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# Peptidergic Neurons of *Aplysia* Lose Their Response to Cyclic Adenosine 3′:5′-Monophosphate during a Prolonged Refractory Period[1]

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## Abstract

**Although the peptidergic bag cell neurons of *Aplysia* are ordinarily silent, they respond to brief electrical stimulation by producing an afterdischarge of about 30 min duration. This afterdischarge is followed by a refractory period lasting many hours during which electrical stimulation either fails to initiate afterdischarges or produces discharges of much shorter duration. Previous work has demonstrated that cyclic AMP plays a role in the genesis of afterdischarge, both in intact bag cell clusters and in isolated cultured bag cells. We have now examined the hypothesis that in the refractory period either the synthesis of cyclic AMP or the response to cyclic AMP is attenuated.**

**Direct measurements of cyclic AMP showed that cyclic AMP levels in the bag cell neurons are elevated to a similar extent after stimulation in refractory and nonrefractory clusters of neurons. We have found, however, that the response to cyclic AMP is altered during the refractory period. The electrophysiological responses of bag cell neurons were first examined in intact clusters of cells within the abdominal ganglion. Cyclic AMP levels were elevated using the adenylate cyclase activator, forskolin, in the presence of theophylline (FT). The duration of a first bag cell afterdischarge could be greatly increased if FT was added before stimulation. The duration of a stimulated second bag cell afterdischarge could also be significantly increased if FT was added within a brief period following the end of the first afterdischarge. Furthermore, at these times the addition of FT often resulted in the onset of spontaneous afterdischarges. In contrast, at 60 min after the end of the first afterdischarge, FT treatment had no effect on second afterdischarge durations and never induced spontaneous discharges. Measurements of cyclic AMP levels in the presence of FT showed that the lack of effect of FT in the refractory period was not due to failure to elevate cyclic AMP levels at this time.**

**Further analysis of the electrophysiological responses of the bag cell neurons was carried out using clusters of bag cell neurons isolated from the abdominal ganglia. Cells in such isolated clusters prepared from control unstimulated ganglia responded to FT with marked oscillations in the membrane potential followed by afterdischarge, accompanied by an enhancement of spike width. In contrast, cells in clusters prepared from ganglia that had been stimulated to afterdischarge and had entered the refractory period failed to demonstrate membrane potential oscillations or afterdischarge in response to FT, although spike enhancement was observed.**

**These data suggest that the responsiveness of the bag cell neurons to cyclic AMP can be profoundly modified in a way that depends on the prior history of activity of these neurons.**

Many changes in excitability in neuronal and neuroendocrine cells are thought to be controlled by the cyclic nucleotide, cyclic AMP, through the activity of cyclic AMP-dependent protein kinase (Greengard, 1978; Castellucci et al., 1980; Kaczmarek et al., 1980; Adams and Levitan, 1982; dePeyer et al., 1982; Alkon, et al., 1983).

One system of neurons in which cyclic AMP plays an important role is that of the bag cell neurons of *Aplysia*, which control egg-laying behavior in this animal. Although these electrically coupled neurons are ordinarily silent, a brief stimulus train to an afferent pathway or to the bag cell cluster elicits a synchronous afterdischarge lasting approximately 30 min (Kupferman and Kandel, 1970). The afterdischarge triggers the complex sequence of egg-laying behaviors through the release of several neuroactive peptides from the bag cell neurons (Arch, 1972; Pinsker and Dudek, 1977; Dudek et al., 1979; Stuart et al., 1980; Strumwasser et al., 1981). Previous work has suggested that the onset of the afterdischarge is the result of changes in the electrical properties of the bag cells controlled by cyclic AMP-dependent protein kinase. Electrical stimulation of an afterdischarge produces a significant increase in cyclic AMP levels within the cluster, and an afterdischarge can be initiated by cyclic AMP analogues in the absence of nerve stimulation and can be prolonged by phosphodiesterase inhibitors (Kaczmarek et al., 1978). Many of the electrophysiological changes that occur at the beginning of an afterdischarge, including both the onset of membrane potential oscillations that may drive a repetitive discharge and an enhancement of action potential width, may be observed in isolated bag cell neurons in cell culture on exposure to cyclic AMP analogues or on intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase (Kaczmarek et al., 1980; Kaczmarek and Strumwasser, 1981). Agents which elevate cyclic AMP levels have been shown to alter the properties of multiple $K^+$ conductances in cultured bag cells (Kaczmarek and Strumwasser, 1984; Strong, 1984; Strong and Kaczmarek, 1984). Moreover, the onset of afterdischarge has been shown to be associated with changes in the phosphorylation state of at least two proteins which are substrates for an endogenous cyclic AMP-dependent protein kinase (Jennings et al., 1982).

Following the afterdischarge, the cells enter a refractory period during which further electrical stimulation either fails to produce a second afterdischarge or produces a much shorter discharge with

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a lower frequency of firing (Kupferman and Kandel, 1970; Kaczmarek et al., 1978, 1982). Recovery from refractoriness occurs gradually over many hours, and full-length afterdischarges can again be elicited 18 to 24 hr after the onset of refractoriness. It is thought that this prolonged refractory period serves *in vivo* to prevent the occurrence of further afterdischarges in the bag cell neurons while the sequence of behaviors triggered by the first afterdischarge is still in progress.

Although evidence suggests that calcium ion entry may play a role in refractoriness (Kaczmarek et al., 1982; Kaczmarek and Kauer, 1983), little is known about the effects of cyclic AMP during the refractory period. We have now examined cyclic AMP levels and electrophysiological responses to elevated cyclic AMP levels in refractory bag cell neurons.

## Materials and Methods

*Materials.* Mature *Aplysia californica* (150 to 400 gm) were obtained from Alacrity Marine Biological Services (Redondo Beach, CA) and were maintained in artificial seawater at 14°C. The pleuroabdominal connectives that join the abdominal ganglion to the head ganglia were cut close to the pleural ganglia, and the abdominal ganglion was then dissected out with the entire length of these connectives. The abdominal ganglia were then placed in an artificial seawater medium (ASW) composed of 460.0 mM NaCl, 10.4 mM KCl, 55.0 mM $MgCl_2$, 11.0 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.8. All experiments were performed in 10 ml of ASW at 14°C.

*Extracellular recordings.* The intact ganglia were placed in a recording chamber at 14°C and a suction electrode for stimulation was placed on the connective nerve about 1 cm from the cell bodies. A recording suction electrode was placed at the rostral end of the corresponding bag cell cluster. The large compound action potentials which can be recorded with this positioning of the recording electrode represent the synchronous firing of most or all of the 200 to 400 cells within the bag cell cluster.

Afterdischarges were stimulated by passing current through the stimulating electrode (20 V, 2.5 msec; 6 Hz, 5 sec). The last compound action potential recorded was taken as the end of the afterdischarge. For stimulation of afterdischarges in the refractory period, a similar pulse train was used. If this stimulus was not sufficient to produce afterdischarge, the stimulating voltage was increased with each train up to 50 V, with about 30 sec between pulse trains. If no afterdischarge occurred, the stimulating electrode was moved closer to or onto the bag cell cluster and stimulation was repeated (Kaczmarek and Kauer, 1983). If no afterdischarge occurred with maximal stimulation, the duration of the afterdischarge was scored as zero.

*Cyclic AMP determinations.* For determination of cyclic AMP levels following electrical stimulation, the intact ganglion was placed in the recording chamber as described above, except that a recording electrode was placed over each bag cell cluster to be sure that the afterdischarge occurred in both clusters. Two minutes into the afterdischarge, bag cell clusters were rapidly dissected away from the abdominal ganglion, and the connective nerves were cut close to the bag cell bodies. In cases where refractory clusters gave no afterdischarge, clusters were removed 4 min after the start of stimulation. For experiments measuring levels of cyclic AMP after treatment with forskolin in the presence of theophylline (FT), the ganglia with connectives were treated with FT for 10 min, after which the bag cell clusters were dissected away as described above. In all experiments, each cluster was homogenized separately in 6% trichloroacetic acid at 0°C. Aliquots were taken for protein determination (Lowry et al., 1951), and cyclic AMP concentrations were measured using a cyclic AMP [$^{125}$I] radioimmunoassay kit (New England Nuclear, Boston, MA).

*Intracellular recordings.* For experiments in intact bag cell clusters, the connective tissue overlying the bag cell clusters was first softened by preincubation of the ganglia for 2 hr in ASW containing 1 mg/ml of collagenase and 0.5 mg/ml of elastase (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 22°C. After rinsing in ASW, the ganglia were placed in the recording chamber at 14°C and attached to extracellular suction electrodes as described above. Glass microelectrodes were filled with 3M KCl and typically had resistances of 30 to 60 megohms. Recordings were made through an S-7100A electrometer (W-P Instruments, Inc., New Haven, CT). Intracellular stimulation was achieved by passing current through the recording electrode, after correcting for bridge imbalance. Action potentials and afterdischarge recordings were stored on polygraph chart records.

*Isolated desheathed clusters.* To prepare clusters of bag cell neurons isolated from the abdominal ganglion and from their neurites in the connective tissue, intact abdominal ganglia were first incubated in the collagenase/elastase solution described above. At this point, refractory clusters were obtained by stimulating the bag cell clusters to afterdischarge; after the end of the afterdischarge, electrical stimulation was repeated until no further discharges were elicited. Control clusters were not electrically stimulated. The clusters were then dissected free of the abdominal ganglion and the connective nerve, and the surrounding connective tissue was peeled away from around each cluster, using fine forceps. Each isolated desheathed cluster was transferred to fresh ASW and placed in the recording chamber where it was positioned for intracellular recording using an empty glass micropipette. Intracellular recordings from cells in isolated desheathed clusters were carried out as described above for cells in intact clusters. The interval between the end of the afterdischarge and the start of intracellular recording from cells in refractory clusters ranged from 1 to 3 hr in different

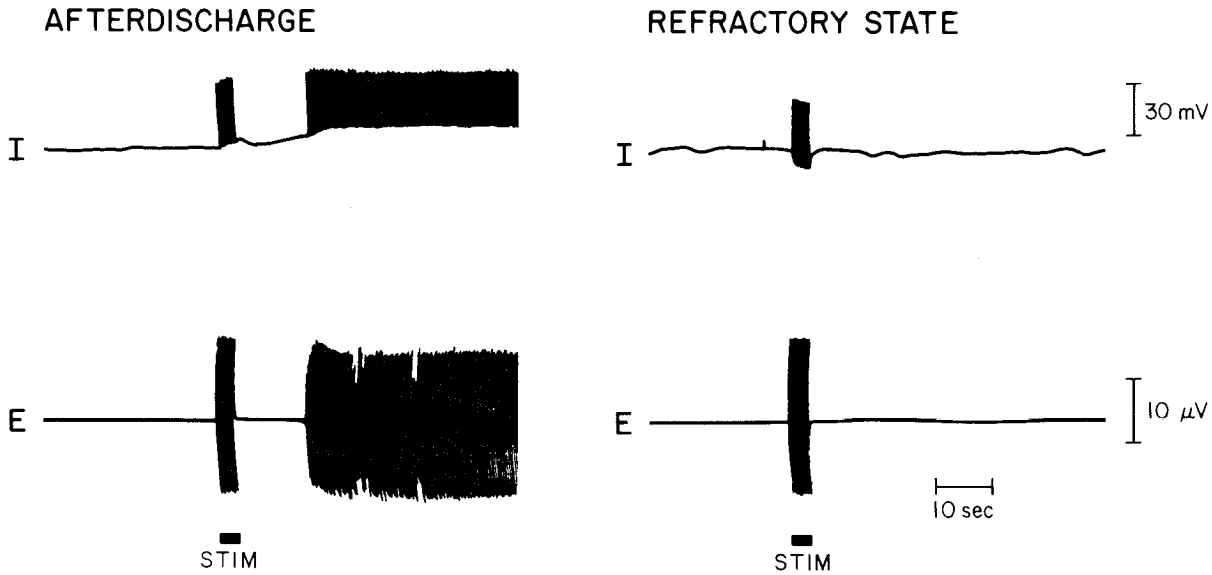


*Figure 1.* Stimulation of bag cell neurons to trigger a first afterdischarge and subsequent stimulation in the refractory period. The pleuroabdominal connective nerve was electrically stimulated (20 V, 2.5 msec, 6 Hz, 4 sec) at the times denoted by the *bars*. In a previously unstimulated bag cell cluster (*left*), the stimulus produces an afterdischarge. Sixty minutes into the refractory period (*right*), the same stimulus does not elicit an afterdischarge. *Top trace* (*I*), intracellular recording from a bag cell. *Bottom trace* (*E*), extracellular recording from the bag cell cluster. Intracellular records have been truncated in amplitude by the chart recorder.

TABLE I

*Cyclic AMP is elevated in bag cell clusters with stimulation of a first afterdischarge and with stimulation in the refractory period*

Control abdominal ganglia were left unstimulated, while experimental ganglia were either stimulated to a first afterdischarge or stimulated to a first afterdischarge and then stimulated again 1 hr after the first afterdischarge; clusters were then removed for assay. Levels of cyclic AMP were measured in unstimulated bag cell clusters (Control), in clusters removed from the ganglia at 2 min into the electrically stimulated afterdischarge (Stim., first AD), and in clusters removed from ganglia after stimulation in the refractory period, 60 min after the end of a first afterdischarge (Stim., refractory period). Results are expressed as picomoles of cyclic AMP per milligram of protein per bag cell cluster (mean ± SEM). Stimulus parameters and assays of cyclic AMP and protein were as described under "Materials and Methods." No phosphodiesterase inhibitor was used in these experiments.

| Condition | Cyclic AMP Level | *N* |
|---|---|---|
| | *pmol of cyclic AMP/mg of protein* | |
| Control | 9.6 ± 0.9 | 16 |
| Stim., first AD | 13.8 ± 1.9 | 16 |
| Stim., refractory period | 16.5 ± 1.5 | 14 |

experiments, but this interval was not observed to affect any of the reported results.

*Reagents.* The adenylate cyclase activator, forskolin (F), was obtained from Calbiochem-Behring Corp. (San Diego, CA) and was kept as a 10 mM stock solution in ethanol. Theophylline (T) (Sigma Chemical Co., St. Louis, MO) was stored as a 10 mM stock solution in ASW until use. For experiments on duration of afterdischarge, 50 to 1000 μl of concentrated stock solutions of forskolin and theophylline were added to the bath containing the intact ganglion at the appropriate time, and after 10 min, electrical stimulation was begun, as described above.

## Results

*Afterdischarge and refractoriness.* In a previously unstimulated ganglion, brief electrical stimulation (5 sec) to a connective nerve or to the bag cell cluster produces a long-lasting afterdischarge of synchronous action potentials in the bag cells. Figure 1 is a record of the beginning of an electrically stimulated afterdischarge showing both intracellular spikes and extracellularly recorded compound action potentials. Following the end of the first afterdischarge, stimulation can elicit second short afterdischarges of low firing frequency, but in many cases no further afterdischarges can be elicited (Fig. 1). The onset of refractoriness is also associated with a failure of bag cell action potentials to invade fully the somata (Dudek and Blankenship, 1977a, b; Kaczmarek et al., 1978).

Whereas the first afterdischarge lasts about 0.5 hr (mean duration of first afterdischarge, 32.3 ± 2.7 min; $n = 34$), second afterdischarges stimulated at 10 or 60 min after first afterdischarge are much briefer (mean duration, 3.2 ± 2.1, $n = 7$, and 4.0 ± 2.2 min, $n = 8$, respectively).

*Cyclic AMP levels in the refractory period.* Previous work has shown that cyclic AMP levels in the bag cell neurons rise during the first 2 min of an afterdischarge and thereafter decline to control levels (Kaczmarek et al., 1978). To determine whether the failure to trigger long-lasting afterdischarges during the refractory period is due to the failure of stimulation to elevate cyclic AMP at this time, the levels of cyclic AMP in bag cell clusters were measured under three conditions: (*1*) in control, unstimulated clusters, (*2*) in clusters stimulated to a first afterdischarge, and (3) in electrically stimulated refractory clusters, which had completed a first afterdischarge 1 hr previously (Table I). Previous measurements of changes in cyclic AMP levels on stimulation of an afterdischarge were carried out in the presence of a phosphodiesterase inhibitor (Kaczmarek et al., 1978). Because refractoriness may result in changes in phosphodiesterase activity, a comparison of control and refractory bag cell neurons could be misleading if carried out in the presence of such an inhibitor. The present determinations were therefore carried out in normal ASW. Table I shows that, even in the absence of a phosphodiesterase inhibitor, a significant increase in cyclic AMP levels in the bag cell neurons was found on stimulation of a first afterdischarge ($p < 0.05$). A similar increase was also observed on

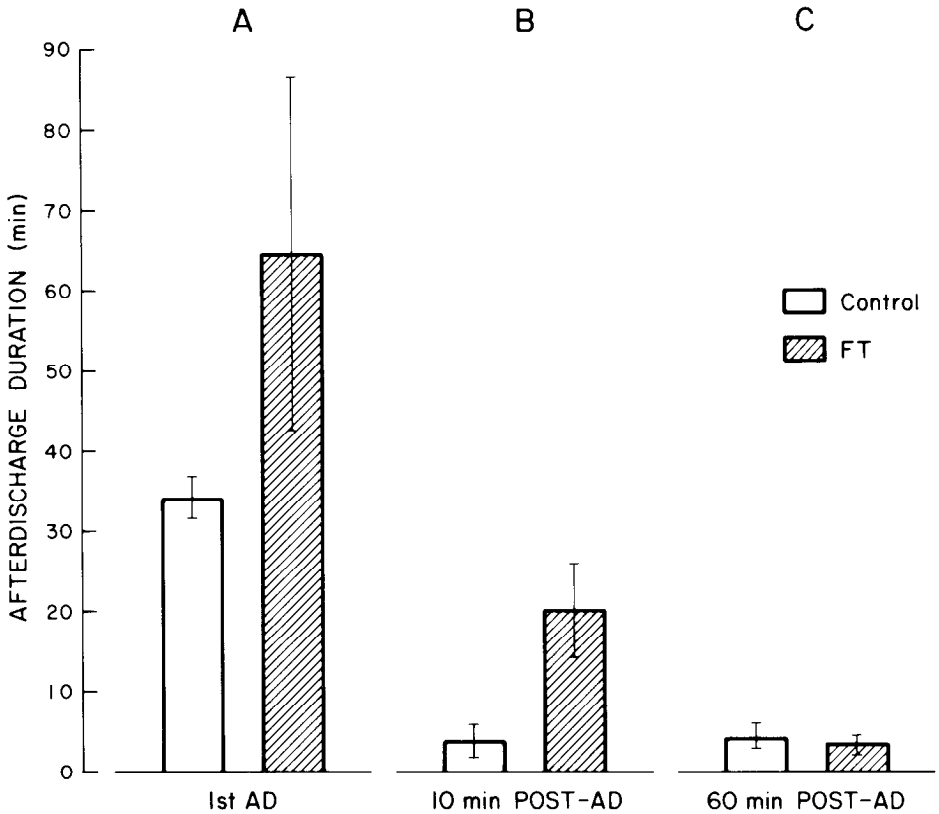


*Figure 2.* Effects of FT treatment on afterdischarge duration in intact abdominal ganglia. The durations of stimulated afterdischarges were measured either in normal ASW (*Control*) or after 50 μM forskolin and 1 mM theophylline had been added to the recording bath 10 min before stimulation (*FT*). Afterdischarge duration was monitored by both extracellular recording and intracellular recording. *A*, Duration of a first afterdischarge (control, $n = 34$; FT, $n = 7$). At 10 min (*B*) or 60 min (*C*) following a first afterdischarge, FT was added to the bathing ASW of experimental ganglia. Ten minutes later, these ganglia were electrically stimulated and the duration of the second afterdischarge was recorded (*10 min post-AD*: control, $n = 7$; FT, $n = 11$; *60 min post-AD*: control, $n = 8$; FT, $n = 7$). In all cases, when an afterdischarge started spontaneously within 10 min of exposure to FT, no electrical stimulation was applied. Results are expressed as mean ± SEM.

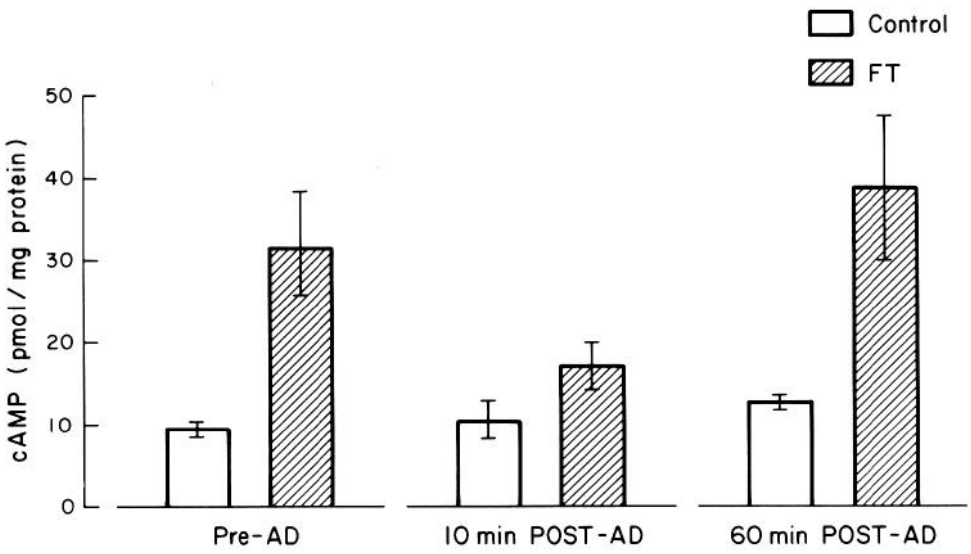


*Figure 3.* The elevation of cyclic AMP levels in bag cell clusters by FT. Levels of cyclic AMP were assayed in bag cell clusters from intact ganglia after a 10-min incubation in ASW either in the absence (*Control*) or presence (*FT*) of 50 μM forskolin and 1 mM theophylline. Ganglia were either left unstimulated (*Pre-AD*) or were stimulated to afterdischarge and exposed to FT 10 or 60 min following the afterdischarge. Bag cell clusters were then dissected out for cyclic AMP assay. The *histograms* to the *left* (*Pre-AD*) show cyclic AMP levels measured in unstimulated ganglia incubated either with or without FT for 10 min (control, $n = 16$; FT, $n = 8$). The *histograms* in the *center* and to the *right* show cyclic AMP levels measured at 10 and 60 min post-AD, respectively; afterdischarges were stimulated in bag cell clusters, and beginning at 10 or 60 min after the end of the afterdischarge, the ganglia were incubated for 10 min either in the absence of presence of FT (10 min post-AD: control, $n = 4$; FT, $n = 8$; 60 min post-AD: control, $n = 4$; FT, $n = 8$). Results are expressed as picomoles of cyclic AMP per milligram of protein per bag cell cluster (mean + SEM). Stimulus parameters and assays for cyclic AMP and protein are as described under "Materials and Methods."

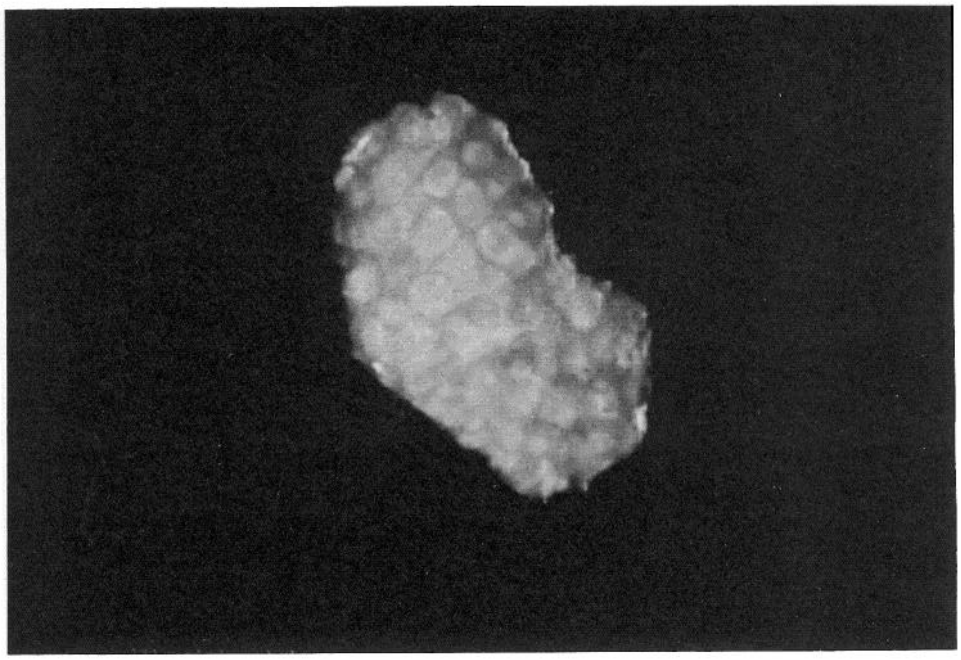

*Figure 4.* An isolated desheathed bag cell cluster preparation which was used in the present experiments to compare the responses of bag cell neurons in the control and refractory states. Isolated desheathed clusters were prepared by removal of the abdominal ganglion and connective nerve and gentle removal of the connective tissue surrounding each cluster.

stimulation in the refractory period. Although these data cannot rule out that changes in cyclic AMP metabolism occur during the refractory period, they argue that the inability of stimulation to trigger long-lasting afterdischarge is probably not due to lower cyclic AMP levels in response to stimulation.

*Effects of FT on bag cell neuronal afterdischarges in intact ganglia.* To determine how the electrophysiological responses of the bag cell neurons to cyclic AMP are altered in the refractory period, we first tested responses of these neurons in intact abdominal ganglia. Cyclic AMP analogues have been shown to trigger and to prolong the duration of first afterdischarges (Kaczmarek et al., 1978). In these studies we elevated cyclic AMP levels with the adenylate cyclase activator, forskolin (50 μM). Because of the high levels of phosphodiesterase activity in abdominal ganglia (Levitan and Norman, 1980), we applied forskolin in the presence of theophylline (1 mM) (FT). We found that exposure to FT before stimulation of a first afterdischarge markedly increased the durations of these first afterdischarges (Fig. 2). In addition, when FT was added 10 min after the end of the first afterdischarge, second afterdischarges stimulated at this time were of significantly greater duration than those without FT treatment. In contrast, exposure to FT 1 hr after the end of the first afterdischarge had no effect on the durations of stimulated second afterdischarges.

In addition, we found that when FT was added before the first afterdischarge or at 10 min following the first afterdischarge, the bag cell neurons would often begin to discharge spontaneously, without electrical stimulation. Such spontaneous discharges were never observed when FT was added 60 min following the afterdischarge.

In order to rule out the possibility that the lack of electrophysiological effects of FT 60 min into the refractory period was due to a

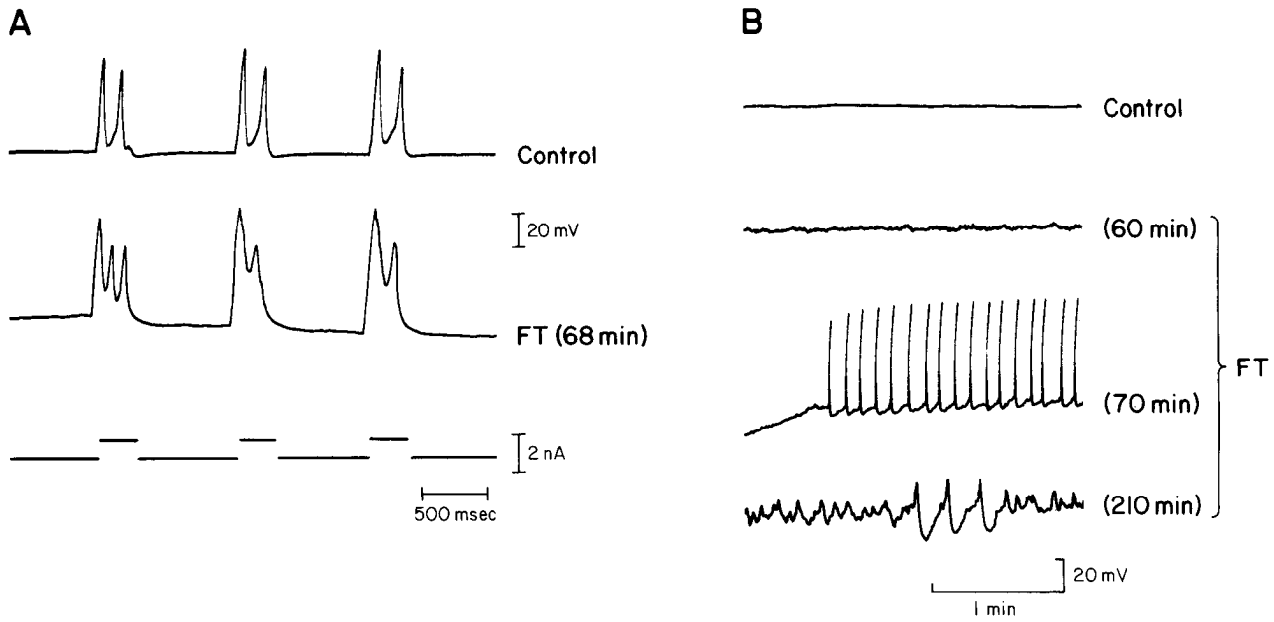


*Figure 5.* Spike broadening, membrane potential oscillations, and afterdischarge induced by FT in control isolated desheathed bag cell clusters. *A*, Action potential response of a bag cell in a control cluster to a train of depolarizing current pulses of fixed intensity (*lower trace*). The *upper* and *middle traces* show the responses before and at 68 min after addition of FT to the bath. *B*, Development of subthreshold membrane potential oscillations and afterdischarge; membrane potential records prior to FT addition and at 60, 70, and 210 min after FT addition. FT concentrations were 50 μM forskolin and 1 mM theophylline. *A* and *B* are records from the same cell.

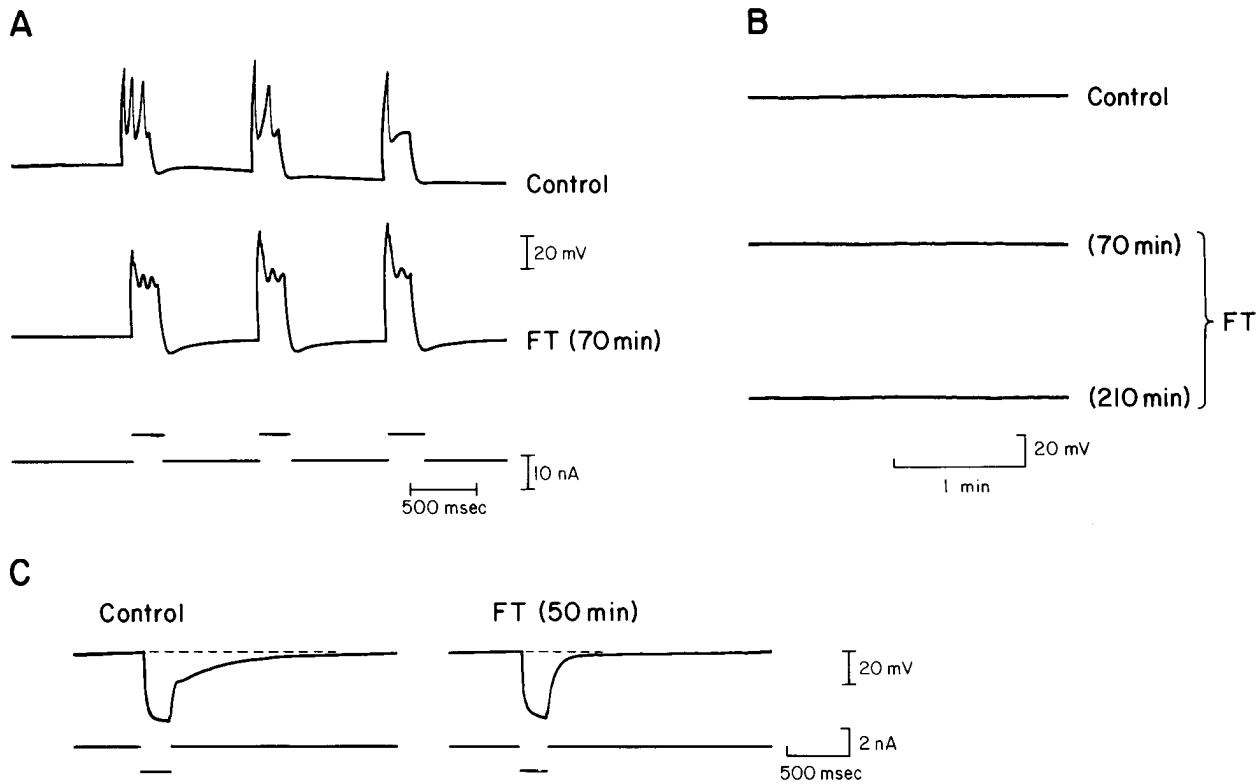


*Figure 6.* Electrophysiological responses to FT in cells within refractory bag cell clusters. Bag cells in intact ganglia were stimulated to afterdischarge until no further afterdischarge could be triggered, after which isolated desheathed clusters were prepared. *A*, Action potential response of a bag cell in a refractory cluster to a train of depolarizing pulses of constant intensity (*lower trace*), before (*upper trace*), and 70 min after (*middle trace*) FT addition to the bath. *B*, Lack of any membrane potential oscillations after FT treatment in a bag cell in a refractory cluster. *A* and *B* are records from the same cell. *C*, Responses of a bag cell from a refractory cluster to a hyperpolarizing current pulse (*lower traces*), before (*left upper trace*), and 50 min after (*right upper trace*) FT addition to the bath. FT concentrations were 50 μMM forskolin and 1 mM theophylline.

failure of FT to elevate cyclic AMP levels at this time, we measured the changes in cyclic AMP levels produced by FT in clusters of bag cell neurons. We found that FT treatment increased levels of cyclic AMP in bag cells at each of the three times tested (Fig. 3), although FT appeared to be less effective at 10 min after a first afterdischarge than before or at 60 min after a first afterdischarge. Thus, although the duration of afterdischarge was responsive to an elevation of cyclic AMP before an afterdischarge and during a short period following the afterdischarge, this responsiveness was lost by 1 hr into the refractory period.

*Isolated desheathed bag cell clusters.* Intracellular recordings from the bag cell neurons within intact abdominal ganglia are complicated by the fact that these cells comprise an electrically coupled network with multiple spike-initiating zones that are distal to the somata (Blankenship and Haskins, 1979; Haskins and Blankenship, 1979; Kaczmarek et al., 1979). For this reason, most investigations of the electrical effects of cyclic AMP in these neurons, including both current clamp and voltage clamp studies, have used single isolated bag cell neurons maintained in cell culture (Kaczmarek and Strumwasser, 1981, 1984; Strong, 1984; Strong and Kaczmarek, 1984). However, such isolated neurons will only afterdischarge in response to cyclic AMP analogues and do not have a clearly defined refractory period. This prompted us to develop a modified preparation, the isolated desheathed cluster, which could be rapidly prepared from intact ganglia after the onset of the refractory period, and which we used for further current clamp analysis of the effects of FT in control and refractory clusters. Bag cell clusters are cut away from the abdominal ganglion and the pleuroabdominal connectives, and the connective tissue surrounding each cluster is gently pulled away. This treatment leaves a cluster of bag cell bodies isolated from most of the bag cell neuritic processes which project into the surrounding connective tissue sheath, and eliminates synaptic and humoral inputs to the bag cells (Fig. 4).

Intracellular recordings were carried out in single cells within these isolated desheathed clusters. The action potentials evoked in response to a train of depolarizing current pulses (0.78 to 7.0 nA, 2.2 msec; 1 Hz, 7 sec) and the membrane potential response to a hyperpolarizing current pulse (1.0 to 4.0 nA, 2.2 msec) were monitored every 5 to 10 min. Once these responses stabilized, FT was added to the bath, and the monitoring of cell response was continued using the same stimulus parameters. This preparation was used to compare the electrophysiological responses of control and refractory clusters to FT treatment.

We recorded from three cells in control clusters and found that all of them responded to FT treatment with electrophysiological changes that appeared identical to those that have been observed in single cultured bag cell neurons on exposure to either cyclic AMP analogues or FT (Kaczmarek and Strumwasser, 1981; Kaczmarek and Kauer, 1983). These changes included broadening of the action potential, oscillations of the membrane potential, and afterdischarge.

Figure 5*A* shows that the shape of the action potential is altered following exposure to FT. The spike is markedly broadened, and a characteristic shoulder develops on the falling phase of the action potential. Figure 5*B* shows the onset of membrane potential oscillations and discharge after the addition of FT. After the addition of FT, all cells in control clusters developed oscillations of the membrane potential, which evolved into an afterdischarge similar to that described in cultured bag cells exposed to cyclic AMP analogues. As the *bottom trace* in Figure 5*B* shows, the oscillations continue and increase in amplitude in these neurons for several hours following the afterdischarge.

Intracellular recordings were also made in isolated desheathed clusters prepared from intact ganglia that had been stimulated to generate bag cell afterdischarges and had entered the refractory period. Even before the addition of FT to the bath, differences could be observed between cells in refractory clusters and cells in control clusters. Cells in refractory clusters often required more current to produce an action potential (control: 0.78 nA ± 0.0, $n = 3$; refractory: 3.43 nA ± 1.01, $n = 7$). Action potentials evoked by depolarizing current pulses in cells within refractory clusters also failed to repolarize to as negative a potential as did action potentials from cells in control clusters, which generally repolarized nearly to the base line resting potential before the next spike occurred (Fig. 6*A*).

Figure 6 demonstrates that, although some electrophysiological responses to elevated cyclic AMP levels are retained in bag cell neurons in refractory clusters, a very important aspect of the cyclic AMP response is lost in refractoriness. Upon FT treatment, six of seven cells in refractory clusters failed to develop membrane potential oscillations or to afterdischarge, even though in some cases FT remained present for 15 hr (Fig. 6*B*).

Broadening of the action potential accompanied by the characteristic shoulder on the falling phase of the spike was observed in response to FT in seven of seven neurons in refractory clusters (Fig. 6*A*). Figure 6*C* shows another FT response which was still observed in cells in refractory clusters. Previous work, using both internally dialyzed and two-microelectrode voltage clamp techniques on isolated cultured bag cell neurons has shown that the fast transient potassium current ($I_A$) is significantly reduced after FT or cyclic AMP analogue treatment (Kaczmarek and Strumwasser, 1984; Strong, 1984). In current clamp recording, this current can be seen as a marked break in the repolarization of the membrane potential following a hyperpolarizing current pulse. The break is followed by a gradual return to the resting potential lasting several hundred milliseconds (Connor and Stevens, 1971). In two of seven neurons recorded from in refractory clusters, the response to a hyperpolarizing current pulse clearly showed such a break in the repolarization. In both of these cells, the break in the repolarization was very much reduced after FT treatment even though the resting membrane potential did not change (Fig. 6*C*). This evidence suggests that the sensitivity of $I_A$ to cyclic AMP is retained in the refractory period.

## Discussion

The prolonged duration of the refractory period in the bag cell neurons is particularly intriguing and suggests that it is controlled by some biochemical, or perhaps even structural, change within these cells. We have now found that the electrophysiological response of the bag cell neurons to pharmacological elevations of cyclic AMP differs in control and refractory cells. Within clusters of cells in intact abdominal ganglia, elevation of cyclic AMP either before the first afterdischarge or very soon after the afterdischarge ends result in both the onset of spontaneous discharge and a significant increase in the duration of afterdischarges. One hour into the refractory period, however, neither the triggering of afterdischarges nor their duration is sensitive to increased cyclic AMP levels. It appears, therefore, that during the first hour following the afterdischarge the bag cells undergo a biochemical and/or biophysical change which prevents the initiation and the extension of the afterdischarge in response to FT. Our experiments with isolated desheathed clusters suggest that, although some of the electrophysiological responses of the bag cell neurons to cyclic AMP are retained in the refractory period, the ability to respond to FT treatment with the production of membrane potential oscillation and afterdischarge is lost after the onset of refractoriness.

One unexpected result was found in measurements of cyclic AMP levels after FT treatment at various times. Whereas FT elevates cyclic AMP to similar levels before and at 60 min after an afterdischarge, FT is only about half as potent at 10 min after the afterdischarge. This suggests that near the end of the afterdischarge, either cyclic AMP synthesis is inhibited, or cyclic AMP breakdown is accelerated. Since at this time the bag cells still respond to elevated levels of cyclic AMP with an extended afterdischarge, control of cyclic AMP synthesis or breakdown may be responsible for terminating the afterdischarge under normal conditions. However, this control must be short-lived and cannot account for refractoriness,

since by 60 min after the afterdischarge, FT treatment is once again fully potent.

There are two plausible explanations for our results. One attractive idea is that one of the currents normally altered by cyclic AMP becomes insensitive to elevations of cyclic AMP in the refractory period. For example, elevation of cyclic AMP results in a region of negative slope resistance in the steady-state current-voltage relations of isolated cultured bag cell neurons which may cause the membrane potential oscillations and repetitive discharge (Kaczmarek and Strumwasser, 1984). This reponse to cyclic AMP may become attenuated following an afterdischarge. Such a specific change in one response to cyclic AMP could occur at the level of the protein kinase, a kinase substrate, a phosphoprotein phosphatase, or the ion channel ultimately influenced by these enzymes. Previous work (Kaczmarek and Kauer, 1983) has shown that calcium ion entry into bag cell neurons causes refractoriness. A change in the sensitivity of one current could therefore be brought about by calcium ions, perhaps acting through the calcium-dependent protein kinase systems present in the bag cell neurons (DeRiemer et al., 1985).

Alternatively, it is possible that each of the ionic channel responses to cyclic AMP proceeds normally but that the refractory period is associated with additional unrelated biophysical and biochemical changes that prevent the expression of the membrane potential oscillations and prolonged discharge. We have, in fact, observed that even before the addition of FT, there are differences in action potential threshold and action potential repolarization levels between cells from control and refractory clusters. Recent evidence has indicated that, in addition to their modulation by cyclic AMP-dependent mechanisms, the electrical properties of the bag cell neurons can be regulated by the calcium/phospholipid-dependent protein kinase (protein kinase C) which may enhance inward currents (DeRiemer et al., 1985). It may be that changes in the latter second-messenger system contribute to the altered electrical properties of the bag cell neurons in the refractory period. These questions may be resolved by voltage clamp analysis if single isolated "refractory" cells can be prepared.

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