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Neurotransmitter modulation, phosphodiesterase inhibitor effects, and cyclic AMP correlates of afterdischarge in peptidergic neurites

(*Aplysia*/bag cell neurons/serotonin/dopamine/plasticity)

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ABSTRACT The neuroendocrine bag cells in the abdominal ganglion of *Aplysia* generate a long-lasting synchronous afterdischarge upon brief stimulation of an afferent pathway. After this afterdischarge the cells become refractory to further synaptic stimulation. We find that synchrony, afterdischarge, and prolonged refractoriness are properties that can be expressed in the isolated asomatic neurites of the bag cells. We have distinguished two independent types of refractoriness. The first (type I) is seen as a failure of action potentials generated in the tips of bag cell neurites to invade cell somata. The second form of refractoriness (type II) controls the duration of afterdischarge such that stimuli after the first afterdischarge produce only very short afterdischarges or fail to elicit an afterdischarge. Type II refractoriness is sensitive to serotonin and certain of its analogues, and to dopamine and the methylxanthine phosphodiesterase inhibitors. Extracellularly applied serotonin suppresses an ongoing afterdischarge while dopamine and the phosphodiesterase inhibitors, when applied at the end of the first afterdischarge, generate a subsequent afterdischarge of long duration without further electrical stimulation. None of these compounds influenced the degree of type I refractoriness. We have shown that both serotonin and dopamine stimulate the formation of cyclic AMP in the bag cell clusters and in the pleurovisceral connectives and that the occurrence of an afterdischarge is associated with a specific increase in total cyclic AMP in bag cell bodies. Moreover, afterdischarges can be generated in unstimulated preparations by extracellular application of the cyclic AMP analogues, 8-benzylthio-cyclic AMP or 8-methylthio-cyclic AMP. Our data suggest that serotonin and/or dopamine may control bag cell activity and that activation of adenylate cyclase is linked to bag cell afterdischarge.

Of special interest to neurobiologists are those cells and synapses where long-lasting changes in excitability take place, particularly when these changes can be related to some aspect of an animal's behavior. The experimental systems in which such changes can be studied with ease are few, and frequently they confound the experimentalist with a complex network of different cell types making it difficult to isolate those individual synapses where the plastic changes occur. Using pharmacological and electrophysiological techniques, we have studied the polypeptide-secreting bag cell neurons from the abdominal ganglion of *Aplysia californica*. These neurons play an important role in egg-laying behavior and display interesting long-term changes of excitability during and after activity. They offer the advantage of relatively simple organization and are morphologically isolated from other neurons in the ganglion.

The neuroendocrine bag cells comprise a cluster of about 400 polypeptide-secreting neurons at the base of each pleurovisceral connective nerve (1, 2). The processes of these cells contain moderately dense core granules (~2000 Å) and extend along the connective tissue of the connective nerves (Fig. 1*A*). Morphological evidence has shown that all bag cell processes terminate within the caudal half of the connectives (1), while electrophysiological studies suggest that the majority of processes terminate within 5.0 mm of the bag cell clusters (3). There is direct evidence in *A. dactylomela* that these processes are electrically coupled, which presumably accounts for the synchrony of their action potentials (4). The granules are thought to contain the polypeptide egg laying hormone, which is synthesized in the cell bodies and is then transported up the processes (2, 5–10). Release of the hormone takes place during a prolonged afterdischarge of synchronous action potentials which precedes the act of egg laying *in vivo* (11). Such afterdischarges may be triggered *in vitro* by electrical stimulation of either the rostral end of a pleurovisceral connective or of a pleurocerebral connective. (An example of an afterdischarge may be seen in Fig. 1*B*.) After their discharge the cells become refractory to further stimulation for up to several hours (12).

In this paper we distinguish two, apparently independent, mechanisms that control the sensitivity of bag cells to afferent stimuli. One of the mechanisms controls the spatial propagation of bag cell action potentials and has previously been described by Dudek and Blankenship (13, 14). The other mechanism determines whether or not the cells will afterdischarge. We have shown that serotonin and dopamine have suppressive and facilitatory effects, respectively, on bag cell afterdischarge and that refractoriness to afterdischarge may be abolished by certain xanthine derivatives. In unstimulated preparations, afterdischarges may be initiated by extracellular addition of cyclic AMP (cAMP) analogues. We have also shown that a specific increase in cAMP accompanies an afterdischarge and have determined its time course.

MATERIALS AND METHODS

A. californica were obtained from Pacific Bio-Marine Laboratories, Inc. (Venice, CA). The animals were kept at 14°, and all electrophysiological experiments were carried out at this temperature. The pleurovisceral connectives that join the abdominal ganglion to the pleural ganglia were cut close to the pleural ganglia. Abdominal ganglia were then dissected out with the entire length of these connectives and placed in a recording chamber containing fresh filtered seawater or artificial medium (460 mM NaCl/10.4 mM KCl/11.0 mM $CaCl_2$/55.0 mM $MgCl_2$/10 mM Tris·HCl, pH 7.8). A suction electrode for stimulation was placed at the pleural end of the right connective. Bag cells could also be stimulated by positioning the electrode on the pleurocerebral connective when this was included in the preparation. Two recording suction electrodes were positioned over each side of the ganglion, one over the bag cell bodies and the other a short distance (~4 mm) from the bodies along the pleurovisceral connectives. To produce isolated asomatic neurite preparations, we dissected the abdominal

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Abbreviation: cAMP, cyclic AMP.

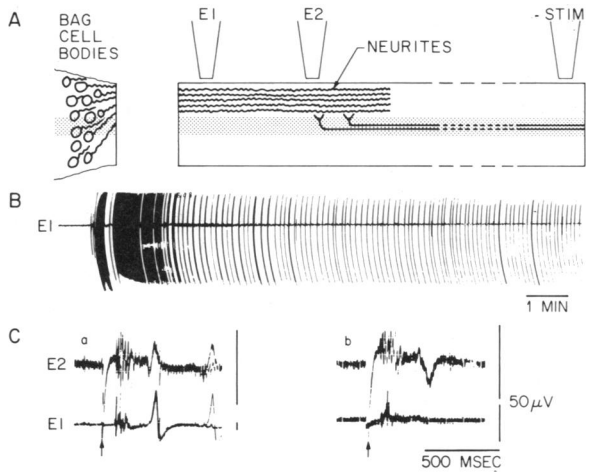

FIG. 1. (*A*) Schematic representation of the isolated pleurovisceral connective preparation. Bag cell neurites are depicted as traveling in the connective tissue (1), where they receive an afferent input from the head ganglia. Suction electrode E1 was placed at the abdominal end of the connective and E2 was situated about 4 mm from E1. (*B*) Onset of afterdischarge in an isolated connective preparation. The afterdischarge was triggered by a brief stimulus train to the pleural end of the connective (20 V, 2.5 msec, 6 Hz, 15 sec). Positive is up in this and all subsequent tracings. (*C*) Spontaneous onset of type I refractoriness during an experiment. The first pair of superimposed tracings (*a*) shows the response of the neurites to stimuli (30 V, 2.5 msec) at the start of the experiment. Stimuli are marked by an arrow and are followed by short-latency, low-threshold multiphasic responses that are unrelated to bag cell activity (judged by intracellular recordings from bag cells in intact ganglion preparations). The first bag cell response is seen on E2 with a latency of 250 msec followed by a larger response on E1. One hour later, after multiple stimuli and afterdischarge, the neurites at E2 may still be activated by stimuli, while no response can be recorded at E1, as seen in *b*.

ganglion with the bag cell somata away from the connectives close to the rostral border of the bag cell clusters and positioned the recording electrodes as in Fig. 1*A*. Since these cells and neurites discharge synchronously, external electrodes record compound action potentials. Each such event probably represents the nearly simultaneous firing of a few hundred bag cell neurites (3, 12).

All pharmacological agents were made up in fresh filtered seawater or artificial medium at pH 7.8. To achieve the final concentrations of agents given in this paper, we added 100–500 μl of a more concentrated solution directly to the extracellular medium (8 ml).

For determination of the effects of serotonin and dopamine on cAMP levels, abdominal ganglia, with the entire length of each pleurovisceral connective attached, were incubated with 1 mM theophylline and 0.2 mM serotonin or dopamine for 5 min at 14°. The region of the bag cell bodies was then rapidly removed and the connectives were dissected into two equal halves. For determination of cAMP levels during an afterdischarge, ganglia were incubated with 1 mM theophylline starting 2 min before electrical stimulation. At various times after the onset of afterdischarge, the region of the bag cell bodies and the proximal (neurite containing) and distal (stimulated) halves of the connectives were again rapidly removed. These were homogenized in 6% trichloroacetic acid at 0°. cAMP concentrations were determined by radioimmunoassay (15). Our preliminary data with crude homogenates showed that exogenously added phosphodiesterase significantly accelerated the degradation of assayable material. Measurements of cAMP in the region of the bag cell bodies showed that total cAMP levels correlated well with the sexual maturity of the animal, as indexed by the weight of the reproductive tract. cAMP levels rose approximately linearly from a mean value of 6.9 pmol/bag cell cluster for animals with 0.5-g reproductive tracts to 35.0 pmol/bag cell cluster for those with 1.4-g reproductive tracts. All experimental and control animals were therefore carefully matched for reproductive tract weight.

RESULTS

We have found that the control of bag cell excitability is located in the neurites of the connective tissue and may be studied in a preparation in which the cell bodies have been cut away leaving the isolated pleurovisceral connective (Fig. 1*A*). Stimulation of the pleural end of a freshly dissected connective may induce a long-lasting afterdischarge in the isolated neurites that is in all respects similar to that recorded with the ganglion intact except that the interactions that can arise between left and right bag cell clusters are eliminated (4) (Fig. 1*B*). As seen in preparations including bag cell somata (3), the compound action potentials evoked by stimuli to the pleural end of the connective as well as the spontaneous potentials during the afterdischarge travel from the distal tips of the neurities towards the abdominal end of the connective (Fig. 1*C*).

The bag cells appear to use two mechanisms to control their excitability and, hence, the amount of hormone that is secreted. Both mechanisms result in a form of refractoriness that sets in after the first afterdischarge of a freshly dissected preparation, and in both cases recovery occurs spontaneously after several hours. The first (type I refractoriness) controls the spatial propagation of action potentials once they have been initiated close to the presumed point of synaptic input. The second (type II refractoriness) controls the duration of time for which the neurites will afterdischarge. Both processes could be observed in both isolated connective and intact ganglion preparations.

Type I Refractoriness. This is seen as a diminution of the extracellularly recorded response at the abdominal (proximal) end of the connective. Stimuli delivered to the bag cell input after the first or second afterdischarge of a freshly dissected preparation will frequently fail to elicit any response at the proximal end of the neurites (3, 13). The response 4 mm up the connective, however, remains unchanged in amplitude although its shape is often altered to give a much larger negative component (Fig. 1*C*). This suggests the presence of a local active current sink at the tips of the neurites when action potentials fail to propagate towards the cell bodies. Although the onset of this block occurred at variable times during or after the first afterdischarge of a freshly dissected preparation, no correlation could be observed between this propagation-block and the ability to afterdischarge. None of the pharmacological agents used in this study could either induce this type of block prematurely or restore propagation towards bag cell somata once type I refractoriness had set in. Spontaneous recovery usually followed after several hours.

Type II Refractoriness. This second form of refractoriness concerns the ability of a stimulus to elicit an afterdischarge. A previously unstimulated preparation will discharge for a mean duration of 30 min after brief repetitive stimulation of the pleural end of the connective. Following this first afterdischarge, subsequent afterdischarges of shorter duration (2–8 min) can sometimes be produced by more intense tetanic stimulation (see Fig. 4, 2nd afterdischarge). After several such short discharges have been produced, further stimulation generally cannot produce any afterdischarge at all. At this time, the ability to elicit a short afterdischarge in the distal tips of the neurites can be partially restored by replacing the extracellular medium by fresh medium prior to stimulation. Although these afterdischarges are very short [mean duration = 4.3 ± 1.0 min (SEM), $N = 8$], they suggest that a buildup of an extracellular

substance may be a minor component of type II refractoriness.

Profound effects on the duration of afterdischarge could be obtained with serotonin (0.125 μM) as well as with tryptamine (625 μM), bufotenine (18 μM), and ergonovine (120 μM). When added to the existing medium at any time during the first afterdischarge, these agents terminated the afterdischarge (Fig. 2*B*). Subsequent stimulation was incapable of producing an afterdischarge, although apparently normal bag cell responses after each stimulus pulse could usually still be seen with serotonin. These suppressive effects could be reversed by rinsing with fresh medium, after which repetitive stimulation could again produce afterdischarges of short duration.

In contrast to the effects of serotonin analogues on type II refractoriness, 0.12 mM dopamine has facilitatory effects on bag cell afterdischarge. When applied during an afterdischarge, dopamine provided a transitory increase in the frequency of firing as well as a small increase in the amplitude of the extracellularly recorded compound action potentials. Much more dramatic, however, were the effects of dopamine when applied to the medium within 1 min of the spontaneous termination of an afterdischarge. After a delay of 40 sec or more, the afterdischarge was restored without electrical stimulation and continued for many minutes, frequently for considerably longer than the duration of the first afterdischarge (Fig. 2*A*). If, however, dopamine was introduced more than 2 min after the end of an afterdischarge, this restorative effect could not be seen. Dopamine, itself, was incapable of initiating an afterdischarge in fresh preparations.

The above results suggest that serotonin and/or dopamine may have a role in controlling the excitability of bag cells. Clearly, further criteria need to be met before these compounds can be definitively established as endogenous transmitters. We have found that the presumed afferent input to the bag cells, activated by stimulation of the pleural end of the connectives, is unaffected by the dopamine antagonist, haloperidol (12.5 μM), as well as by *d*-tubocurarine (625 μM), hexamethonium (625 μM), and atropine (625 μM). A complete block of the stimulus evoked response could, however, be obtained with strychnine (625 μM) and, also, occasionally with bufotenine (18 μM, three out of five experiments) and with tryptamine (625 μM, three out of twelve experiments). In these experiments the blockage was readily reversed by rinsing and could be repeated a number of times.

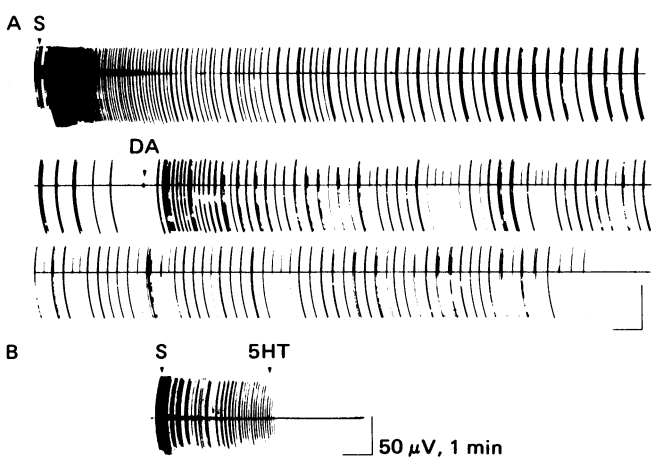

FIG. 2. (*A*) Effect of dopamine (0.12 mM) when introduced in the extracellular medium at the end of the first bag cell afterdischarge in a previously unstimulated intact abdominal ganglion. Dopamine (DA), added at the end of the first afterdischarge, after a latency of about 40 sec generates a subsequent afterdischarge of long duration. (*B*) Effect of serotonin on bag cell cluster afterdischarge. At the arrow marked 5HT, serotonin (1.2 mM) was introduced in the extracellular medium, causing an abrupt termination of afterdischarge. The threshold for this effect was found to be 10^{-7} M serotonin, although at lower concentrations the delay between bath application and the end of the afterdischarge increased to about 1 min. S, stimulation.

Changes in cAMP Levels. Serotonin and dopamine as well as electrical stimulation have been shown to increase the formation of cAMP in *Aplysia* abdominal ganglia and in the pleurovisceral connectives (16–18). We decided therefore to investigate how changes in the adenylate cyclase system might be linked to bag cell activity. The effects of a 5-min incubation with (0.2 mM) serotonin or dopamine on cAMP levels are shown in Table 1. This table gives data for ganglia from animals with reproductive tract weights between 0.5 and 0.6 g. Both serotonin and dopamine markedly increased cAMP levels in the region of the bag cell bodies and in both the proximal and distal halves of the pleurovisceral connectives. The effect of dopamine at the bag cell bodies was, however, rather variable and therefore not statistically significant ($P < 0.1$). Our data confirm the results of Cedar and Schwartz (17) on the effects of these compounds on cAMP levels in the abdominal ganglion and extend them to the bag cells themselves.

Using juveniles with a mean body weight of 110 ± 40 (SD) g [mean reproductive tract weight, 0.98 ± 0.56 (SD) g, $N = 30$], we also measured cAMP levels in connectives and in the region of bag cell bodies before and after electrical stimulation of the distal connective. This distribution of body and reproductive tract weights provided a population of animals that was on the borderline of sexual maturity (6). Although in most cases bag cells could be made to fire by stimulation of the distal end of the pleurovisceral connectives, not all were able to provide an afterdischarge. cAMP levels were measured at 2 min after the end of electrical stimulation. Stimulus trains that evoked bag cell firing but did not result in a sustained afterdischarge gave rise to increases in cAMP concentrations in connectives but not in the bag cell bodies (Table 2). In those cases where similar stimulus trains did evoke an afterdischarge, however, there was a significant increase ($P < 0.025$) in the region of the bag cell bodies and a further small increase in the proximal half of the connective that contains the bag cell neurites (not significant, $P < 0.2$). Moreover, in three cases in which the afterdischarge was confined to one cluster only, bag cell clusters from the same ganglion could be compared. Mean cAMP levels were 3.1 times greater in the afterdischarging cluster ($P < 0.025$). This increase in cAMP is therefore specific to the generation of a bag cell afterdischarge.

An estimate of the time course of the cAMP change during an afterdischarge was made by measuring cAMP levels in the

Table 1. Effect of incubation with serotonin or dopamine on cAMP levels

	Bag cell bodies	Connectives	
		Proximal	Distal
Control	6.9	5.7	7.5
Serotonin	42.3	61.5	62.7
Dopamine	23.9	37.5	37.5

All experiments were at 14° in the presence of 1 mM theophylline. Experimental ganglia were incubated for 5 min with 0.2 mM serotonin or dopamine, after which they were dissected and assayed for cAMP. Results are in pmol of cAMP and are the mean of two to five experiments. The increase in cAMP in the region of the bag cell bodies after serotonin treatment is significant at $P < 0.01$ ($N = 3$). The effect of dopamine at the bag cell bodies was rather variable and is not statistically significant ($P < 0.1$, $N = 5$). The increase in cAMP levels in the proximal and distal connectives, after serotonin or dopamine treatment, are all significant at, at least, $P < 0.05$. All data are from animals with reproductive tract weights of 0.5–0.6 g.

Table 2. Effect of afterdischarge on cAMP levels

	Bag cell bodies	Connectives	
		Proximal	Distal
Control	27.3	10.3	15.7
Electrical stimulation, no afterdischarge	22.0	43.8	53.3
Electrical stimulation, afterdischarge (2 min)	63.7	61.5	55.1

All experiments were at 14° in the presence of 1 mM theophylline. Experimental ganglia were stimulated at the distal end of the pleurovisceral connectives for up to 40 sec (20 V, 2.5 msec, 6 Hz via a suction electrode) or until an afterdischarge was generated. After 2 min the bag cell bodies were cut away and the connectives were cut into proximal and distal halves for the determination of cAMP levels by radioimmunoassay. Results are in pmol of cAMP and are the mean of four to nine experiments. The increase in cAMP at the bag cell bodies during an afterdischarge is significant at $P < 0.025$. The mean reproductive tract weights for the three groups were: controls, 0.81 g; stimulation with no afterdischarge, 1.07 g; stimulation with afterdischarge, 0.92 g.

region of the bag cell bodies at various times following the onset of afterdischarge (Fig. 3). Data points for this figure were expressed as percent change relative to the control cAMP level for animals of the same reproductive tract weight. The concentration of cAMP in the bag cell clusters appears to reach a maximum about 2 min after the onset of afterdischarge and thereafter to decline to control levels.

cAMP Analogues and Methylxanthines. We could detect no effects of extracellularly applied cAMP or of dibutyryl-cAMP either on the amplitude of bag cell spikes or on the duration of the afterdischarge. The cAMP analogues, 8-benzylthio-cAMP and 8-methylthio-cAMP (0.5 mM) were, however, capable of initiating a bag cell afterdischarge in a previously unstimulated ganglion or isolated neurite preparation. Such 8-substituted derivatives are phosphodiesterase-resistant (19), and 8-benzylthio-cAMP has been shown to be effective on the nervous system of *Aplysia* (20). After addition of either analogue to the extracellular medium, bag cell afterdischarges, apparently similar in all respects to those triggered by electrical stimulation, started spontaneously after a delay of between 2.5 and 25 min.

The methylxanthines theophylline (1.25 mM), isobutylmethylxanthine (1.25 mM), and caffeine (1.25 mM) also had profound effects on afterdischarge. At these concentrations, these compounds are potent inhibitors of phosphodiesterase (21). Their effects appeared very similar to those of dopamine. When applied to the medium within 1 min of the end of the

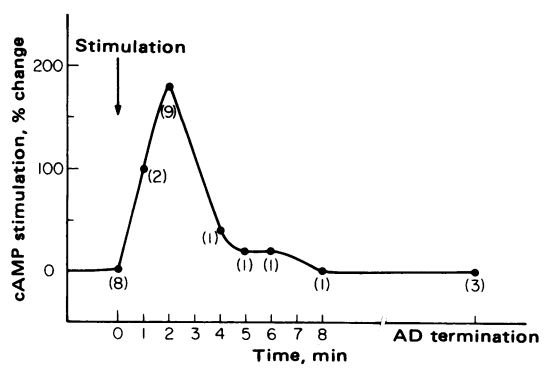

FIG. 3. Time course of change in cAMP levels of the bag cell clusters following the onset of afterdischarge. The percent change in cAMP levels is expressed relative to control levels for bag cell clusters from animals with the same reproductive tract weight. The number of determinations at each time point is given in parentheses. AD, afterdischarge.

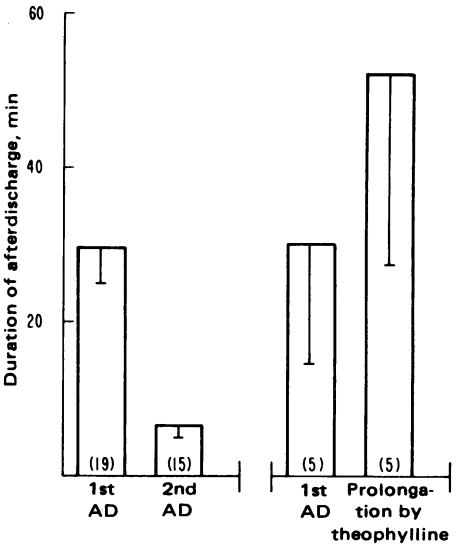

FIG. 4. Duration of afterdischarges in isolated connective preparations. The first two histograms give the result of experiments in which an afterdischarge (1st AD) was triggered by a stimulus train to the distal end of a previously unstimulated connective. Within 10 min of the termination of this first afterdischarge, a second stimulus train (20 V, 2.5 msec, 6 Hz, 15 sec) was given which produced much shorter afterdischarges (2nd AD). The third and fourth histograms show the result of experiments in which only the first afterdischarge (1st AD) was triggered by a stimulus train and in which 1.2 mM theophylline was introduced to the extracellular medium within 60 sec of the spontaneous termination of the first afterdischarge. The fourth histogram gives the mean duration of bag cell firing from the time of theophylline introduction to termination of this "second afterdischarge." Bars show SEM; number of experiments is shown in parentheses.

first afterdischarge, these compounds restored the afterdischarge without any electrical stimulation and generally maintained it for a duration longer than that of the first afterdischarge (Fig. 4). Note that the mean duration of the theophylline-restored afterdischarges was 7.5 times longer than those which could be generated by electrical stimulation at this time. Another phosphodiesterase inhibitor, papaverine (0.125 mM) also had similar effects, although these were not seen in all experiments. These effects were independent of the degree of type I refractoriness. If a block of propagation towards the bag cell somata had already set in by the end of the first afterdischarge, these compounds would restore the afterdischarge to the distal tips of the bag cell neurites only (electrode position E2, Fig. 1 *A* and *B*). They were, by themselves, however, incapable of initiating an afterdischarge.

DISCUSSION

We have shown that the bag cells use two independent processes (spatial and temporal) which diminish their sensitivity to afferent stimuli following a long-lasting afterdischarge. Both processes occur within the neurites of these cells at a distance from the cell bodies and may be observed and studied in an asomatic preparation. These processes may be designed to limit the amount of egg laying hormone that can be released from the neurites.

Our work suggests roles for serotonin and dopamine or closely related compounds in the control of bag cell activity but does not provide definitive evidence that these are the actual transmitters in this system. Invertebrate neurons frequently possess multiple receptors, with mixed excitatory and inhibitory mechanisms, for the same transmitter and receptors may be

differentially distributed between the cell soma and neurite regions of a cell (22, 23). Thus, the method of bath application cannot determine whether these compounds would act as excitatory or inhibitory when applied focally, as by an iontophoretic pulse (23). In fact, our preliminary experiments with iontophoresis on dissociated bag cell somata in cell culture indicate serotonin has a slow depolarizing and excitatory action (24), although its effect on bag cell neurites remains to be tested.

The bag cell afterdischarge is closely correlated with a stimulation of cAMP synthesis. The results with the cAMP analogues suggest that this increase in cAMP is related to the genesis of the afterdischarge. It is also possible that the stimulation of cAMP formation has important effects that are less directly related to afterdischarge; e.g., mediating metabolic changes such as increased hormonal synthesis. The results with the phosphodiesterase inhibitors provide the hypothesis that the termination of an afterdischarge may result from increased phosphodiesterase activity. This increase of phosphodiesterase activity may also prevent cAMP concentrations from rising to levels that may generate or facilitate an afterdischarge upon subsequent stimulation (type II refractoriness). Two points about these interpretations deserve comment. First, it is puzzling that serotonin and dopamine, which both elevate cAMP levels in the region of the bag cells, had opposing affects on afterdischarge. The bag cell body region is not entirely free of neuropile, axons of passage, and glia. Elevated cAMP levels may therefore reflect, at least in part, the stimulation of these components by serotonin or dopamine, or even, during an afterdischarge, by the released egg laying hormone. Second, the methylxanthines are not entirely specific as phosphodiesterase inhibitors and may be acting on some other aspects of the cells metabolism, such as calcium binding and sequestration. Their effects also appear very rapid in onset compared with changes in the electrical activity of other *Aplysia* neurons induced by phosphodiesterase inhibitors (20), warning against an exclusive interpretation in terms of an intracellular inhibition of phosphodiesterase.

The simplicity of the bag cell system, compared with other systems where long-term changes in neuronal excitability take place, offers the hope that further studies will provide an unambiguous explanation of these mechanisms and of the role of cAMP.

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