prevented establishing an adequate control period. And, there were three healthy preparations from which we elicited good bag cell discharges, but saw no changes in R15 bursting. All three of these negative cases occurred in February and March and in relatively small animals (170, 230 and 320 grams). It is possible that time of year and/or animal size could account for these failures. Further experience should reveal whether there is seasonal frequency of nonresponding animals. No other differences between these three animals and the animals giving positive responses were noted.

G. Evidence for hormonal mediation

At present there is no conclusive evidence for hormonal mediation of the changes in R15 burst behavior. Clearly, the bag cell discharge is implicated as the initiating event, but synaptic or indirect mediation cannot be excluded.

We feel that neural mediation is very unlikely for several reasons.

The latency and long duration of the response is more consistent with a hormonal action than a neural action. And, we have seen no evidence of unusual neural input that could explain the response (figure 15). Our electrical stimulation cannot be the initiating event because comparable stimulation without a bag cell discharge has never evoked the response (figure 9), and because two of the reported responses followed spontaneous bag cell bursts. It could be argued that the spontaneous bursts were initiated by some neural event, or that there are synaptic connections between the bag cells and R15, but the evidence does not support these explanations. Figure 14 shows a very high gain record of R15 during the first spikes in a spontaneous bag cell discharge. There is no visible neural input to R15 either directly from the bag cells or concurrent with their activation. And, if some neural event caused the bag cells to fire, we might expect to see unusual neural activity in other cells in the ganglion. No such activity has been seen in R15, the left upper quadrant cells or the RB cells we have monitored during spontaneous bursts.

The possibility that the bag cells cause the R15 changes indirectly via some other cell (either neural or hormonally mediated) will be investigated in later studies, but we have no reason to suspect this is the case.

We have two pieces of more direct experimental evidence that the effect we are studying is hormonal. We have been attempting to do an experiment where two ganglia are pinned in the same dish so that exciting the bag cells in one ganglion might stimulate a response in R15 in the other ganglion, thus establishing humoral mediation. While technical difficulties have delayed these results, we have had one partially successful experiment. R15 was not healthy, however, and after a small encouraging response, went on to die. In a second recent experiment, bag cell extract was added to a desheathed preparation and a small, longer latency response was observed.

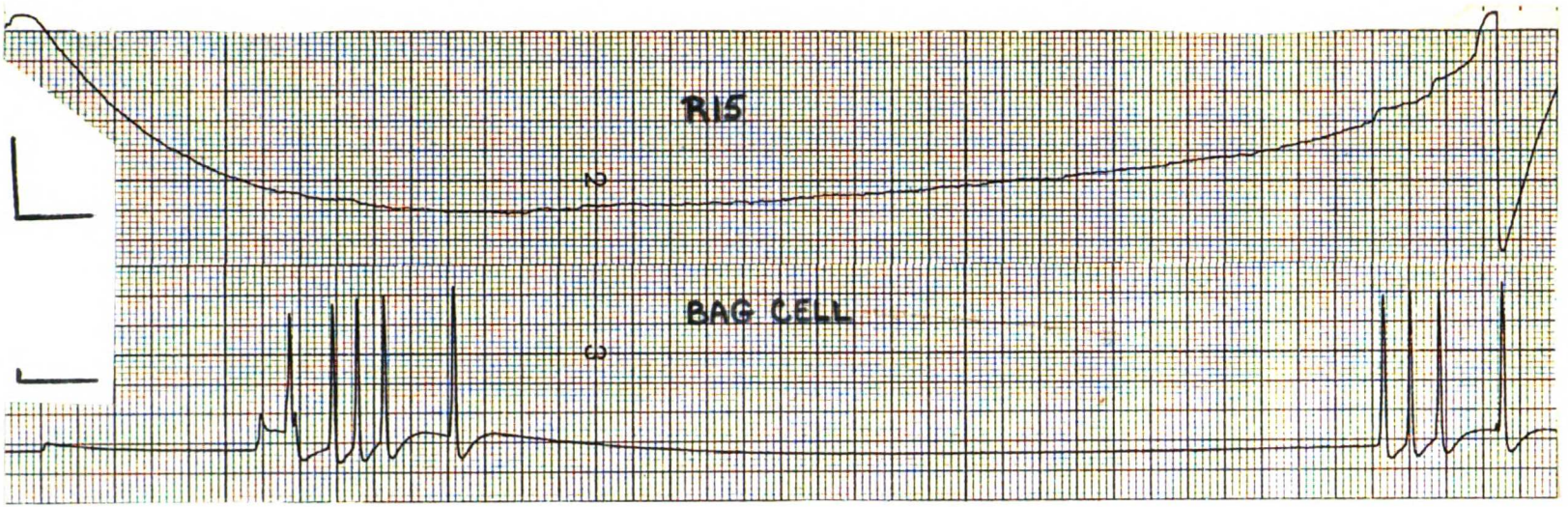


FIGURE 14 Simultaneous recordings of R15 and a bag cell during the first spikes of a spontaneous bag cell discharge. R15 is shown at high gain to demonstrate that no synaptic input to R15 is visible concurrent with the discharge. The two small epsps near the end of the record are from L10 and had been seen regularly during the control period. Calibration is 10 mv, 400 msecs.

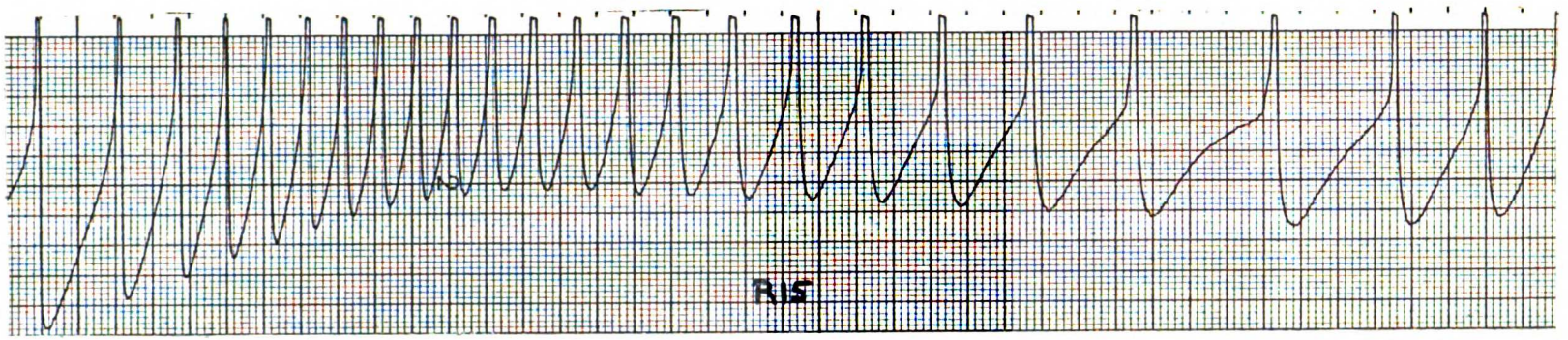


FIGURE 15 High gain record of R15 at the end of a burst that started a sustained spiking episode. There is no apparent excitatory input that would explain the prolonged spiking. Calibration is same as figure 14. Both records from August 31, 1974.

The slow potential increased 15% and the average spikes per minute increased 23% by one hour. All response characteristics returned to control levels within two hours. Efforts in this direction are continuing.

Finally, previous work supports our theory of hormonal mediation of the R15 response. As discussed earlier, stimulated release of a biologically active bag cell hormone has been demonstrated (34), and this hormone is likely to be a small polypeptide (2); Recently Barker et al (5) have shown that the small peptide hormones vasopressin and oxytocin can induce changes in R15 bursting similar to the bag cell response.

H. Response mechanisms

Although the goal for this series of experiments was simply to characterize the R15 response to bag cell activation, a few conclusions can be made about the mechanisms underlying the response.

The increase in hyperpolarizing excursion might at first appear to be a simple hyperpolarization of the cell, so that one might theorize that bag cell hormone just increases the resting potential of R15. Figure 16 points out the differences between the bag cell response and hyperpolarization. Notably, hyperpolarization alone does not increase the number of spikes in a burst but if anything will decrease the number. Imposed hyperpolarization does not lead to prolonged spiking episodes or increased spike velocity. And hyperpolarization consistently leads to an increase in interburst interval, which might not be true of the bag cell response. The slow potential increase, then is probably in both the hyperpolarizing and depolarizing directions. An explanation for the sustained spiking might be that the steady spiking episodes are caused by excitatory input. Figure 15 shows a high gain record of R15 at the end of a burst that started a sustained spiking episode, and no synaptic input is visible. In fact, it is interesting to look back at the sustained spiking shown in figure 8 to notice that the spiking still follows the usual burst pattern (speeding up

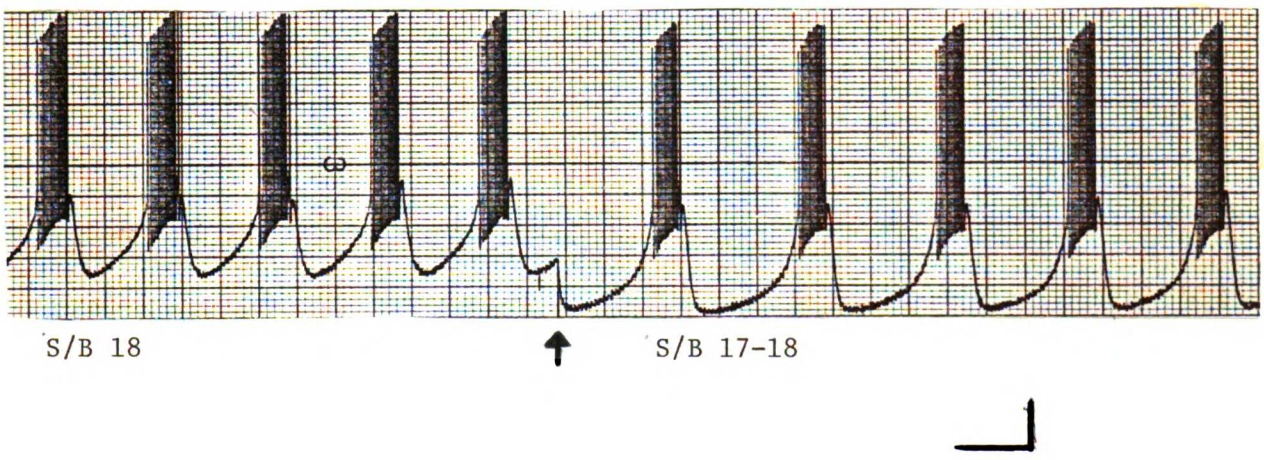


FIGURE 16 Effect of hyperpolarization on R15 burst properties. At arrow, 5 nAmps of hyperpolarizing current were passed to cause a 30% increase in the amplitude of the slow potential. (Cell was impaled with a double barreled microelectrode, one barrel passing current, the other recording). Simple hyperpolarization causes a decrease or no change in the number of spikes in a burst, no change in the spiking velocity during the burst, and an increase in the time between bursts (up 42% in this case). The action of bag cell hormone, then is not a simple change in membrane potential since the bag cell response is not consistent with the hyperpolarization response. In particular, hyperpolarization does not cause an increase in spikes per burst or spike velocity. Calibration is 20 mv, 10 seconds.

and slowing down of spikes for example) except that there are no silent periods. Something would seem to be preventing the usual large potassium current from taking over and effecting the silent phase.

The increased spiking velocity during the bursts of the response period is not due to a change in the spike duration, but in the interspike interval. This can be demonstrated even in the output shown in figure 10. Spike duration is not measureable in this figure, but the decrease in interspike interval is obvious.

A clue about conductance changes during the response was revealed in a before/after current-voltage relation study done on the February 12 response. More work needs to be done before these results can be presented, but our initial investigation shows a large conductance increase occurring after the bag cell discharge when current voltage relationship is examined at the beginning of a burst of spikes.

Some recent work in other labs may also be relevant to the bag cell response mechanisms. First, a report by Parnas (41) says that prolonged stimulation of excitatory input via the right connective interneuron (XIII) can cause long term effects on R15 bursting properties. We have not been able to duplicate his results as described in his paper, but we do not feel that the response he sees is comparable to the response we see. His response has some differences from ours, and most notably, he can reverse his effects by hyperpolarizing R15 for several minutes. Figure 12 demonstrates that strong hyperpolarization for 8 minutes has no effect on the bag cell response.

Barker and Gainer et al have done some studies on bursting cells which support and enhance our findings (4, 5, 26). These studies showed that normally silent neurons in the land snail and in Aplysia (the giant cell R2, see figure 3) start bursting when divalent cation concentrations are altered (4). And, when the peptide hormones vasopressin or oxytocin

are added to the bathing medium of isolated Aplysia or land snail ganglia (5), or when a specific protein fraction of head ganglia homogenate is added to the medium (26), the amplitude of the slow potential of R15 or land snail 'cell 11' (a cell which is homologous to R15 in many ways) becomes enhanced. When larger doses of hormone are added, R15 falls into steady depolarizations. The work with divalent cation concentrations shows that a certain range of Ca^{++} ions allows bursting behavior. Too little or too much calcium inhibits bursting. The calcium concentration changes needed to alter burst activity are not necessarily physiologic, but the effect is interesting when considering mechanisms of the hormone action. Barker et al's changes in burst behavior (4, 5, 7, 26) seem very similar to the bag cell effect on R15. His figures look much like ours and he reports that one to four hours are required to wash out the effects of the peptide hormones. We would hope that the bag cell effect on R15 is a physiologic example of the same effects Barker has seen with externally applied peptide hormones.

I. Other bag cell effects

During the same months that the experiments on R15 were conducted, other Aplysia neurons in the abdominal ganglion were studied for changes in burst behavior and/or synaptic potential size. Left upper quadrant cells, RB cells, R2, L10 and others have been observed under the same experimental conditions described earlier.

Preliminary evidence indicates that bag cell discharges change burst behavior in other bursters in addition to R15. L10 is usually a beating cell early in the day and may change to burst mode in the afternoon. Bag cell discharges seem to encourage L10 bursting. The left upper quadrant cells can fire in either beat or burst mode. In many preparations (especially in the summer) we have seen spontaneous bag cell discharges lead to hyperpolarization of left upper quadrant cells (unpublished data). This response has a short latency (within a half second) and may be restricted

to the specific left upper quadrant cell called L3 (20). The inhibition produces decreased burst activity for 15 to 30 minutes. Note that the response in this case is inhibitory, unlike the effects on R15 and perhaps L10. Recent studies indicate that the normally silent neuron R2 can be induced to burst under unphysiologic conditions (4, 13). We have not seen R2 burst in response to a bag cell discharge.

In addition to inducing changes in burst behavior, bag cell discharges probably induce increases in the size of synaptic potentials generated by the interneuron L10 (Mayeri and Simon, unpublished). This effect has primarily been found in the RB cells of the cardiovascular system circuitry. R15 receives a small (0.25 - 1.0 mv) excitatory input from L10; the EPSP is normally too small to influence R15 spiking. We have seen cases where the L10 EPSPs in R15 have increased following bag cell discharges. For example, during the August 31 experiment presented in this paper, the L10 EPSP rose from 0.5 mv to 1.5 mv and then fell to control level again during the hour and a half following the spontaneous bag cell discharge. An RB cell that was monitored simultaneously showed a similar increase over the same time course. L10 produces inhibitory synaptic potentials in the left upper quadrant cells (20) and these potentials have also been seen to increase, but more variably and over a longer time interval.

J. Future studies

Because this study is limited in scope, it is appropriate to summarize some of the work that still needs to be done on the bag cell - R15 system:

1. We are currently conducting experiments to decide whether the bag cell effects on R15 are in fact mediated by a hormone.

2. More responses during the egg laying season will need to be observed and seasonality of response evaluated. If the response is found to be seasonal, we can further characterize the features which vary over the year, and study dose-response relationships according to season.

3. A series of current-voltage graphs to determine conductance changes at various points in the burst cycle, and at various times during the response would allow a closer look at the ionic conductance changes that control enhancement of burst behavior.

4. Effects of oxytocin and vasopressin on R15 should be observed under our experimental conditions to better compare Barker and Gainer's work with ours, and to provide evidence that our response might be mediated by a bag cell polypeptide. Seasonal studies of the actions of vasopressin and oxytocin might reveal whether in fact there is a seasonal ability to respond to a given dose of hormone.

5. Since a polypeptide hormone might be involved, cyclic nucleotides could be involved in the response mechanisms. Bath application and microinjection of cAMP and cGMP are planned.

6. More work must be done to determine the functional role of the R15 response. For example, bag cell extract should be injected during egg laying season to determine if an animal gains weight (retains water) as well as lays eggs. This would imply that the excitement in R15 causes a physiologic response (36). Better mapping of R15 processes is necessary to understanding its functional significance.

7. Some data on bag cell discharge effects on other Aplysia burster cells has already been collected. Further analysis is warranted.

8. Further efforts will be made to correlate the information on bag cell induced changes in burst behavior with bag cell induced changes in L10 synaptic potentials.

V. SUMMARY

Activation of the Aplysia neurosecretory bag cells has been discovered to have effects on the nerve cells of the abdominal ganglion, and especially on the bursting neurosecretory cell R15. Within fifteen minutes of a spontaneous or electrically stimulated bag cell discharge, the slow potential governing R15 burst behavior becomes exaggerated and R15 occasionally slips out of burst mode into periods of sustained spiking. The number of spikes in each burst and the rate of spiking during the burst are greatly increased. The result is an overall large increase in R15 spiking activity for at least an hour following bag cell activation. There is evidence that this response may be hormonally mediated.

If hormonal mediation can be established, this simple invertebrate system allows, perhaps for the first time, an opportunity to study humoral communication between two types of neurosecretory cell under close to physiologic conditions. Behavioral implications of this communication and cellular mechanisms underlying the phenomenon are very approachable by virtue of the simplicity of the Aplysia preparation.

The cellular mechanisms of this response might have wide application to general problems of hormonal activation of neural circuitry and more particularly to effects of peptides on the nervous system. Applications to hormonal control of circadian rhythms or neuroendocrine releasing factors are also possible. Certainly the system provides further information on the nature and control of bursting neurons.

In the future, this laboratory will continue exploring the mechanisms mediating the communication between the bag cells and R15 and continue determining the role of these and other nerve cells in Aplysia egg laying behavior.

REFERENCES

1. Alving, B.O.: Spontaneous activity in isolated somata of Aplysia pacemaker neurons, J. Gen. Physiol. 51: 29, 1968.
2. Arch, S.: Polypeptide secretion from the isolated parietovisceral ganglion of Aplysia californica, J. Gen. Physiol. 59: 47, 1972.
3. Bailey, D.F. and Laverack, M.D.: Central nervous responses to chemical stimulation of a gastropod osphradium, Nature 200: 1122, 1963.
4. Barker, J.L. and Gainer, H.: Bursting pacemaker activity in a normally silent neuron, Brain Res. 65: 516, 1974.
5. Barker, J.L. and Gainer, H.: Peptide regulation of bursting pacemaker activity in a molluscan neurosecretory cell, Science 184: 1371, 1974.
6. Barker, J.L. and Gainer, H.: Studies on bursting pacemaker potential activity in molluscan neurons. I. Membrane properties and ionic contributions, Brain Res. 84: 461, 1975.
7. Barker, J.L. and Gainer, H.: Studies on bursting pacemaker potential activity in molluscan neurons. II. Regulation by divalent cations, Brain Res. 84: 479, 1975.
8. Brown, H.F.: Electrophysiological investigations of the heart of Squilla mantis, J. Exp. Biol. 41: 723, 1964.
9. Bullock, T.H. and Horridge, G.A.: Structure and function in the nervous system of invertebrates, San Francisco, 1965, W.H. Freeman and Company.
10. Chen, C.F., von Baumgarten, R. and Harth, O.: Metabolic aspects of the rhythmogenesis in Aplysia pacemaker neurons, Pflügers Arch. 345: 179, 1973.

11. Coggeshall, R.E.: A cytologic analysis of the bag cell control of egg laying in Aplysia, J. of Morphology 132: 461, 1970.

12. Coggeshall, R.E.: The muscle cells of the follicle of the ovotestis in Aplysia as the probable target organ for bag cell extract, Am. Zoologist 12: 521, 1972.

13. David, R.J., Wilson, W.A. and Escueta, A.V.: Voltage clamp analysis of pentylenetetrazol effects on Aplysia neurons, Brain Res. 67: 549, 1974.

14. Dickinson, A.: Pituitary neuropeptides and behavioural processes, Nature 253: 499, 1975.

15. Dyer, R.G. and Dyball, R.E.: Evidence for a direct effect of LRF and TRF on single unit activity in the rostral hypothalamus, Nature 252: 486, 1974.

16. Eales, N.B.: Aplysia, Liverpool Mar. Biol. Comm. Mem., no. 24, 1921, University Press.

17. Faber, D. and Klee, M.: Membrane characteristics of bursting pacemaker neurones in Aplysia, Nature New Biol. 240: 29, 1972.

18. Fingerman, M.: Endocrine mechanisms in marine invertebrates, Fed. Proc. 32: 2195, 1973.

19. Ford, D.H., editor, Influence of hormones on the nervous system, Basel, 1971, S. Karger.

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

21. Gainer, H. and Wollberg, Z.: Specific protein metabolism in identifiable neurons of Aplysia californica, J. Neurobiol. 5: 243, 1974.

22. Gerschenfeld, H.M.: Transmitters in invertebrate synapses, Physiol. Rev. 53: 1, 1973.

23. Greengard, P. and Kebabian, J.W.: Role of cAMP in synaptic transmission in the mammalian PNS, Fed. Proc. 33: 1059, 1974.

24. Hagadorn, Irvine R.: Neuroendocrine mechanisms in invertebrates, in Neuroendocrinology, Martini, L. and Ganong, W.F., editors, New York, 1967, Academic Press.

25. Harris, G.W., Michael, R.P. and Scott, P.P.: Neurological sites of action of stilbesterol in eliciting sexual behavior, In Symposium on the neurological basis of behavior, Wolstenholme, G.E.W. and O'Connor, C.M., editors, Boston, 1958, Little, Brown & CO.

26. Ifshin, M., Gainer, H. and Barker, J.: Peptide factor extracted from molluscan ganglia that modulates bursting pacemaker activity, Nature 254: 72, 1975.

27. Jahan-Parwar, Smith, M. and von Baumgarten, R.: Activation of neurosecretory cells in Aplysia by osphradial stimulation, Amer. J. Physiol. 216: 1246, 1969.

28. Kandel, E.R., Frazier, W.T., Waziri, R. and Coggeshall, R.E.: Direct and common connections among identified neurons in Aplysia, J. Neurophysiol. 30: 1352, 1967.

29. Kandel, E.R. and Kupfermann, I.: The functional organization of invertebrate ganglia, Ann. Rev. of Physiol. 33: 193, 1970.

30. Katz, B.: Nerve, muscle and synapse, New York, 1966, McGraw Hill.

31. Klee, M., Faber, D. and Heiss, W.: Strychnine- and pentylentetrazol-induced changes of excitability in Aplysia neurons, Science 179: 1133, 1973.

32. Konoshi, S. and Masanori, O.: Excitatory action of hypothalamic substance P on spinal motoneurones of newborn rats, Nature 252: 734, 1974.

33. Kupfermann, I. and Kandel, E.: Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of Aplysia, J. Neurophysiol. 33: 856, 1970.

34. Kupfermann, I.: Stimulation of egg laying by extracts of neuroendocrine cells (bag cells) of abdominal ganglion of Aplysia, J. Neurophysiol. 33: 877, 1970.

35. Kupfermann, I.: Studies on the neurosecretory control of egg laying in Aplysia, Am. Zoologist 32: 513, 1972.

36. Kupfermann, I. and Weiss, K.: Water balance regulation by the neurosecretory cell R15 in Aplysia californica, IRCS 2: 1695, 1974.

37. Libet, B.: Generation of slow inhibitory and excitatory postsynaptic potentials, Fed. Proc. 29: 1945, 1970.

38. Macginitie, G.E.: The egg laying activities of the sea hare, Tethys californicus, Biol. Bull, 67: 300, 1934.

39. Mayeri, E., Koester, J. and Liebeswar, G.: Functional organization of the neural control of circulation in Aplysia, Amer. Zool 14: 943, 1974.

40. Milburn, N.S. and Roeder, K.D.: Control of efferent activity in the cockroach terminal abdominal ganglion by extracts of corpora cardiaca, Gen. & Comp. Endocrinology 2: 70, 1962.

41. Parnas, I., Armstrong, D. and Strumwasser, F.: Prolonged excitatory and inhibitory synaptic modulation of a bursting pacemaker neuron, J. Neurophysiol. 37: 594, 1974.

42. Parnas, I. and Strumwasser, F.: Mechanisms of long-lasting inhibition of a bursting pacemaker neuron, J. Neurophysiol. 37: 609, 1974.

43. Peretz, B. and Moller, R.: Control of habituation of the withdrawal reflex by the gill ganglion in Aplysia, J. Neurobiol. 5: 191, 1974.

44. Pfaff, D.W.: Luteinizing hormone-releasing factor potentiates lardosis behavior in hypophysectomized ovariectomized female rats, Science 182: 1148, 1973.

45. Prosser, C.L.: Comparative Animal Physiology, Philadelphia, London, Toronto, 1973, W.B. Saunders Co.

46. Rojakovick, A.S. and March, R.B.: The activation and inhibition of adenyl cyclase from the brain of Madagascar Cockroach, Comp. Biochem. & Physio. 43: 1B, 1972.

47. Schwartz, J.H., Castelucci, V.F. and Kandel, E.R.: Functioning of identified neurons and synapses in abdominal ganglion of Aplysia in absence of protein synthesis, J. Neurophysiol. 34: 939, 1971.

48. Smith, T.G., Barker, J.L. and Gainer, H.: Requirements for bursting pacemaker potential activity in molluscan neurons, Nature 253: 450, 1975.

49. Steiner, F.A., Ruf, K. and Akert, K.: Steroid sensitive neurons in rat brain, anatomical localization and response to neurohumours and ACTH, Brain Res. 12: 74, 1969.

50. Stinnakre, J. and Tauc, L.: Central neuronal response to the activation of osmoreceptors in the osphradium of Aplysia, J. Exp. Biol. 51: 347, 1969.

51. Strumwasser, F.: Types of information stored in single neurons, in Invertebrate Nervous Systems, Wiersma, C.A.G., editor, Chicago, 1966.

52. Strumwasser, F.: Membrane and intracellular mechanisms governing endogenous activity in neurons; In Physiological and Biochemical Aspects of Nervous Integration, Francis D. Carlson, ed., Englewood Cliffs, N.J., Prentice Hall Inc., 1968.

53. Strumwasser, F., Jacklet, J. and Alvarez, R.: A seasonal rhythm in the neural extract induction of behavioral egg-laying in Aplysia, Comp. Biochem. Physiol. 29: 197, 1969.

54. Tauc, L.: Transmission in invertebrate and vertebrate ganglia, Physiol. Rev. 47: 521, 1967.

55. Thompson, T.E. and Bebbington, A.: Structure and function of the reproductive organs of three species of Aplysia, Malacologia 7 (2-3): 347, 1969.

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

57. Toevs, L.A.: Doctoral thesis, California Institute of Technology, Pasadena, Calif., 1970.

58. Truman, J.W.: How moths 'turn on': A study of the action of hormones on the nervous system, Amer. Sci. 61: 700, 1973a.

59. Woodbury, D.M. and Vernadakis, A.: Influence of hormones on brain activity, in Neuroendocrinology, Martini, L. and Ganong, W.F., editors, New York, 1967, Academic Press.

60. Yano, S., Kumagai, A. and Yamamura, Y.: Inhibitory effect of oxytocin upon catecholamine action on smooth muscle, in Symposium on Neuroendocrinology, Hokkaido Univ School of Medicine, 1967, Buneido Co. Ltd., Sapporo.