Studies of Excitability in a Model Peptidergic System: The Roles of Cyclic AMP, Protein Phosphorylation and Serotonin During Afterdischarge in the Bag Cell Neurons of Aplysia californica.

Thesis by

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To my parents

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ABSTRACT

The polypeptide hormone-secreting bag cell neurons from the abdominal ganglion of Aplysia can be induced to fire repetitively when triggered by a brief electrical stimulus to the afferent pathway. This thesis investigates the mechanism of this afterdischarge by employing biochemical, pharmacological and electrophysiological approaches.

The description of bag cell afterdischarge, its modulation by the transmitters serotonin and dopamine and evidence for the role of cyclic AMP in the genesis of afterdischarge is presented in Chapter 1. Bag cell afterdischarge is shown to be inhibited by the application of serotonin and lengthened by the application of dopamine or the methylxanthine phosphodiesterase inhibitors. Cyclic AMP undergoes a 2-3 fold increase in the bag cell clusters during an electrically-stimulated afterdischarge but not in matched controls where equivalent electrical stimulation did not elicit afterdischarge. As further evidence for a role for cyclic AMP in the genesis of afterdischarge, afterdischarges were obtained in unstimulated preparations by the extracellular application of the cyclic AMP analogues, 8-benzylthio-cyclic AMP and 8-methylthio-cyclic AMP.

Chapter 2 describes protein phosphorylation in bag cell tissues under a number of different conditions. The presence of an endogenous, cyclic AMP-dependent protein kinase activity is demonstrated in crude membranes prepared from bag cells and the substrate specificity for this activity is shown to be similar to that of protein kinase catalytic subunit prepared from bovine heart. Increases in phosphorylation of a 33,000 dalton and 21,000 dalton phosphoprotein are shown to occur during electrically-stimulated afterdischarge in bag cells. The 21,000 dalton substrate is shown to be apparently specific to bag cell tissues and an amino acid composition and partial amino acid sequence of this protein is presented.

Chapter 3 presents evidence that serotonin, within the physiological range reported by other workers for Aplysia (0.1—1.0 μM) brings about a rapid inhibition of an ongoing afterdischarge. This inhibition is antagonized by the stereospecific blocker of serotonin action, D-butaclamol but not its inactive isomer, L-butaclamol. Serotonergic inhibition is shown to be associated with decreased bag cell action potential duration and height and an increased threshold to spike generation. Evidence is presented that the second, calcium-dependent phase of bag cell afterdischarge is most sensitive to the action of the transmitter and that the potassium channel blocker, tetraethylammonium can overcome serotonin's inhibitory effect. This raises the possibility that serotonin may cause inhibition of bag cell afterdischarge by increasing potassium conductance. The possible functional role of serotonin inhibition of egg-laying is discussed.

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1. INTRODUCTION

The nervous system has traditionally been thought of as being involved with very rapid events taking place on a millisecond time scale. The endocrine system has been associated with the slower, longer-lasting events in regulation (Pfaff, 1979). This thesis deals with a class of neurons that display a characteristic pattern of long-lasting electrical behavior and secrete a peptide hormone that controls egg-laying in the mollusc, Aplysia californica. This type of neuron, termed a neurosecretory cell, has been studied most extensively in vertebrates. The research of Kandel (1964) on the hypothalamic neurons of the goldfish provided an early description of the characteristic electrical events recorded intracellularly from such neurons. Neuro-secretory cells from different phyla share many of the same electrical characteristics (Maddrell and Nordmann, 1979). The action potentials of these neurons are in the range of 80 mV and the inward current appears to be carried by both sodium and calcium ions (Hagiwara and Byerly, 1981). The action potentials of neurosecretory cells are of longer duration (50-100 msec) than those found in non-endocrine nerve cells. These lengthened action potentials are thought to reflect an enhanced calcium current in these neurons and are associated with the major activity of these cells: hormone secretion (Cooke, 1977). There is evidence from studies conducted on peptidergic cells that the characteristic electrical activity of such neurosecretory cells causes the release of peptide hormone. Studies employing tungsten microelectrode recordings in the hypothalamus of the monkey (Hayward and Vincent, 1970) have demonstrated neurons that respond with an increased firing rate to the injection of hypertonic saline solutions into the carotid artery, a hyperosmotic stimulus that results in enhanced vasopressin secretion (Dunn et al., 1973). The secretion of insulin from peptidergic β cells in response to an elevation of blood glucose is associated with the occurrence of electrical discharge activity (Matthews and O'Connor, 1979).

Release of the egg-laying hormone (ELH) has also been shown to be associated with long-lasting afterdischarge in the peptidergic bag cells of Aplysia (Stuart et al., 1980).

A common electrical behavior shared by many peptide secreting cells including the β-cells of the pancreas (Meissner and Atwater, 1976), vasopressin neurons (Poulain et al., 1977) and the bag cell neurons is burst activity and spike broadening.

In these cells a repetitive discharge associated with secretory activity is induced by the appropriate stimulus. In the case of bag cell neurons, following brief electrical stimulation of the afferent pathway (or the application of purified peptides from the atrial gland) an afterdischarge with an average duration of thirty minutes occurs (Kaczmarek et al., 1978). In recordings made from the β cells of mouse islets in vitro, a sustained exposure to glucose at physiological concentrations results in repetitive oscillations of the membrane potential for the duration of the exposure (Matthews and O'Connor, 1979). Bursts of action potentials occur, whose duration and frequency are a function of the glucose concentration. In vitro recordings made from hypothalamic neurons in cell culture (Gahwiler and Dreifuss, 1979) demonstrate bursting behavior and provide evidence that the discharge is generated by an endogenous mechanism residing in the cell and is not an outcome of synaptic actions.

It has been demonstrated in vasopressin-secreting neurons in vitro that this characteristic phasic activity leads to a greater secretion of vasopressin than an equal number of stimuli presented in a tonic pattern (Nordmann and Dreifuss, 1972; Dyball and Thompson, 1977). These effects were found to be calcium-dependent. One possible mechanism for enhanced secretion during burst activity is frequency-dependent spike broadening. Spike broadening was first demonstrated in the neurosecretory neuron, R15 of Aplysia (Strumwasser, 1967). The spike broadening associated with burst activity has been postulated to result from inactivation of the delayed rectifier potassium channel (Thompson, 1980) and/or the calcium-activated potassium

channel (Strumwasser et al., 1981; Kaczmarek and Strumwasser, 1981). Profound spike broadening occurs during the course of afterdischarge in bag cells (Strumwasser et al., 1981 and appendix II) and appears to parallel the increase in cyclic AMP levels reported in the first chapter of this thesis. Evidence obtained from bag cell neurons grown in cell culture suggest that cyclic AMP (Kaczmarek and Strumwasser, 1981) and protein phosphorylation (Appendix I and Chapter 2) may mediate spike broadening by decreasing potassium conductance. Longer duration action potentials would lead to a greater calcium ion influx per action potential resulting in enhanced secretion of peptide. The all-or-none nature of the bag cell afterdischarge ensures that once triggered, the afterdischarge (with its attendant spike broadening) is self-generative and does not require the continued presence of a secretagogue, in contrast to the example of β cells presented above. Thus, while the bag cells may be a model system for the study of such time-dependent phenomena as spike frequency, spike duration, peptide-secretion and their biochemical correlates it is not suited to the study of dose-dependent effects (which can be investigated in the insulin-secreting β-cell system).

In addition to broadened action potentials, endogenously generated discharges of long duration (afterdischarge) appear to be common to neurosecretory cells of both vertebrates and invertebrates (Maddrell and Nordmann, 1979). This thesis investigates the biochemical and electrical events of afterdischarge in a model system—the bag cell neurons of Aplysia.

Among the possible mechanisms responsible for excitability changes in the nervous system is a biochemical one mediated by cyclic adenosine-3',5'-monophosphate. Cyclic AMP has been postulated to act as a second messenger for transmitter and hormone action (Greengard, 1978) in both the central and peripheral nervous system of vertebrate species. The only known mode of action of cyclic AMP in eukaryotic cells is through the stimulation of protein kinase enzyme activity. Although some

investigators maintain that there may be direct intracellular actions of cyclic AMP that do not involve protein phosphorylation in organisms as advanced as vertebrates (Weller, 1979) there is no experimental evidence to support this. Kuo and Greengard (1969) were the first to describe the presence of cyclic AMP-dependent protein kinase in a wide range of phyla including Mollusca. In more recent years a number of researchers have investigated the role of cyclic AMP in nervous system functioning in the mollusc Aplysia californica. Cedar and co-workers (Cedar and Schwartz, 1972; Cedar, Kandel and Schwartz, 1972) showed that stimulation of the pleurovisceral connective nerves or application of transmitter substances resulted in an increased cyclic AMP formation in the abdominal ganglion. In the course of investigating the cyclic AMP changes occurring in bag cell neurons during afterdischarge it was found that both electrical stimulation and the application of the transmitter substances, serotonin and dopamine increased total cyclic AMP levels in the pleurovisceral connective nerves as well as the bag cell cluster region (Chapter 1) confirming and extending the findings of these earlier workers. Other workers have implicated cyclic AMP in a long-lasting hyperpolarization produced in the parabolic burster neuron (R15) of the abdominal ganglion (Drummond et al., 1980). Another possible site of action of cyclic AMP is in sensitization of the gill-withdrawal reflex in Aplysia (Klein and Kandel, 1978). Recent research on serotonin responses in the snail, Helix aspersa have demonstrated a slow, long-lasting inward current brought about by the action of cyclic AMP on a calcium-sensitive potassium conductance (Deterre et al., 1981).

In all of these cases (with the possible exception of R15 in Aplysia and the Helix neurons) the tissues under study are a heterogenous population of neurons and it has been difficult to clearly demonstrate that cyclic AMP is having its actions in a single neuron or homogenous group of neurons. For these same reasons, very little progress has been made in investigating protein phosphorylation in neurons of Aplysia. Levitan and Barondes (1974) demonstrated a transmitter-stimulated increase in

phosphorylation of an 120,000 dalton protein in the abdominal ganglion; however, the long incubations required (22 hours) raised questions about the physiological relevance of this finding. Ram and Ehrlich (1978) demonstrated the presence of a cyclic GMP-dependent protein kinase in the pleurovisceral connective nerves but did not relate it to any known physiological function.

Chapter 1 of this thesis presents evidence that cyclic AMP is involved in the genesis of the long-lasting excitability change (afterdischarge) in the bag cell neurons. The data of Frazier and co-workers (Frazier et al., 1967) indicated that the bag cell clusters consisted of a single morphological class of neurons and a supporting population of glia. The more recent work of Chiu (Chiu, 1981; Chiu and Strumwasser, 1981) has convincingly demonstrated that all the neurons comprising a bag cell cluster display ELH-immunoreactivity and thus constitute a homogenous population of peptidergic neurons. The advantage of employing bag cells as a model system in these studies is that this high degree of cellular homogeneity allows one to investigate in parallel the electrophysiological and biochemical events taking place in a single class of neurons.

In the second chapter it is shown that there is a cyclic AMP-dependent protein kinase present in bag cell tissues and a characterization is presented of the protein substrates of this kinase and their regional distribution within the nervous system of Aplysia. Two phosphoproteins are described which undergo an increase in phosphorylation during afterdischarge and one of these (a 21,000 dalton phosphoprotein, termed BC-2) that appears to be relatively specific to bag cell tissues is chemically characterized. The evidence presented in Chapter 1 for the involvement of cyclic AMP in afterdischarge is the first example of a long-lasting endogenous burst mechanism in Aplysia mediated by cyclic nucleotides. Models have been proposed postulating a role for cyclic AMP in the mechanism of endogenously bursting neurons (Rapp and Berridge, 1977) but the evidence to date has suggested that cyclic AMP may

only have a modulatory influence on the burst generation mechanism (Levitan et al., 1979). In mammals, there is evidence that cyclic AMP may be involved in a burst-generating mechanism during glucose-stimulated discharge in pancreatic β-cells (Valverde et al., 1979).

The phosphoproteins described in Chapter 2 may play a role in the genesis of afterdischarge in the bag cells (such as altering calcium or potassium conductances; Appendix I) or may be involved with altered rates of synthesis or secretion of the peptide hormone, egg-laying hormone. The biosynthesis of peptide hormones in neurosecretory cells appears to be functionally linked to their electrical activity (Gainer et al., 1977). Recent research by Berry and Arch (1981) has demonstrated an enhanced synthesis (30% increase) of the precursor to ELH in the bag cell neurons in response to a depolarizing stimulus (100 mM potassium) lasting 4 hours. These authors speculate that the enhanced biosynthesis of the peptide hormone observed may be one of the biochemical sequelae of the 3-fold increase in cyclic AMP observed during afterdischarge (Chapter 1). There is no evidence available on the effects of high potassium depolarization on cyclic AMP levels in the bag cells.

In mammalian peptidergic systems secretory activity is subject to control by peptide factors or monoaminergic transmitters (Macleod, 1976). In the case of the mammotrophs of the anterior pituitary, secretory activity is stimulated by thyrotropin releasing factor and inhibited by the monoamine, dopamine. An analogous dual control of excitability appears to act in the bag cell system described in Chapter 3. In the bag cells, peptides purified from the atrial gland are able to initiate afterdischarge activity and the monoamine transmitter serotonin is able to suppress this discharge and the associated egg-laying behavior.

Serotonin has been identified as the 'arousal transmitter' in Aplysia in both the gill withdrawal reflex (Klein and Kandel, 1978) and the biting response (Weiss et al., 1975). Evidence is presented in Chapter 3 that serotonin inhibits afterdischarge

by acting on serotonin receptors at concentrations in the range reported to be physiologically active in Aplysia. It may be that suppression of egg-laying and its attendant behaviors through serotonergic inhibition of bag cell afterdischarge occurs during arousal in Aplysia; thus serotonin may act to suppress some behavioral programs while activating others in a coordinated manner.

The mechanisms described in this thesis for the control of excitation and inhibition in peptidergic neurons of Aplysia may be generally applicable across phyla and may eventually lead to an understanding of other long-lasting neural events such as learning and memory.

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CHAPTER 1

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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.

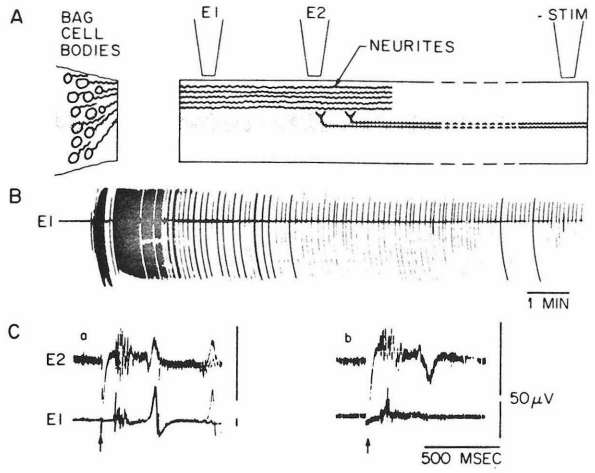

FIG. 1. (*A*) Schematic representation of the isolated pleurovisceral connective preparation. Bag cell neurites are depicted as traveling in the connective tissue (1), where they receive an afferent input from the head ganglia. Suction electrode E1 was placed at the abdominal end of the connective and 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.

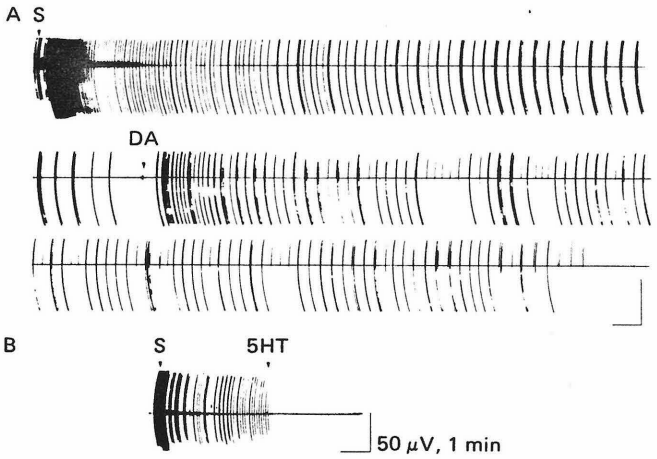

FIG. 2. (*A*) Effect of dopamine (0.12 mM) when introduced in the extracellular medium at the end of the first bag cell afterdischarge in a previously unstimulated intact abdominal ganglion. Dopamine (DA), added at the end of the first afterdischarge, after a latency of about 40 sec generates a subsequent 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

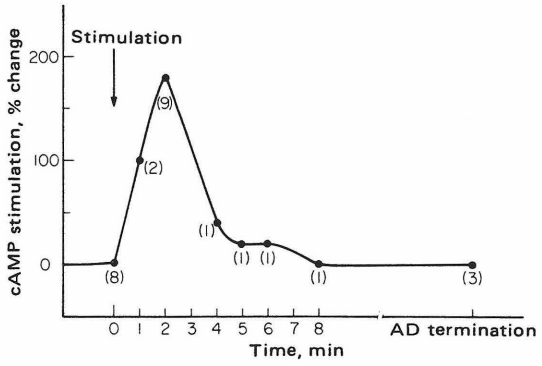

FIG. 3. Time course of change in cAMP levels of the bag cell clusters following the onset of afterdischarge. The percent change in cAMP levels is expressed relative to control levels for bag cell clusters from animals with the same reproductive tract weight. The number of determinations at each time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

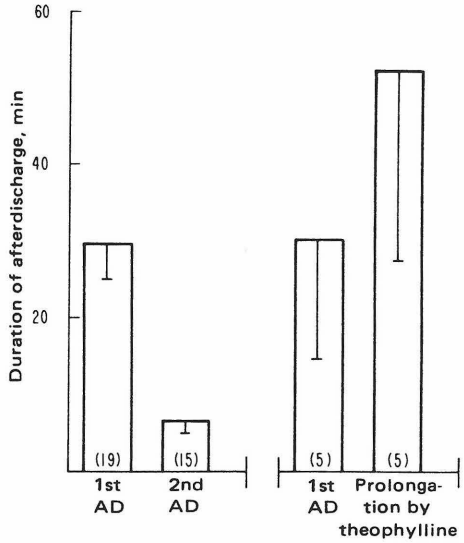

FIG. 4. Duration of afterdischarges in isolated connective preparations. The first two histograms give the result of experiments in which an afterdischarge (1st AD) was triggered by a stimulus train to the distal end of a previously unstimulated connective. Within 10 min of the termination of this first 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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CHAPTER 2

PROTEIN PHOSPHORYLATION DURING AFTERDISCHARGE IN PEPTIDERGIC NEURONS OF APLYSIA

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Abstract

The bag cells of Aplysia are a group of peptidergic neurons that respond to brief electrical stimulation by producing a prolonged afterdischarge of synchronous action potentials. We have previously demonstrated that the cyclic nucleotide, 3',5'-cyclic adenosine monophosphate plays a role in the genesis of the bag cell afterdischarge and that the microinjection of the catalytic subunit of cyclic AMP-dependent protein kinase increases the excitability of bag cell neurons in culture.

In this paper we present evidence that the phosphorylation state of at least two proteins (Proteins BC-1 and BC-2) are enhanced after the onset of afterdischarge. One of these proteins, BC-2 (approximately 21,000 daltons), undergoes cyclic AMP-stimulated phosphorylation in cell-free extracts in vitro and is the major substrate in bag cell tissues for exogenous cyclic AMP-dependent protein kinase catalytic subunit. This protein undergoes a substantial change in phosphorylation at 20 min into the afterdischarge using both bag cells prelabeled with $Na_2H^{32}PO_4$ and a 'back phosphorylation' (postlabeling) procedure. No change could, however, be detected early (2 min) in the afterdischarge with either technique for this protein. We have determined that BC-2 phosphoprotein is apparently specific to bag cell tissue and is not the precursor to bag cell neurosecretory peptide previously described. We have also determined the amino acid composition and present a partial sequence of the N-terminus obtained from 100 picomoles of protein eluted from an SDS-polyacrylamide gel band.

Using the method of prelabeling phosphoproteins in bag cells with $Na_2H^{32}PO_4$, we observed an 82% increase in phosphorylation of a 33,000 dalton protein (BC-1) at 2 min and a 69% increase at 20 min into the afterdischarge. In vitro labeling with [γ-^{32}P]ATP demonstrated that this protein undergoes cyclic AMP-dependent phosphorylation and that its phosphorylation was also markedly enhanced by exogenous cyclic AMP-dependent protein kinase. BC-1 does not appear to be a phosphoprotein

nique to bag cells and from Coomassie blue staining is a minor component in this tissue.

We have described two proteins in bag cell tissues whose phosphorylation state is altered by both cyclic AMP with in vitro labeling and by afterdischarge in the intact bag cell cluster. A change in phosphorylation state of these proteins may be related to the electrical events of afterdischarge or to its biochemical sequelae.

Introduction

Many neuronal and neuroendocrine cells undergo excitability changes that are thought to involve cyclic nucleotides or calcium ions. In eucaryotic cells, the only known means by which cyclic nucleotides act is by stimulating the activity of endogenous protein kinase enzymes which leads to the phosphorylation of specific protein substrates. Changes in the state of protein phosphorylation have been hypothesized to be responsible for altering the ionic conductances and hence the state of excitability of individual nerve cells (Greengard, 1978).

It has been previously shown that the cyclic nucleotide, cyclic AMP, is involved in the excitation of the peptidergic bag cell neurons of Aplysia (Kaczmarek et al., 1978). The bag cells comprise 2 symmetrical clusters of neurons at the base of each pleurovisceral connective nerve. They are anatomically distinct from the remainder of the abdominal ganglion and their processes extend rostrally for about 1 cm anteriorly into the pleurovisceral connective nerve. Following brief electrical stimulation of this nerve the bag cells are triggered into a long-lasting ($\sim$30 min) afterdischarge that is synchronous in all the cells of a cluster (Kupfermann and Kandel, 1970). This afterdischarge is associated with a 200% increase in cyclic AMP levels within the cluster, can be initiated by cyclic AMP analogs in the absence of nerve stimulation and is prolonged by phosphodiesterase inhibitors (Kaczmarek et al., 1978). We have also shown that isolated bag cells in cell culture respond to microinjection of cyclic AMP analogs or the catalytic subunit of the cyclic AMP-dependent protein kinase with enhanced calcium action potentials and that this change is associated with an increase in input resistance (Kaczmarek et al., 1980; Kaczmarek and Strumwasser, 1980).

Recently alterations in protein phosphorylation correlated with long-term potentiation of electrically evoked activity in the hippocampal slice (Browning et al.,

1979) as well as with serotonin application to the isolated facial motor nucleus (Dolphin and Greengard, 1981) have been described in mammals.

Although protein phosphorylation has been examined in whole abdominal ganglia from Aplysia by previous workers (Levitan and Barondes, 1974), a characterization of all the major substrate phosphoproteins of protein kinase within a homogeneous population of cells such as the clusters of bag cell neurons has not previously been carried out. We have, therefore, investigated the substrates of protein phosphorylation in the neuroendocrine bag cells of Aplysia californica.

We present evidence that there exists endogenous cyclic AMP-dependent protein kinase in a crude membrane fraction prepared from bag cell tissues. The protein kinase catalytic subunit from bovine heart, previously demonstrated by intracellular injection to enhance calcium action potentials in cultured bag cell neurons (Kaczmarek et al., 1980), is shown to phosphorylate the same protein substrates that are phosphorylated by the endogenous kinase.

We find that the major substrate of protein phosphorylation in bag cell tissue is an apparently bag cell specific, membrane-associated, phosphoprotein of molecular weight approximately 21,000 daltons. This protein (BC-2) undergoes a major change in phosphorylation state during afterdischarge and may therefore be involved in either the changes in excitability that occur during afterdischarge or in some other aspect of neurosecretion or cellular metabolism.

In addition another protein, BC-1 (33,000 daltons), in bag cells pre-labeled with ^{32}P as sodium orthophosphate for 22-24 hr undergoes an early increase in phosphorylation with afterdischarge.

Materials and Methods

Protein kinase catalytic subunit phosphorylation studies. For the measurement of substrates for catalytic subunit in bag cell homogenates, bag cell organs (intact

bag cell clusters and surrounding connective tissue) were dissected away from the remainder of he abdominal ganglion and the pleuroabdominal connective nerves. Clusters were homogenized in 50 mM sodium acetate buffer (pH 6.5) containing 10 mM magnesium chloride, 1 mM zinc acetate and 1 mM theophylline on ice. Zinc was included to inhibit endogenous phosphatase activity (Weller, 1979). The reaction was initiated within 1-2 min of homogenization by adding 50 µl of homogenate to plastic tubes containing 20 µl of 70 µM [γ-^{32}P] ATP (25 Ci/mM, ICN) and 25 units catalytic subunit of protein kinase (Sigma) at 24°C. Controls contained an equivalent volume of buffer replacing protein kinase. After one minute the reaction was terminated by the addition of 75 µl of a solution containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol and 0.001% bromphenol blue followed by heating at 90°C for 15 min.

Gel electrophoresis. The solubilized proteins were separated on 10 cm long SDS polyacrylamide (10%) slab gels using Tris-glycine buffer as described by Rudolph and Kreuger (1979). Electrophoresis was carried out at 100 volts (constant voltage) for 4-5 hrs. Molecular weight standards (lysozyme, β-lactoglobulin, trypsinogen, pepsin, ovalbumin, bovine serum albumin) were run on a separate lane in every experiment in order to ascertain the molecular weights of the phosphoprotein substrates. The gels were stained with Coomassie blue to localize proteins, destained and dried under vacuum. A linear regression analysis was conducted on the 120 molecular weight standard data points obtained from these gels and the plotted mean standard curve (Fig. 1) was found to approximate a straight line with a regression coefficient: R^2 = 0.86. The dried polyacrylamide gels were subjected to autoradiography using Kodak XR-OMAT X-ray film.

The stimulation of ^{32}P incorporation into individual phosphoprotein bands was quantitated by scanning of the autoradiograms on a Joyce Loebl microdensitometer.

Cyclic AMP stimulated phosphorylation studies. For the assay of endogenous

cAMP-dependent protein kinase activity in bag cell tissues we prepared a crude membrane fraction. Exploratory experiments with bag cell homogenates did not give consistent cyclic AMP-stimulated phosphorylation and therefore further experiments were restricted to the crude membrane preparation. The bag cell organs from 6-10 animals were dissected away from the remainder of the abdominal ganglion and the bag cell somata (and associated glia) were squeezed out from the surrounding connective tissue. This bag cell tissue was then homogenized in 50 mM sodium acetate buffer (pH 6.5) containing 10 mM magnesium chloride, and 1 mM theophylline on ice, transferred to a polycarbonate tube at 4°C and centrifuged for 45 min at 100,000 g. The pellet obtained (crude membrane pellet) was resuspended in a small volume of the sodium acetate buffer described above. The phosphorylation reaction was initiated by adding 50 µl of the crude membrane suspension to plastic tubes containing 70 µM [γ-^{32}P]ATP and the sodium salt of 3',5' cyclic adenosine monophosphate at the appropriate concentration. The subsequent treatments were identical to those described under protein kinase catalytic subunit above.

Inorganic ^{32}P labeling studies. Whole abdominal ganglia with attached pleuro-visceral connective nerves were incubated at 14°C in 2 ml of (pH 7.8) filtered seawater (FSW) containing 0.5 mCi $Na_2H^{32}PO_4$ (NEN) for 22-24 hr. This incubation period was chosen because previous reports (Levitan and Barondes, 1974) indicated that shorter incubations resulted in highly variable phosphoprotein patterns. After incubation the ganglia were removed and transferred to a recording chamber containing 2 mM theophylline in FSW at 14°C. Experimentals were electrically stimulated to after-discharge by passing current (22V, 2.5 msec, 6Hz, 5-10 sec) through an extracellular suction electrode applied to the distal end of one pleuroabdominal connective nerve. Controls received no stimulation. At 2 or 20 min into the afterdischarge the ganglion was removed, the bag cell organs dissected free and immediately homogenized with a ground glass homogenizer in 10% trichloracetic acid on ice. The resulting precipitate

was collected by centrifugation, washed twice with trichloracetic acid and extracted with acetone-ethanol to remove lipids. The pellet was resuspended in 0.6 ml of 0.2 M sodium phosphate pH 6.4 containing 100 μg/ml ribonuclease A (Sigma) and incubated at 37°C for 2 hr. Trichloracetic acid was added to precipitate the protein and the pellet washed twice with 10% trichloracetic acid, rinsed with acetone and resuspended in sample buffer containing 10% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol and 0.001% bromphenol blue for electrophoresis as above.

Afterdischarge-dependent phosphorylation. A second method that was used to investigate changes in the state of phosphorylation during afterdischarge was a back phosphorylation or postlabeling procedure similar to that described by Dolphin and Greengard (1981). Large animals (reproductive tract weights 3.0-7.5 g) were employed for this purpose due to the prominence of BC-2 protein in their bag cell tissues.

Abdominal ganglia were dissected in the recording chamber at 14°C to prepare hemiganglia consisting of separate right and left sides and associated bag cell clusters. The cut preparation was rinsed 2-3 X with fresh FSW at 14°C and then either the left or right bag cell cluster was afterdischarged by stimulating the pleurovisceral connective with an extracellular suction electrode (22V, 2.5 msec, 6Hz, for 5-10 sec). The remaining cluster was employed as a control. In some experiments, bag cell clusters in whole abdominal ganglia were triggered to afterdischarge and matched ganglia from animals with similar reproductive tract weights were used for controls. At either 2 or 20 min into the afterdischarge the experimental and control hemiganglia were removed from the recording chamber, the bag cell organs quickly dissected free and homogenized separately in ground glass homogenizers containing 50 mM sodium acetate buffer (pH 6.5), 10 mM magnesium chloride, 1 mM zinc acetate and 1 mM theophylline on ice. The control and experimental bag cell organ homogenates were then incubated for 1 min with 25 units protein kinase catalytic subunit as described above.

Quantitation by densitometry of autoradiograms. The phosphorylation changes occurring in the different experimental conditions described in this paper were quantitated by means of microdensitometry. Densitometer scans of the autoradiograms were carried out on a Joyce-Loebl microdensitometer, routinely using a 0-1 O.D. scale. In all cases peak area was quantitated by cutting out and weighing of the peak in question from the densitometer tracing on copy paper as described by Rudolph and Krueger (Rudolph and Krueger, 1979). Cyclic AMP and PKC effects were quantitated directly using peak area. In the case of tracings from pre-labeled preparations, BC-1 and BC-2 areas were expressed as a ratio relative to the area of an internal reference peak at 28,000 daltons (see Fig. 3) that could be routinely identified in all the preparations and did not appear to undergo a change in phosphorylation state.

In the case of the post-labeling technique, BC-1 and BC-2 peak areas were expressed as a ratio relative to the combined areas of three routinely identifiable reference peaks at 45,000, 62,000 and 68,000 daltons (Fig. 2). The two methods of choosing internal reference peaks in pre- and post-labeling conditions were different because the high optical density background in the high molecular weight region of the autoradiograms resulting from the former technique made it difficult to identify the 45,000, 62,000 and 68,000 peaks in every preparation, therefore the major phosphoprotein at 28,000 was employed as a reference peak when pre-labeling. The three higher molecular weight peaks (45,000, 62,000 and 68,000) were clearly identifiable in every post-labeling preparation whereas the 28,000 dalton phosphoprotein was only a minor phosphoprotein under these experimental conditions.

Protease digestion experiments. To establish the proteinaceous nature of the phosphorylated bands observed on the SDS-polyacrylamide gels, experiments were conducted with trypsin and pronase. Pronase (Calbiochem) at a concentration of 100 µg/ml and trypsin (Sigma, Type XII) at a concentration of 50 µg/ml were incubated with crude membrane preparations, prelabeled by incubation with protein

kinase catalytic subunit and [γ-^{32}P] ATP. Similar experiments were carried out on bag cell proteins prelabeled with $Na_2H^{32}PO_4$. Controls were conducted with the same treatment using heat-inactivated enzymes.

Pulse-chase experiment. In order to demonstrate that the major phosphoprotein in bag cell tissue was not the prohormone precursor to the egg-laying hormone reported by Arch (1976), a double-label experiment was conducted. Pleurovisceral ganglia were incubated for 1 hr at 14°C in artificial sea water medium (Arch, 1972) supplemented with 20 mM glucose and containing 0.5 mCi ^{3}H-leucine. A crude membrane fraction was prepared from the bag cell clusters as described above and then reacted with [γ-^{32}P] ATP and protein kinase catalytic subunit. The reaction was stopped after 1 min by the addition of the sample buffer described above. An SDS 10%-polyacrylamide gel was run and the gel tracks sliced into 0.25 mm segments with a razor blade. These segments were then counted in Wilson's cocktail (Ward et al., 1970) for ^{32}P and ^{3}H using a Beckman LS-230 scintillation counter, corrections being made for count crossover.

Amino-terminal amino acid sequence analysis of protein BC-2. In order to characterize the bag cell specific substrate of phosphorylation (BC-2) as fully as possible from the small amount of material available, we employed a new, highly sensitive protein sequencer developed at Caltech. This device is two orders of magnitude more sensitive than the next most sensitive protein sequenator in existence and allows the determination of amino acid sequence from a single protein band excised from an SDS-polyacrylamide gel (Hewick et al., 1981). A crude membrane pellet was prepared from bag cell clusters pooled from 30 animals. This pellet was resuspended in 200 µl sample buffer, heated at 90°C for 15 min. Sodium thioglycolate was added to a final concentration of 5 mM. The sample was then electrophoresed on an SDS 10%-polyacrylamide gel, followed by Coomassie blue staining. The Coomassie blue stained BC-2 band was excised from the gel and eluted electrophoretically

by the method of Hunkapiller and Hood (1981): The excised gel fragments in 0.05 M Tris acetate buffer (pH 7.8) containing 0.01% SDS were placed in an apparatus for the electrophoresis (60V, 16 hr) of the protein into a collection tube capped with a disc of Spectrapor dialysis membrane (Spectrum Medical Industries, California, 6000-8000 dalton cutoff). The 200 µl of sample concentrate was salt exchanged by electrodialysis (50V, 8h) against 0.05 M ammonium bicarbonate containing 0.1% SDS (Hunkapiller and Hood, 1981). An aliquot of the eluted sample was run on an SDS-12% polyacrylamide gel and stained with Coomassie blue to ascertain purity. A single band was observed with an approximate molecular weight of 20,000-22,000 daltons. An aliquot of the eluted protein was divided into two samples, one of which was hydrolyzed, the other untreated and these were then subjected to amino acid analysis (Smyth et al., 1963) on a Durrum D500 amino acid analyzer. Fifty µl of the eluted sample was loaded into the cartridge of the new highly sensitive gas-liquid solid phase sequenator developed at Caltech (Hewick et al., 1981), and sequenced for 22 cycles. We estimate (based on amino acid analysis) that the sample used for sequencing contained approximately 100 pmols protein.

Results

Afterdischarge-dependent phosphorylation. When bag cells are stimulated to afterdischarge in FSW at 14°C there is a significant change in phosphorylation of two proteins, BC-1 and BC-2. One of these, BC-2, a protein of molecular weight 20,600 (±400) daltons, undergoes a significant increase in phosphorylation with both pre- and post-labeling techniques. The range of molecular weights determined for phosphoprotein BC-2 from 24 separate SDS-10% polyacrylamide gels determined from experiments over a 2 year period was 18,500-24,000 daltons.

This afterdischarge-dependent phosphorylation change was seen as a 73% (±9%) decrease in phosphorylation of the BC-2 protein substrate in bag cell organ homogenates

from abdominal ganglia allowed to afterdischarge for 20 min when back phosphorylated with protein kinase catalytic subunit. No change in phosphorylation of the minor phosphoprotein band BC-1 was detected in these experiments. The result from a single hemiganglion experiment is shown in Figure 2. The change in phosphorylation state of BC-2 with afterdischarge was significant ($P < 0.05$, $N = 4$) with a paired t-test analysis. There was no difference in the density of the Coomassie blue stained BC-2 protein band from the afterdischarged and control bag cell organs upon visual inspection. In two cases out of four a change in phosphorylation of a protein running near the front (approximately 12-14,000 daltons) was also observed (see Fig. 2). These back phosphorylation labeling studies conducted with exogenous protein kinase indicate that BC-2 undergoes a substantial increase in phosphorylation during afterdischarge and consequently there are fewer sites available for phosphorylation upon incubation with radiolabeled ATP. Experiments were also conducted at a time point 2 min into the afterdischarge, when cyclic AMP concentrations are known to peak (Kaczmarek et al., 1978), but no detectable change in phosphorylation of any of the bands was observed ($N = 4$). There was no significant change in phosphorylation of BC-2 at this time point ($25 \pm 17\%$ increase).

Experiments were also conducted with whole abdominal ganglia pre-labeled for 22-24 hr with inorganic ^{32}P. The pattern of phosphoproteins of bag cell organs, labeled in this manner, was similar but not identical to that obtained under *in vitro* labeling conditions with protein kinase. Bag cell organs prelabeled in this manner were electrically stimulated to afterdischarge, dissected at time points of 2 min and 20 min into the afterdischarge and their protein phosphorylation patterns compared to unstimulated controls. The two phosphoproteins that appeared to undergo reproducible changes in phosphorylation were a 33,000 dalton ($32,500 \pm 600$) and the BC-2 protein (Fig. 3). The 33,000 dalton band (BC-1) increased in phosphorylation by 82% ($P < 0.005$) at 2 min into the afterdischarge and it appeared to remain elevated at 20 min (Table 1).

The BC-2 protein was observed to undergo a change (92%, $P<0.025$) in phosphorylation at 20 min into the afterdischarge, but no significant change at the 2 min time point. A control band of 45,000 daltons analyzed in a similar manner did not undergo a significant change in phosphorylation at either time point (Table 1). High molecular weight (>100,000 dalton) phosphoproteins were difficult to quantitate as a result of the often intense but diffuse labeling of these bands with long ^{32}P-prelabeling incubation periods.

Protein kinase catalytic subunit stimulated phosphorylation. In a series of 5 separate experiments, the effect of exogeneously added protein kinase catalytic subunit on phosphorylation of bag cell proteins in a homogenate of bag cell organs was studied (Fig. 4). All of the major labeled bands were found to be trypsin and pronase sensitive. The three protein substrates showing the greatest increase in phosphorylation (as quantitated by increase in the densitometric area of individual peaks of an autoradiogram) were the BC-1, BC-2, and 100,000 dalton bands. We repeated this procedure for the other regions of the nervous system and found that the 100,000 dalton phosphoprotein occurs in the connective tissue surrounding the cluster but not in the bag cell tissues (Table 2). This phosphoprotein may be the 100,000 dalton paramyosin described in Aplysia muscle (Mandelbaum, 1980). The BC-2 substrate is a major band both on gels stained for protein with Coomassie blue and on autoradiograms showing the basal phosphorylation state. The regional distribution of this band (Table 2) shows that it is found within the bag cell cluster as well as the pleurovisceral connective nerve where bag cells processes extend and appears to be relatively specific to these tissues. Treatment with PKC results in a significant increase in the phosphorylation of this band on autoradiograms relative to other major bands such as the 62,000 and 68,000 dalton bands. The BC-1 phosphoprotein is a minor band on both protein stained gels and autoradiograms of the basal phosphorylation state in bag cell organs but undergoes a substantial increase in phosphorylation

upon treatment with PKC. The increase was difficult to quantitate due to the very small amount of phosphorylation observed in this band under basal conditions (Fig. 5).

Cyclic AMP-stimulated phosphorylation. In a study (N = 7) of endogenous cyclic AMP-stimulated protein kinase in crude membrane preparations from bag cell tissues, a cyclic AMP dose-dependent increase in phosphorylation of all the major bands was observed (Fig. 5 and 6). The phosphoproteins undergoing the greatest increase in phosphorylation were the BC-2 and 68,000 dalton bands. As shown in Table 2, the 68,000 dalton phosphoprotein is widely distributed throughout the nervous system and is not enriched in bag cells. Moreover, recent studies of the regional distribution of phosphoproteins show that the 68,000 dalton substrate is not found in the connective tissue sheath of the pleuroabdominal connective nerve but is enriched in the nerve trunk (unpublished observations). This phosphoprotein may be the one described earlier in pleurovisceral connective nerve by Ram and Ehrlich (1978). The BC-1 protein appeared to undergo the smallest cyclic AMP stimulated phosphorylation change observed although the magnitude of this change was difficult to quantitate for the reasons mentioned above.

The protein phosphorylation reaction was also conducted in a sodium acetate buffer medium containing 1 mM EGTA in the presence or absence of 4 mM calcium chloride. Calcium was found to inhibit both basal and cAMP-stimulated protein phosphorylation activity, a finding that has also been reported in other systems (Lemay et al., 1974). Cyclic AMP stimulated phosphorylation was inhibited by more than 50% under these conditions. EGTA alone enhanced the phosphorylation rate over controls lacking EGTA. Under the reaction conditions used to study the kinase activity in bag cell tissues, it may be that endogenous calcium ion inhibits the observed basal phosphorylation rate.

Characterization of the BC-2. The BC-2 protein, which we have shown to undergo a significant increase in phosphorylation during afterdischarge and which

is the major substrate for both endogenous and exogenous protein kinase in bag cell tissues was further characterized. As noted above, it was detectable as a distinct phosphoprotein on autoradiograms prepared from pleuroabdominal connective nerves and bag cell clusters but not pleural-pedal or cerebral ganglia (Table 2). An SDS-polyacrylamide gel was run with bag cell tissues labeled by the two methods ([γ-^{32}P]-ATP and ^{32}P as sodium orthophosphate) and the bands identifiable as BC-1 and BC-2 appeared to run with identical electrophoretic mobilities under both labeling schemes.

In order to demonstrate that BC-2 was not identical to the 29,000 dalton ELH precursor earlier reported by Arch (1976), double-label experiments were conducted using ^{3}H-leucine to follow newly synthesized protein. The ^{32}P-labeled phosphoprotein migrated faster and ran separately from the ^{3}H-labeled protein described as Pro-ELH by Arch (Fig. 7B).

The BC-2 protein was isolated from an SDS-polyacrylamide gel and analyzed on a gas-liquid solid phase sequenator as described under methods. A partial sequence of the first 23 residues of the N-terminal region of 100 picomoles of the protein was obtained (Fig. 7A). This analysis was repeated on a second, independently isolated BC-2 band, confirming this partial sequence. This region seems to be hydrophobic in character. The protein sample was also subjected to amino acid analysis twice and the amino acid compositions from each run are shown in Table 3. Much less sample was available for the second amino acid analysis (run 2) and therefore the values obtained are less reliable. The data from the two runs were not averaged but are shown separately in Table 3. A computer search was conducted to determine if the partial sequence obtained was similar to that found in any previously described proteins. There was no significant homology between the partial sequence of the first 23 residues of this protein and any of the 1,612 sequenced proteins examined in the search.

Discussion

Cyclic AMP-stimulated phosphorylation is brought about by the action of the tetrameric enzyme, cyclic AMP-dependent protein kinase (Rubin and Rosen, 1975). On binding cyclic AMP to the two regulatory subunits, the tetramer dissociates releasing the two catalytic subunits which then transfer the terminal phosphate of ATP to serine and threonine residues of substrate proteins. Many tissues in diverse organisms have been shown to possess this enzyme (Greengard, 1978). Of particular interest to the neurobiologist are those systems where protein kinase action is thought to bring about a change in the state of membrane excitability.

A number of neural systems, both invertebrate (Paris et al., 1980; Neary et al., 1980) and vertebrate (Browning et al., 1979; Greengard, 1978) have been investigated in an attempt to address the role of protein phosphorylation in membrane excitability. One of the major difficulties in studying neural systems such as these is the heterogeneity of the cellular population under investigation. Non-neural preparations such as the turkey erythrocyte (Greengard, 1978) and cultured adrenal medulla cells (Amy and Kirshner, 1981) have provided relatively homogenous populations of cells for the study of the relationship between phosphorylated proteins and their putative functions such as ion flux and secretion. The bag cells of Aplysia represent a neural system where the cellular homogeneity makes it particularly suitable for the study of biochemical events and their relationship to membrane excitability (Strumwasser et al., 1981). All neurons in the bag cell cluster are electrically coupled by means of gap junctions (Kaczmarek et al., 1979) and afterdischarge synchronously in response to brief electrical stimulation. We have previously presented biochemical and electrophysiological evidence for a role of cyclic AMP in the excitability changes during afterdischarge.

In this paper, we have investigated protein phosphorylation in the bag cell neurons and its relationship to afterdischarge. We have demonstrated that cyclic AMP can act on an endogenous kinase in bag cell tissue to stimulate the phosphorylation

of specific proteins. Two proteins were shown to undergo significant changes in phosphorylation state associated with afterdischarge. The regional distribution of all the major phosphoproteins found in bag cell tissues was investigated and only BC-2 appeared to be relatively specific to bag cell tissues (Table 2). This protein was the major substrate for both cyclic AMP-stimulated phosphorylation and phosphorylation stimulated by added catalytic subunit of cyclic AMP-dependent protein kinase. Whether the change in phosphorylation state which occurs in this protein during afterdischarge is a direct consequence of the increase in cyclic AMP levels during afterdischarge (Kaczmarek et al., 1978) cannot however, be determined at present. Although a protein may serve as a substrate for cyclic AMP-dependent protein kinase in vitro, this does not ensure that its phosphorylation in vivo is solely under the control of this enzyme. Other mechanisms, such as calcium-dependent phosphorylation may be the primary regulators of phosphorylation state for a particular protein (Schulman and Greengard, 1978a,b). It is possible that the BC-2 protein which did not undergo an increase in phosphorylation at 2 min into the afterdischarge (when cyclic AMP levels peak) may undergo a later Ca^{2+}-dependent phosphorylation. In the bag cell studies reported here, we found that exogenous calcium did alter phosphorylation in cell-free preparations in vitro. Studies are planned to investigate this phenomenon in more detail.

Under both of the labeling paradigms that we have used, prolonged prelabeling with ^{32}P-orthophosphate and rapid 'back-phosphorylation' with PKC, we observed a phosphoprotein with an apparent molecular weight of 33,000 (BC-1). Although the former method showed the phosphorylation state to alter at the onset of afterdischarge, no change in phosphorylation could be detected using the latter method. The small amount of phosphorylation of the BC-1 band in a homogenate of bag cell tissues under basal conditions may account for the difficulty of quantitating this change with the back phosphorylation technique.

The bag cell neurons undergo a number of characteristic changes in excitability following brief electrical stimulation. The onset of afterdischarge is composed of a one to two minute bout of high frequency firing followed by a prolonged period of lower frequency firing of broad action potentials with a predominant calcium component (Strumwasser et al., 1981; Acosta-Urquidi and Dudek, 1981). Upon cessation of afterdischarge, these neurons remain refractory for a period of many hours (Kupfermann and Kandel, 1970). The extracellular application of cyclic AMP analogues can trigger this complete sequence without electrical stimulation (Kaczmarek et al., 1978). The fact that BC-2 undergoes a large change in phosphorylation at 20 min into the afterdischarge but no significant change at 2 min suggests that it may be associated with one of the later phases of afterdischarge. It will be interesting to determine the complete time course of phosphorylation changes for both the BC-1 and BC-2 proteins.

Whether or not these proteins that we have studied are indeed substrates for the electrical changes induced by cAMP and protein kinase in bag cells, cannot be determined at present. They could also be involved in a host of other cellular functions including peptide processing, intermediary metabolism or secretory function, although we have shown that BC-2 is not the precursor to ELH previously described (Arch 1976; Loh et al., 1977). That the BC-2 protein is not a secreted one is suggested by the fact that there is no apparent change in the density of the Coomassie blue protein staining of the band on polyacrylamide gels of bag cell clusters made to afterdischarge.

In testing the hypothesis that a cyclic AMP dependent phosphorylation of protein controls the excitability of bag cell neurons, we have previously shown that microinjection of the catalytic subunit of cyclic AMP-dependent protein kinase into individual bag cells enhances their calcium action potentials in a manner consistent with a decrease in membrane conductance to potassium ions (Kaczmarek et al., 1980; Kaczmarek

and Strumwasser, 1981a). Similar results have been reported for the enhancement of spike duration in sensory neurons initially primed with TEA in Aplysia (Castellucci et al., 1980). We have demonstrated a parallel increase in both cyclic AMP levels and intracellularly recorded spike broadening during afterdischarge in the intact bag cell clusters (Strumwasser et al., 1981). Recent studies (L. K. Kaczmarek, and F. Strumwasser, 1981b) on voltage clamped individual bag cell neurons in culture suggest that cyclic AMP acts by decreasing a calcium-dependent potassium conductance. It may be, therefore, that one of the two phosphorylation substrates reported in this paper (BC-1 and BC-2) is functionally linked to a potassium conductance and may play a role in spike broadening and enhancement of calcium influx. It is therefore of interest that Alkon and colleagues (Alkon, 1980; Neary et al., 1980) have characterized a long-lasting depolarization in type B photoreceptors of the mollusc, Hermissenda associated with behavioral conditioning. They attribute this depolarization to a decrease in potassium conductance and find that it correlates with a change in the phosphorylation of 2 proteins of molecular weight 20,000 and 23,000 daltons. In view of the similar electrical changes in these cells and in bag cells, it will be important to determine if these phosphoproteins are related to the approximately 21,000 dalton protein we observe in bag cells. The sequence data presented in this paper may make such a comparison possible.

BC-2 could also be related to the 22,000 dalton protein phospholamban that has been found in cardiac sarcoplasmic reticulum (Tada et al., 1975). Phospholamban appears to play a role in the control of intracellular calcium ion concentrations by altering the activity of Ca^{2+}-ATPase. Control of intracellular calcium would be expected to be very important during bag cell afterdischarge, and could influence the potassium conductance indirectly through calcium activated potassium channels (Meech, 1972).

We have met many of the requirements that are needed to demonstrate that protein phosphorylation mediated by cyclic AMP influences the electrical properties of the bag cell neurons (Kaczmarek et al., 1980). It remains to be determined which phosphoproteins are causally related to the electrical events of afterdischarge and subsequent refractoriness and whether these substrates are uniquely distributed among different classes of neurons.

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Figure 1. Averaged molecular weight standard curve. The abcissa represents the relative mobility (Rm) of the protein band relative to the gel front. The ordinate shows the molecular weight. The molecular weight standard curve represents the mean values (± S.D.) of the mobility of the molecular weight markers (β-lactoglobulin, trypsinogen, pepsin, ovalbumin and bovine serum albumin) from 24 separate SDS-10% polyacrylamide gels run over a 2 year period. The plotted straight line represents the best fit linear regression to this data ($R^2 = 0.86$). The mean molecular weights obtained for BC-1 and BC-2 were calculated from their relative mobilities on the same 24 polyacrylamide gels, as the standard curve. (BC-1 represents the mean calculated from the 21 polyacrylamide gels on which it was visible.)

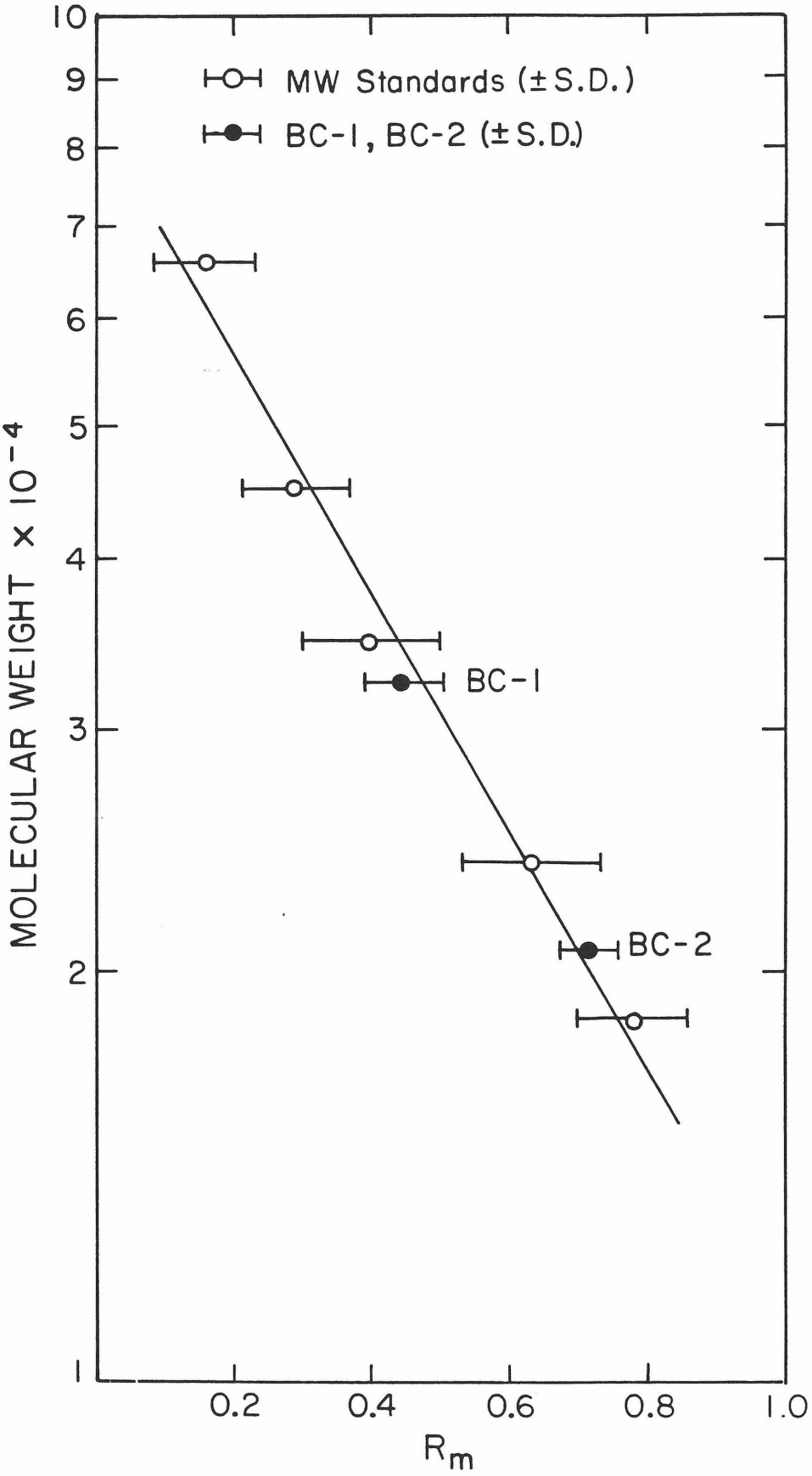
MW Standards (± S.D.)
BC-1, BC-2 (± S.D.)
BC-1
BC-2
MOLECULAR WEIGHT x 10⁻⁴
10
9
8
7
6
5
4
3
2
1
0.2
0.4
0.6
0.8
1.0
Rm

Figure 2. Afterdischarge-dependent phosphorylation: post-labeled preparation. Hemiganglion preparation where the left bag cell cluster was electrically stimulated to afterdischarge, then dissected at 20 min into the afterdischarge and 'backphosphorylated' with protein kinase. The densitometer traces shown illustrate the change in phosphorylation of BC-2 with afterdischarge (AD) compared with the control (C) from the undischarged cluster. The inset photograph shows the autoradiogram (left 2 tracks) from which the densitometer traces were made and the corresponding Coomassie blue protein stained gel (right 2 tracks). The 3 reference phosphoprotein bands used in quantitating the changes in BC-2 (see Methods) are denoted by an asterisk (*).

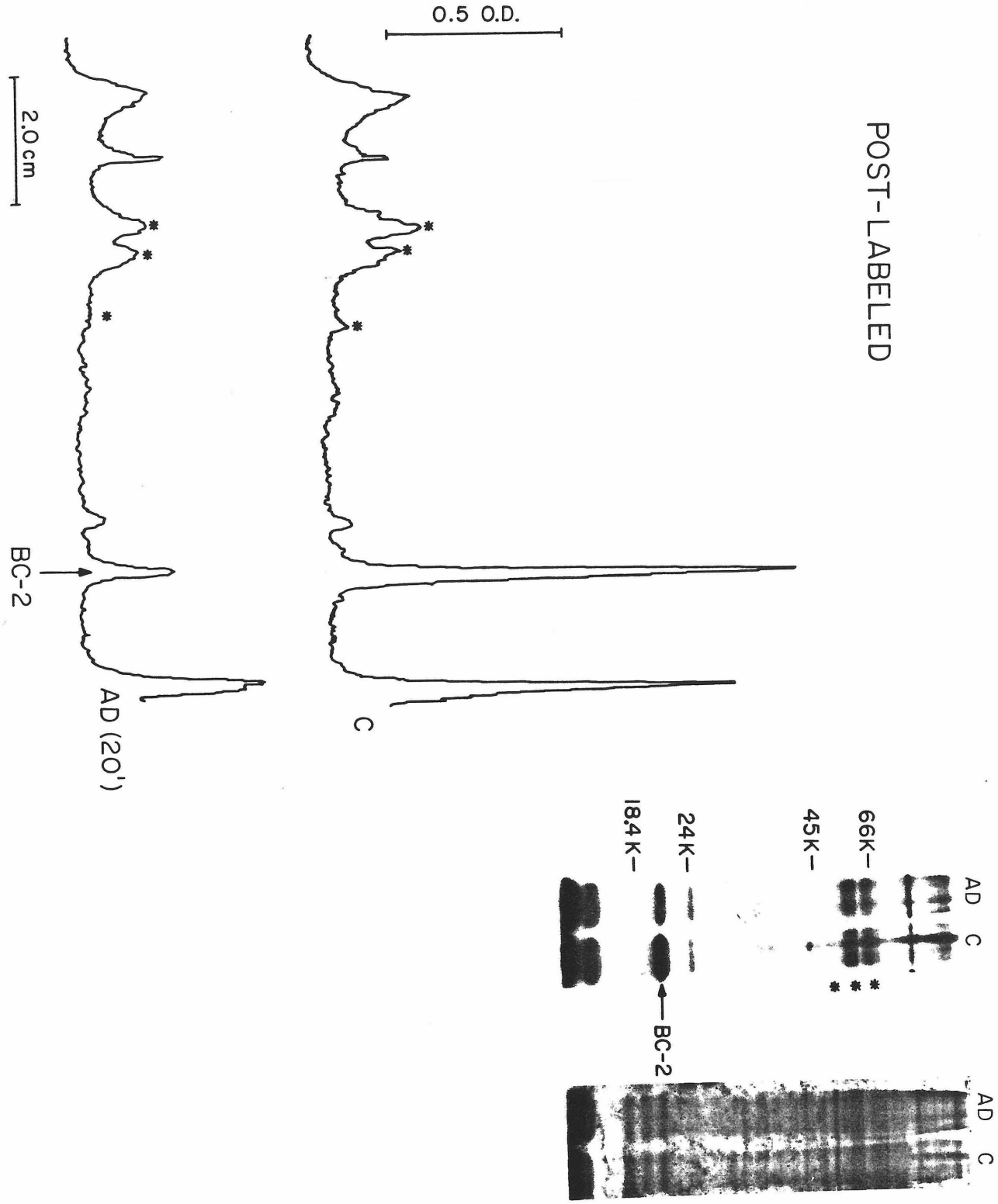
POST-LABELED
0.5 O.D.
C
AD (20')
2.0 cm
BC-2
AD C
66K–
45K–
24K–
18.4 K–
BC-2
AD C

Figure 3. Afterdischarge-dependent phosphorylation:pre-labeled preparation. The densitometer traces shown illustrate the change in phosphorylation of BC-1 and BC-2 with afterdischarge (AD) compared with the undischarged control (C) in abdominal ganglia pre-labeled with ^{32}P as Na_2HPO_4 for 22-24 hrs. the inset photograph shows the autoradiogram from which the densitometer traces were made. An asterisk (*) is used to denote the reference peak (see Methods) employed to quantitate the changes in BC-1 and BC-2.

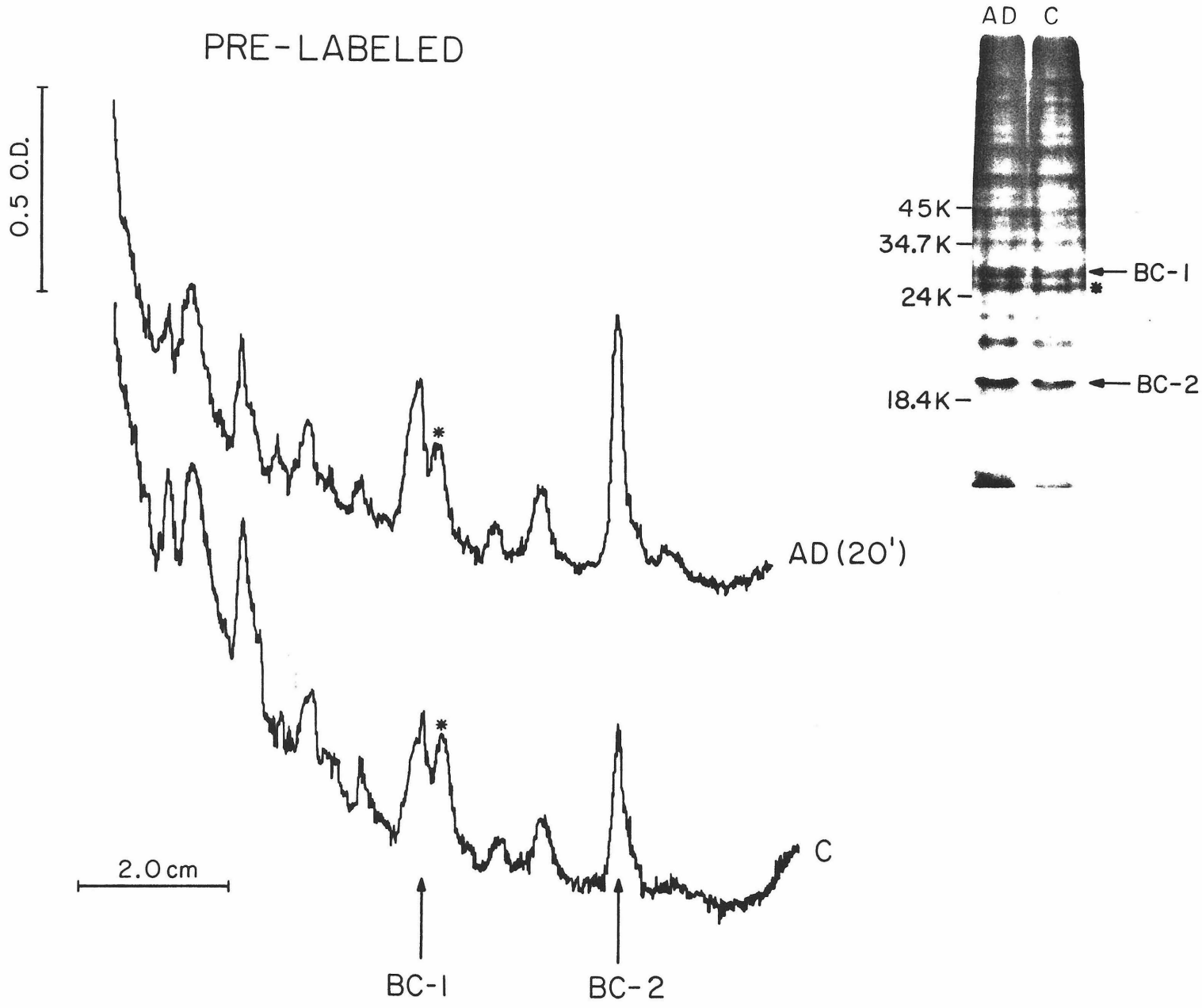
PRE-LABELED
0.5 O.D.
AD (20')
C
2.0 cm
BC-1
BC-2
AD
C
45K
34.7K
24K
18.4K
BC-1
BC-2

Figure 4. Protein kinase catalytic subunit stimulation of protein phosphorylation the increase in area under the peak on a densitometer scan of the autoradiogram. The histogram represents the data from N = 5 separate experiments (± S.E.M.).

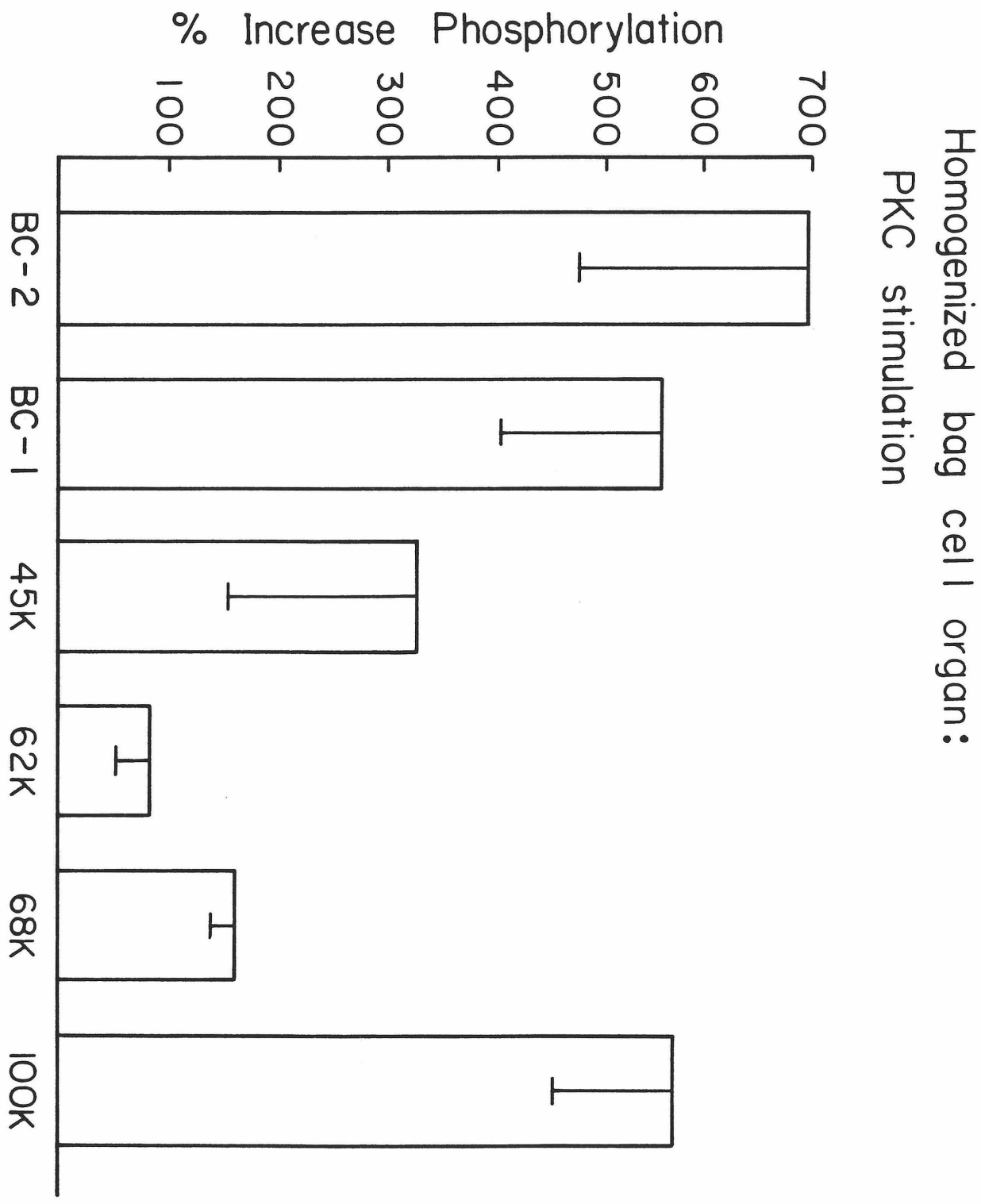
Homogenized bag cell organ:
PKC stimulation
% Increase Phosphorylation
100
200
300
400
500
600
700
BC-2
BC-1
45K
62K
68K
100K

Figure 5. Cyclic AMP and protein kinase stimulated phosphorylation. The densitometer traces show the increase in phosphorylation with 10^{-4} M cyclic AMP of proteins in a crude membrane preparation of bag cell tissues. The inset photograph shows the autoradiogram from which the densitometer traces were made. In addition, the left-most track shows the effect of a high concentration (50-100 units) of protein kinase catalytic subunit on the phosphorylation of these substrate proteins on the same gel.

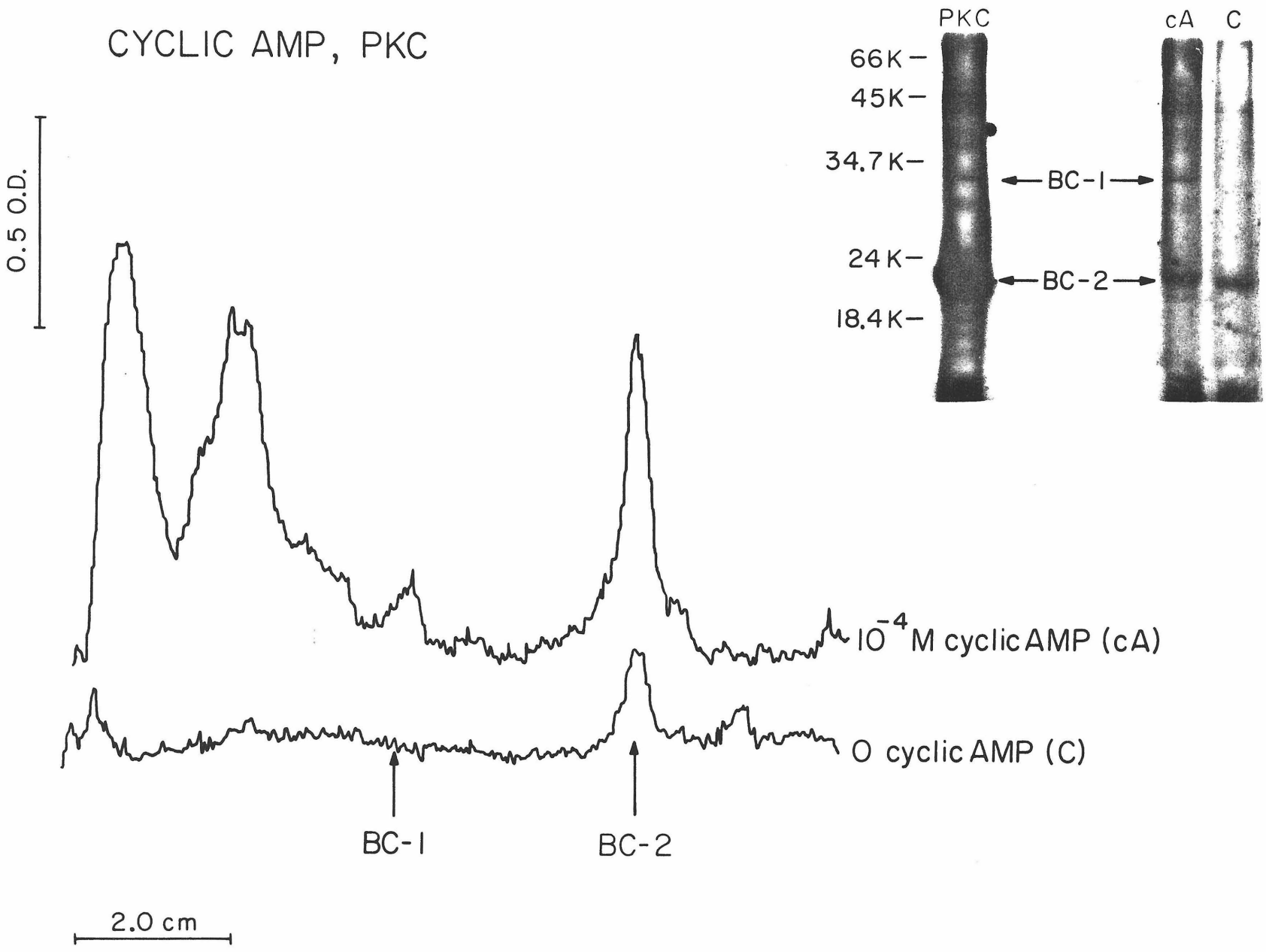
CYCLIC AMP, PKC
PKC
cA
C
66K
45K
34.7K
24K
18.4K
BC-1
BC-2
0.5 O.D.
10^{-4} M cyclic AMP (cA)
O cyclic AMP (C)
BC-1
BC-2
2.0 cm

Figure 6. Cyclic AMP stimulation of protein phosphorylation in bag cell membrane preparations. The percentage increase in phosphorylation represents the increase in area under the peak in a densitometer scan of the autoradiogram. The histogram represents the data from N = 7 separate experiments (± S.E.M.).

Bag cell membrane preparation:
cAMP stimulation

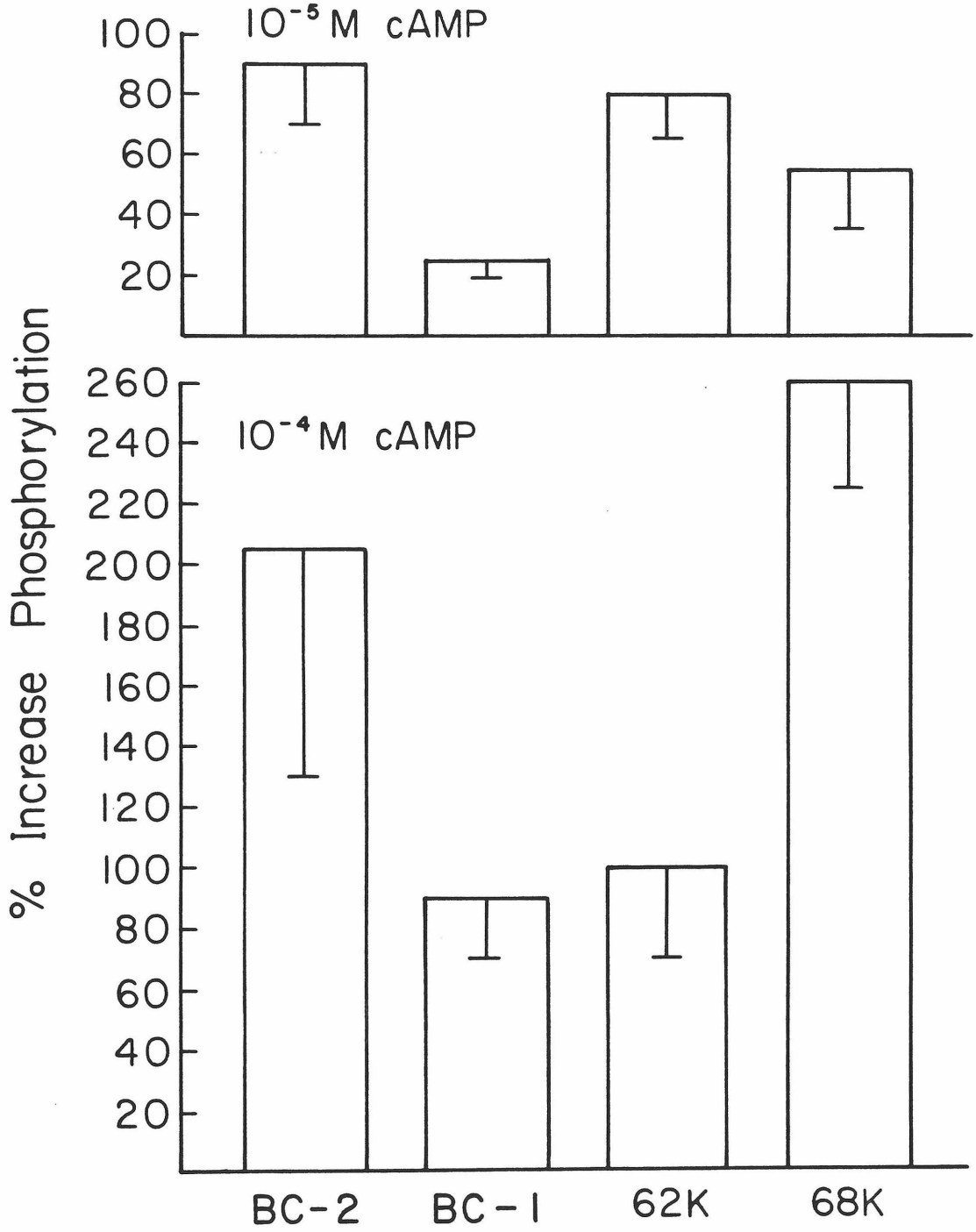

Figure 7. Characterization of the BC-2. 7A) amino acid sequence of the BC-2 phosphoprotein isolated from crude membrane preparations of bag cell tissues. The data presented are the result of two separate experiments. 7B) pulse chase experiment with ^{3}H-leucine labeling of the precursor to ELH. The radioactivity profile obtained on a SDS-10% polyacrylamide gel by sectioning is illustrated. The solid line shows the ^{3}H counts obtained, the dotted line the counts due to ^{32}P label. The approximate molecular weights (based on molecular weight standards) of the two labeled proteins are shown. The data shown represent one of three separate experiments with similar results.

A.

H_2N-( )-( )- Val - His - Gly - Lys - Asn - Phe - Ala -(Arg)

- Asn -(Arg)- Ala - Val - Lys -( )-( )-( )-( )- Phe

Val - Val -(Leu) . . . . . .

B.

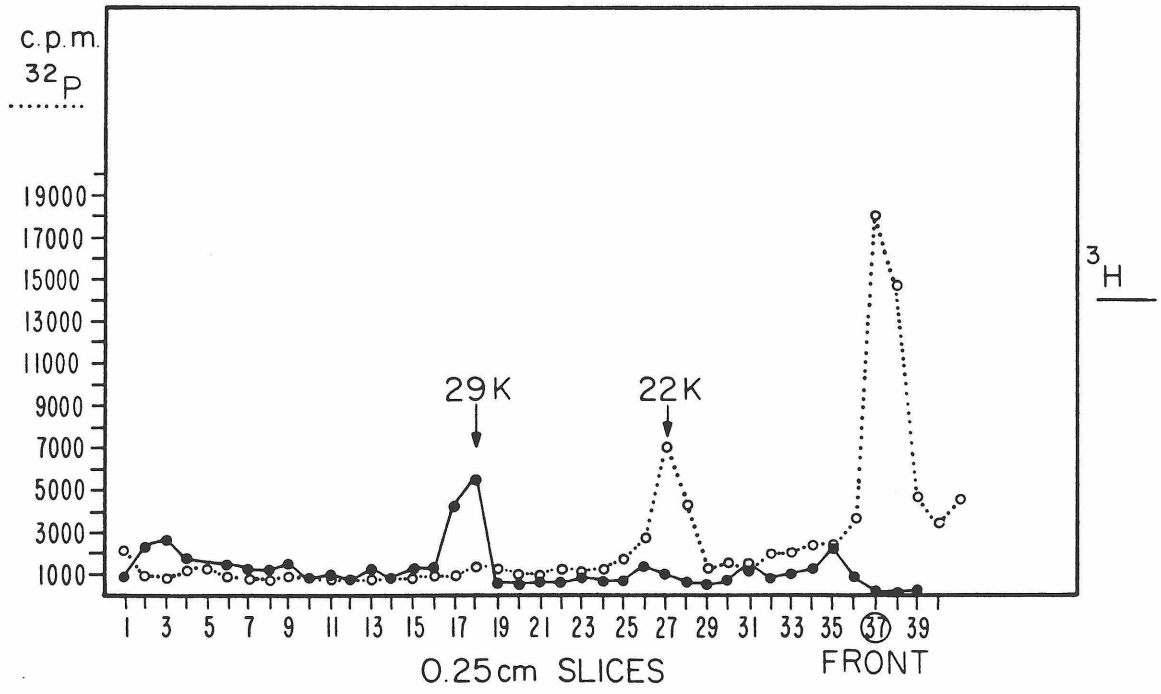

Table Legend

Table 1. The results of a study of phosphorylation changes during afterdischarge of phosphoproteins pre-labelled for 22-24 hrs with inorganic ^{32}P are shown in this table. Data are plotted as percentage change (±S.E.M.) in the area under the peak on a densitometer scan relative to a 28,000 dalton reference peak observed not to change with afterdischarge. The number of separate experiments is shown in parentheses. The BC-1 and BC-2 phosphoproteins were observed to undergo significant increases in phosphorylation during afterdischarge. The 45,000 dalton phosphoprotein is included as a representative phosphoprotein that did not undergo a change in phosphorylation during afterdischarge. Statistical significance was calculated using a one-tailed paired t-test.

Table 1

Protein (m.w.)	% Phosphorylation Change	
	2 minutes	20 minutes
BC-2	-19 ± 28% (5)	+92 ± 23%** (4)
BC-1	+82 ± 14%*** (8)	+69 ± 43%* (4)
45K	-11 ± 12% (8)	-9 ± 32% (4)

*P<0.05

**P<0.02

***P<0.005

Table Legend

Table 2. Homogenates were prepared of each of the regions of the Aplysia nervous system and phosphorylated with [γ]-ATP and catalytic subunit of protein kinase as described under Materials and Methods. Bag cell organs consisted of the bag cell cluster and surrounding connective tissue including axons of passage in the underlying connective nerve. Isolated bag cell clusters consisted of bag cell somata separated from the surrounding connective tissue. The other head ganglia were prepared from ganglion homogenates separated from surrounding connective tissues. The approximate amount of the phosphoprotein detected in autoradiograms is denoted by the number of "+" symbols; a "-" indicates that the band could not be detected.

Table 2

M.W.	Isolated* Bag Cell Cluster	Bag Cell Organ	Pleuro-visceral Connective	Pleural* Pedal Ganglia	Cerebral* Ganglion
122K		+	+	+	+
100K	-	++	++	-	-
68K	+	+++	+++	+++	+
62K	++	+++	+++	+++	+
45K	+	+	+	+	
BC-1	+	+	+	+	-
BC-2	+++	++	+	-	-

*connective tissue removed.

Table 3

Amino Acid Composition of the BC-2 Protein

	Moles/100 moles			Moles/100 moles	
	Run 1	Run 2		Run 1	Run 2
Asx	13.20	11.82	Met	0.69	0.95
Thr	1.80	3.43*	Ile	2.80	4.28
Ser	2.76	4.20*	Leu	7.64	9.20
Glx	11.48	13.04*	Phe	4.68	4.53
Pro	4.92	4.24	Tyr	2.08	0.91
Gly	9.52	n.d.	His	2.12	3.09
Ala	11.52	10.29	Lys	6.76	6.04
Val	8.20	7.59	Arg	9.52	5.37
			Cys	n.d.	n.d.

The data shown in this table were calculated by subtracting the values obtained for the unhydrolyzed sample from the hydrolyzed sample. Run 1 shows the amino acid composition data from the aliquot that was also subjected to protein sequencing (see Fig. 7A). Run 2 shows the composition data obtained from a separate sample containing much less material.

*These values are anomalously high in Run 2 due to the presence of substantial contamination by these residues in the unhydrolyzed protein sample (see Methods).

CHAPTER 3

SEROTONERGIC INHIBITION OF AFTERDISCHARGE IN PEPTIDERGIC BAG CELLS

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ABSTRACT

The neuroendocrine bag cells of Aplysia generate a long-lasting afterdischarge causing the release of the peptide egg-laying hormone (ELH) into the hemolymph (Stuart and Strumwasser, 1980). This afterdischarge activity can be elicited in vitro by electrical stimulation of the pleurovisceral connective (PVC) nerve. It has previously been shown that bag cell afterdischarge and egg-laying behavior can also be induced by a purified neuroactive peptide isolated from the atrial gland in the reproductive tract (Heller et al., 1980). We find that the afterdischarge can be inhibited in a dose-dependent manner by the extracellular application of low concentrations of serotonin (5×10^{-7} M). This inhibitory action of serotonin is blocked by the serotonin antagonist, (D)-butaclamol. We find that serotonin also prevents egg-laying (and associated behaviors) induced by this atrial gland peptide in vivo but not that induced by injection of ELH.

Afterdischarge activity in bag cells consists of a short initial phase during which the spike frequency is high and a longer, secondary phase in which the spike frequency is much lower (Kupfermann and Kandel, 1970). Intracellular recordings during serotonin inhibition of afterdischarge indicate significant decreases in spike amplitude (15%) and duration (18%) compared with controls. In addition the latency and threshold of directly elicited spikes, in the absence of afterdischarge, were considerably increased as a result of serotonin. We present arguments from these data that serotonin may be preventing the conductance decrease associated with the long-lasting second phase of afterdischarge and that this effect of serotonin can be overcome by application of the potassium channel blocker, tetraethylammonium (TEA). Although a serotonergic input to the bag cell neurons has not yet been described the pleurovisceral connective nerves, known to contain bag cell processes, have the highest concentration of serotonin receptors of any nervous tissue in Aplysia

(Drummond et al., 1980a). It remains to be determined whether these represent serotonergic synapses on bag cell processes. We speculate that serotonin, considered a transmitter mediating sensitization (arousal) in other systems in Aplysia, may act to suppress an ongoing peptide hormone release, which would have triggered a behavioral program in the freely behaving animal, thus allowing some degree of behavioral plasticity as a result of changed conditions in the external environment.

INTRODUCTION

The peptidergic bag cells from the abdominal ganglion of Aplysia californica undergo long-lasting changes in neural excitability associated with the triggering of egg-laying behavior (Kupfermann and Kandel, 1970). Such characteristic long-lasting excitability changes appear to be shared by many peptidergic systems in both vertebrates and invertebrates (Simon et al., 1979). The insulin-secreting β cells of the pancreas, for example, produce repetitive electrical discharge activity when stimulated with glucose (Meissner and Atwater, 1976). The peptides secreted by these peptidergic systems have an important role to play in determining the behavioral state of the organism. It is therefore of great interest to elucidate the means by which this peptide secretory activity is controlled.

A number of peptidergic cell systems have been found to be under both excitatory and inhibitory control. The peptidergic prolactin-secreting cells of the anterior pituitary gland represent one model system where this dual control by TRF and dopamine has been demonstrated (Macleod, 1976). It would appear that similar types of control may also exist in some invertebrate neuroendocrine systems (Kaczmarek et al., 1978). The peptidergic bag cell clusters of Aplysia in organ culture have been shown to become spontaneously active upon exposure to purified peptides from the atrial gland, a part of the reproductive tract (Heller et al., 1980). It has been shown previously that the electrically initiated afterdischarge of the bag cell neurons can be abruptly terminated by the extracellular application of concentrations of serotonin as low as 10^{-7} M (Kaczmarek et al., 1978). Like the peptidergic cells of the mammalian anterior pituitary, the bag cell neurons from the abdominal ganglion of Aplysia appear, therefore, to be under both excitatory and inhibitory control. Using pharmacological, behavioral and electrophysiological techniques, we have further explored the basis of the inhibitory control of bag cell activity.

In recent years a number of studies have been published detailing the action of serotonin on neuronal (Klein and Kandel, 1980; Pellmar and Carpenter, 1980b) and putative neuroendocrine (Drummond et al., 1980b) cells in Aplysia. In many of these cases, the function of these cells is unknown. In this paper we describe the effects of serotonin on a well defined population of peptidergic neurons that are involved in controlling reproductive behavior in Aplysia. These cells have been extensively characterized both electrophysiologically (Kupfermann and Kandel, 1970; Kaczmarek et al., 1978) and biochemically. Recent work has presented a complete characterization of the peptide egg laying hormone (Chiu et al., 1979) and considerable progress towards an understanding of the biochemical mechanism underlying the long-lasting changes in membrane excitability associated with after-discharge (Kaczmarek et al., 1978; Kaczmarek et al., 1980; Strumwasser et al., 1981; Jennings et al., 1981). In view of the importance of modulation of neuroendocrine activity by transmitters in vertebrates, studies of similar systems in invertebrates may provide insights into common electrophysiological and biochemical mechanisms.

The function of bag cell afterdischarge is to release in an all-or-none manner a large quantity of egg-laying hormone (ELH) which acts on multiple target sites to bring about egg-laying and its associated behaviors (Strumwasser et al., 1980). We show in this paper that serotonin at apparently physiological concentrations (Table 1) can act to inhibit an atrial peptide-induced or electrically-stimulated bag cell afterdischarge and that serotonin can suppress the egg-laying behavior induced by injection of purified peptides from the atrial gland in vivo. We present evidence that this in vivo effect of serotonin is not acting by suppressing egg-laying hormone action but would appear to be acting by preventing the release of this hormone from its source, the bag cells.

MATERIALS AND METHODS

A. californica was collected locally (Southern California Coast by Mr. John Scotese). The animals were kept at 14°C for electrophysiology experiments and 19°C for egg-laying assays. All electrophysiological experiments were carried out at 14°C.

Electrophysiology. The butaclamol pharmacology experiments were conducted with extracellular recording techniques and bath application of drugs. Animals employed for these experiments had a range of reproductive tract weights of 1-2 g. The pleurovisceral connective nerves that join the abdominal ganglion to the pleural ganglia were cut close to the pleural ganglia. Abdominal ganglia were than dissected out with the entire length of these connectives and placed in a 9 ml recording chamber containing filtered seawater (pH 7.8). A suction electrode was placed at the pleural end of the right connective. Recording suction electrodes were placed over the right and left bag cell clusters. Appropriate pharmacological reagents were made up in fresh filtered seawater and applied extracellularly in 100-200 µl aliquots to the bath. Afterdischarge was initiated by electrical stimulation of the right pleuro-visceral connective at ∿23V, 2.5 msec at 6 Hz for 10-15 sec. The serotonin antagonist, (D)-butaclamol (Drummond et al., 1980b) was a gift from Ayerst Labs.

Intracellular recording experiments were performed on animals having a range of reproductive tract weights of 0.5-6 g. After cutting the pleurovisceral connectives near the pleural ganglia, the abdominal ganglion was placed in filtered seawater at 14°C before transfer to a plastic petri dish (Falcon) containing 230 U/ml collagenase (Worthington) and 20 U/ml elastase (Sigma) in filtered seawater at 21°C for a 2 hr incubation. At the end of 2 hr the ganglion was removed from the enzyme solution and pinned to the base of a recording chamber. The preparation

was immersed in filtered seawater at 14°C in a recording chamber that was grounded with a platinum wire electrode. Microelectrodes used for intracellular recording (10-50 MΩ) were filled with 2 M potassium citrate and connected through a bridge circuit in a WPI-M401 electrometer which had negative-capacitance feedback. Records were taken photographically from a Tektronix oscilloscope and from a Grass chart recorder. Serotonin was bath applied in small aliquots to the 9 ml recording chamber.

Egg-Laying Behavior. Animals were isolated in individual baskets in seawater at 19 ± 0.5°C. Crude atrial gland homogenates were prepared as described (Heller et al., 1980). Purified peptide "B" was prepared as described (Heller et al., 1980) and then dialyzed (Spectrum Medical Industries, M.W. cutoff 3,500) against 10 mM sodium phosphate buffer pH 7.4 overnight at 4°C to remove urea prior to injection. Atrial peptide B or tissue homogenates in 10 mM sodium phosphate buffer pH 7.4 were injected together with an equal volume of serotonin solution in the same buffer. The injections were made through the foot into the hemocoel (Strumwasser et al., 1969). Control injections consisted of the tissue homogenate or peptide alone. Egg-laying was determined to have occurred when the egg strand appeared within a 3 hr time period following injection.

RESULTS

The bag cell afterdischarge can be triggered in vitro by electrical stimulation of the rostral end of a pleurovisceral connective. The mean afterdischarge duration was previously measured in 19 preparations as 30 min (Kaczmarek et al., 1978). The spike frequency during the afterdischarge has a characteristic time course (Kupfermann and Kandel, 1970; Strumwasser et al., 1981). There exist two distinct phases; an initial phase of high spike frequency (up to 4 Hz) lasting 2-3 min and a longer second phase in which spike frequency (0.5 Hz) is much lower. We have

found that these two phases of the bag cell afterdischarge can be dissected pharmacologically and have differing ion conductance requirements (Kaczmarek, Jennings and Strumwasser, in preparation).

Serotonin suppresses bag cell afterdischarge. We have found that serotonin has a profound inhibitory effect on an ongoing bag cell afterdischarge. Serotonin at concentrations reported to be active on other neurons in Aplysia (Table 1) inhibits both electrically stimulated and atrial peptide B induced afterdischarge activity in the bag cells. In a series of experiments in which bag cell afterdischarge was initiated by the bath application of approximately 100 nm quantities of peptide B, 1 μM serotonin reproducibly inhibited the ongoing afterdischarge within 30 sec (N = 4).

Unlike the atrial peptides which appear to act through intermediary neurons in the head ganglion, serotonin appears to act directly on the bag cell organ. Multiple-chamber experiments were conducted in which the abdominal ganglion, pleurovisceral connectives and head ganglia were separated from one another by fluid-impermeable vaseline bridges (but still attached by nerve trunks). Serotonin (2×10^{-5} M) did not inhibit afterdischarge when added to the head ganglia chamber but did inhibit an afterdischarge when added to the chamber containing the pleurovisceral connective nerves or the chamber containing the abdominal ganglion (and associated bag cell clusters). This study demonstrates that it is sufficient for serotonin to act on the pleurovisceral connective alone to inhibit an ongoing afterdischarge. We have determined the dose-response sensitivity of the bag cells to serotonin (Fig. 1) and find that the 0.1 μM-1.0 μM concentration range of serotonin required to inhibit bag cell afterdischarge is similar to that reported for serotonergic modulation of buccal mass muscles and the parabolic burster, R15 in Aplysia (Table 1).

In addition, the bag cell afterdischarge can be dissected into two distinct phases by serotonin or the serotonergic agonists bufotenine and tryptamine. The initial phase of afterdischarge, where firing frequency is high, is not noticeably

inhibited by serotonergic agonists at concentrations that are effective in the later, second phase of the afterdischarge. Serotonin must be applied at concentrations of 10^{-5} M and above to block the initiation of afterdischarge.

The second phase of the bag cell afterdischarge displays a much lower firing frequency and can be seen from intracellular recordings to have bag cell spikes with longer spike widths (increased 30%) than the initial phase (Strumwasser et al., 1981). We find this phase to be much more susceptible to serotonergic agonists than the first phase. Serotonin applied at concentrations of 5×10^{-7} M or above in this second phase typically blocks afterdischarge within 30 seconds (N = 7). The same result was obtained with bufotenine at 5×10^{-5} M (N = 2) and tryptamine at 5×10^{-4} M (N = 5). Lower dosages of serotonin (1×10^{-7} M) cause a marked slowing of spike frequency with an onset latency of less than 30 seconds (N = 7).

The differential sensitivity to serotonin agonists in the two phases of afterdischarge was also demonstrated by preincubating the bag cells in a medium containing serotonin at a concentration of 1×10^{-6} M and then electrically stimulating the bag cells to afterdischarge. The cells were responsive and displayed the high spike frequency characteristic of the first phase of afterdischarge, however in 3 cases out of 4 the afterdischarge terminated at the onset of the slow firing phase of the afterdischarge (mean duration = 4.3 min, N = 4).

To determine the specificity of this suppressive effect of serotonin, we tested the effects of the serotonin antagonist, butaclamol. Drummond et al. (1980b) have shown that butaclamol is an effective stereospecific antagonist of serotonin in Aplysia nervous tissue. Serotonin was bath applied during the second phase of the afterdischarge to bag cell preparations preincubated with either the active (D) or inactive (L) isomer of butaclamol. In preparations incubated with (D)-butaclamol, serotonin failed to produce its characteristic rapid inhibition of afterdischarge (Fig. 1). The duration of afterdischarge was significantly longer (93%, $P<0.01$)

in (D)-butaclamol preparations treated with serotonin than controls incubated with the inactive isomer (L)-butaclamol. There was no significant difference between (D)-butaclamol treated preparations and those receiving no treatment.

It has recently been shown (Kaczmarek and Strumwasser, 1981) that afterdischarge in single bag cell neurons in culture may result from a decreased conductance to potassium. In the present series of experiments we have found that the potassium channel blocker, TEA, at a concentration of 100 mM induces spontaneous discharge in intact bag cell clusters, within 1 to 20 minutes (N = 6) which resembles an electrically stimulated afterdischarge. Serotonergic inhibition of afterdischarge can also be overcome by the application of this potassium channel blocker. Bag cell preparations were preincubated in 10^{-5} M serotonin and shown to be incapable of producing an electrically stimulated afterdischarge. Upon subsequent addition of 100 mM TEA, a very vigorous spontaneous discharge was induced within 1 min (Fig. 2).

Serotonin effects on bag cell spike waveform

Intracellular recordings were made of afterdischarges induced by extracellular stimulation and the effects of bath applied serotonin on spike shape were examined. We observed that bath application of 3.5×10^{-5}-1×10^{-4} M serotonin caused a slowing in the frequency of firing and termination of the afterdischarge in a single bag cell neuron. Such a slowing in the frequency was exactly paralleled by the extracellular recordings of the whole cluster. This serotonin induced termination of an ongoing afterdischarge was associated with a small but significant decrease in both spike height and width (Fig. 3). This decrease in height and width is similar to the changes in bag cell spike shape associated with the normal termination of an electrically stimulated afterdischarge which occurs, on the average, 30 minutes after initiation. The largest change was observed in spike width which decreased as much as 18% in the presence of 10^{-4} M serotonin immediately before the afterdischarge terminated.

In an attempt to determine if these effects could be observed in single bag cell neurons independently of an ongoing afterdischarge, we employed a single microelectrode with a bridge circuit to pass DC depolarizing and hyperpolarizing pulses. DC depolarizing currents of 2-10 nA were capable of eliciting bag cell spikes from the impaled neuron. Single spikes were elicited at 2 min intervals until the cell was observed to be producing stable responses.

Bath application of 5×10^{-5} M serotonin had a rapid effect on DC induced spikes. The spike latency increased over 50% within 2-4 min after application of serotonin and the spike was abolished completely within 4-6 min (Fig. 4). This effect was observed clearly in 7 out of 8 experiments. Increasing the depolarizing current passed could produce re-appearance of the spike - although significantly higher currents were required than before the addition of serotonin. In 6 out of 8 experiments the addition of serotonin was associated with a 4-8 mV hyperpolarization of the membrane potential, occuring within 1 min of addition. Preliminary evidence with DC hyperpolarizing responses suggest that this change in latency may be associated with a decrease in input resistance of the cell.

Serotonin suppresses egg-laying behavior

The above results suggest that serotonin may have a role in controlling the excitability of bag cells. In order to determine whether serotonin can act to prevent egg-laying in the intact animal we conducted a series of behavioral experiments with the peptides from the atrial gland of Aplysia that are able to induce bag cell afterdischarge (Fig. 5). When 10 μmoles serotonin were coinjected with the equivalent of one atrial gland equivalent of purified atrial peptide B, egg-laying was prevented. The typical behavior associated with egg-laying in Aplysia (Strumwasser, 1971) such as climbing a vertical surface, cessation of locomotion and the initiation of head weaving were also not observed in these experiments. If an approximate blood volume of 100 ml is assumed for these experimental animals, then assuming

no diffusional barriers, filtering or other constraints to the free and rapid dispersal of serotonin, then the final concentration of the transmitter in the blood may have been as high as 10^{-4} M.

Serotonin by itself (or with peptides) was observed to have effects on the locomotory behavior of the animal, causing what appeared to be a lack of balance that made it difficult for the animal to adhere to and climb the wall of its tank.

When egg-laying was induced by the bag cell hormone ELH in crude extracts, coinjection of serotonin (10 μmoles) did not prevent the egg-laying or its associated behaviors. Although there was some delay in the onset of egg-laying (2-4 hr), coinjection of crude atrial gland homogenate and 10 μmoles serotonin resulted in egg-laying in 5 out of 10 experiments. This egg-laying behavior in the presence of serotonin resembled that obtained with ELH and serotonin coinjections. Recent evidence obtained in Aplysia without an abdominal ganglion (Strumwasser et al., 1981) suggests that crude atrial gland homogenates may contain a factor capable of inducing egg-laying without the mediation of bag cells. This factor may very well be a hybrid peptide consisting of ELH-like and atrial peptide-like sequences (Schlesinger et al., 1981). Our interpretation of these findings is that serotonin can act to prevent or suppress atrial gland peptide-induced afterdischarge of the bag cells in vivo before a sufficient amount of ELH has been released to induce the fixed behavioral act of egg-laying. Thus, serotonin is acting to "countermand" the atrial peptide signal activating the neuroendocrine bag cell organ.

These results also suggest that serotonin at these concentrations is not acting at the peripheral targets at which the egg-laying hormone acts, but at its central release site only.

DISCUSSION

It has become evident in recent years that serotonin may act as a transmitter or modulator on a diverse population of neurons in Aplysia. In this paper we report that serotonin can act to suppress afterdischarge in the peptidergic bag cell neurons. The concentrations at which serotonin acts is well within the physiological range reported for this organism (Table 1) and for this reason we feel that our result is unlikely to be of just pharmacological interest. In some systems in Aplysia, serotonin has been reported to have a sensitizing (Kandel, 1979) or excitatory effect (Pellmar and Carpenter, 1980) on neurons, while in other systems such as the presumed neuroendocrine cell R15, the effects of serotonin are inhibitory (Drummond et al., 1980b).

We have presented evidence that in the neuroendocrine bag cells of Aplysia serotonin can act to inhibit afterdischarge. There is evidence that ELH is secreted during the later stages of bag cell afterdischarge. There is no detectable immunoreactive ELH released into a 2 ml bathing medium during the first 2 min of an afterdischarge while at later times substantial ELH is detectable (A. Chiu, personal communication). This second phase of afterdischarge occurs subsequent to the increase in cAMP and spike width previously reported (Kaczmarek et al., 1978; Strumwasser et al., 1981). We have recently characterized (Kaczmarek, Jennings and Strumwasser, unpublished observations) the ionic dependence of this second phase of afterdischarge and find that the broadened action potentials are calcium spikes. The action of serotonin we reported in this paper serves to prevent the second (lengthy) phase of the bag cell afterdischarge during which the greatest amount of ELH is likely to be released.

Egg-laying behavior in Aplysia californica appears to be a relatively fixed act with a prescribed series of behaviors and the release of an egg strand from the ovotestis. The bag cell hormone (ELH) released during afterdischarge is believed

to act at a number of sites (Stuart and Strumwasser, 1980; Branton et al., 1978) to elicit this program of behavior and the extrusion of an egg strand. It would appear that serotonin does not act on these multiple target sites to suppress egg-laying (Fig. 5 and Strumwasser et al., 1981) but acts directly on the bag cells. This was illustrated in a series of experiments where serotonin was shown to be incapable of blocking egg-laying behavior induced by a bag cell extract containing ELH. Figure 5 also illustrates that there is some factor in atrial gland homogenates that can induce egg-laying directly without mediation of bag cells. This has been confirmed recently (Strumwasser et al., 1981; Schlesinger et al., 1981) and it is now believed that an ELH-like peptide occurs in the atrial gland in addition to the atrial peptides earlier reported.

In an attempt to characterize the serotonin inhibition of bag cell afterdischarge, pharmacological and electrophysiological experiments were conducted. Only serotonin and serotonin analogs, among many transmitters investigated (Kaczmarek et al., 1978), were effective at inhibiting afterdischarge. This inhibition was shown to be serotonergic in character with stereospecific antagonists (Fig. 1). Extracellular application of serotonin during afterdischarge produced significant decreases in action potential height and width (Fig. 3), a very different result from the changes observed with serotonin in sensory neurons (Kandel, 1979). Serotonin also produced significant increases in spike latency to depolarizing stimuli. Interestingly, it has recently been shown (Kaczmarek and Strumwasser, 1981) that one of the effects of cyclic AMP in producing afterdischarge in single bag cell neurons in culture is a significantly decreased spike latency. It may be that serotonin acts on a similar mechanism in an opposite manner to bring about the inhibition of afterdischarge.

TEA, a well characterized blocker of potassium channels, enhances the width of action potentials in molluscan neurons (Hagiwara and Saito, 1959). We have presented evidence that TEA will overcome the suppressive effects of serotonin

when electrical stimulation is incapable of inducing an afterdischarge. In contrast, the effect of serotonin on dishabituation of the gill withdrawal reflex is enhanced by TEA. In this latter system, serotonin acts to reduce the potassium current of the action potential (Klein and Kandel, 1980). It is possible therefore, that in the bag cells serotonin acts in an opposite manner, to suppress the calcium component or enhance potassium currents in bag cell afterdischarge. We have presented evidence in this paper that serotonergic inhibition of an afterdischarge is associated with decreases in spike height and width - this finding would be consistent with a direct action of serotonin on ion conductances. It has recently been shown (Kaczmarek et al., 1980; Kaczmarek and Strumwasser, 1981) that a decrease in potassium conductance may be associated with the increased excitability of bag cell neurons during afterdischarge. It is tempting to propose that serotonin exerts its effects by increasing potassium conductance and thereby directly opposing this excitatory conductance change. The findings presented in this paper are in accord with this tentative hypothesis. Voltage clamp studies on single bag cell neurons in culture are in progress to test this hypothesis (L.K. Kaczmarek, unpublished).

We have no evidence as to the source of the serotonergic inhibitory input to the bag cell system. However, it has recently been reported by Drummond et al. (1980a) that the pleurovisceral connectives have the highest concentration of serotonin receptors of any nervous tissue in Aplysia. These authors speculate that these serotonin receptors may be associated with bag cell processes in the pleurovisceral connective nerve. These results are supported by our findings with the multiple chamber experiments reported in this paper that application of serotonin to the isolated pleurovisceral connective nerve is capable of inhibiting an afterdischarge.

We have identified a putative inhibitory control of both afterdischarge of the neuroendocrine bag cells and atrial gland peptide induced egg-laying behavior

in vivo. We have characterized the pharmacological stereospecificity of serotonin inhibition of bag cell afterdischarge and described the changes in electrical properties of these neurons resulting from the actions of serotonin. In addition we have presented evidence that serotonin can inhibit afterdischarge by acting on receptors in the pleurovisceral connective nerve. We feel that the study of the action of serotonin on bag cell neurons may lead to an understanding of transmitter modulation of peptidergic function in Aplysia. The similarity between the dual excitatory-inhibitory control of this neuroendocrine system and analogous systems in mammals such as the control of prolactin release in mammotrophs may make it possible to identify a shared biochemical mechanism for the modulation of hormone release in these two systems.

It is intriguing that the effects of serotonin on the bag cells appear to be directly opposite in character to its effects on sensory neurons of the gill-withdrawal reflex (Klein and Kandel, 1980). Recently, Ter Matt and Lodder (1980) reported that acetylcholine will inhibit afterdischarge in the ovulation-hormone producing cells in Lymnaea stagnalis and that this inhibition is thought to be associated with a reaction to adverse conditions. We speculate that the serotonergic control of many different neuronal circuits including bag cells may be associated with a specific behavioral state such as arousal and in Aplysia, serotonin may act to sensitize certain neural circuits while suppressing others.

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FIGURE LEGENDS

Fig. 1. Serotonergic inhibition of bag cell afterdischarge. Abdominal ganglia were preincubated for 30 min at 14°C in a recording chamber containing either filtered seawater alone or filtered seawater containing 10^{-5} M (D)- or (L)- butaclamol. Afterdischarge was initiated by electrical stimulation to the right pleurovisceral connective. Afterdischarge was recorded by suction electrodes placed on the right and left bag cell clusters. At 4 min into the afterdischarge (and 2 min intervals thereafter) 100-200 µl aliquots of a stock serotonin solution were applied extracellularly to a final concentration as illustrated. (D)-butaclamol [but not its inactive isomer (L)-butaclamol] was an effective antagonist of the serotonergic inhibition of bag cell afterdischarge ($P < 0.01$).

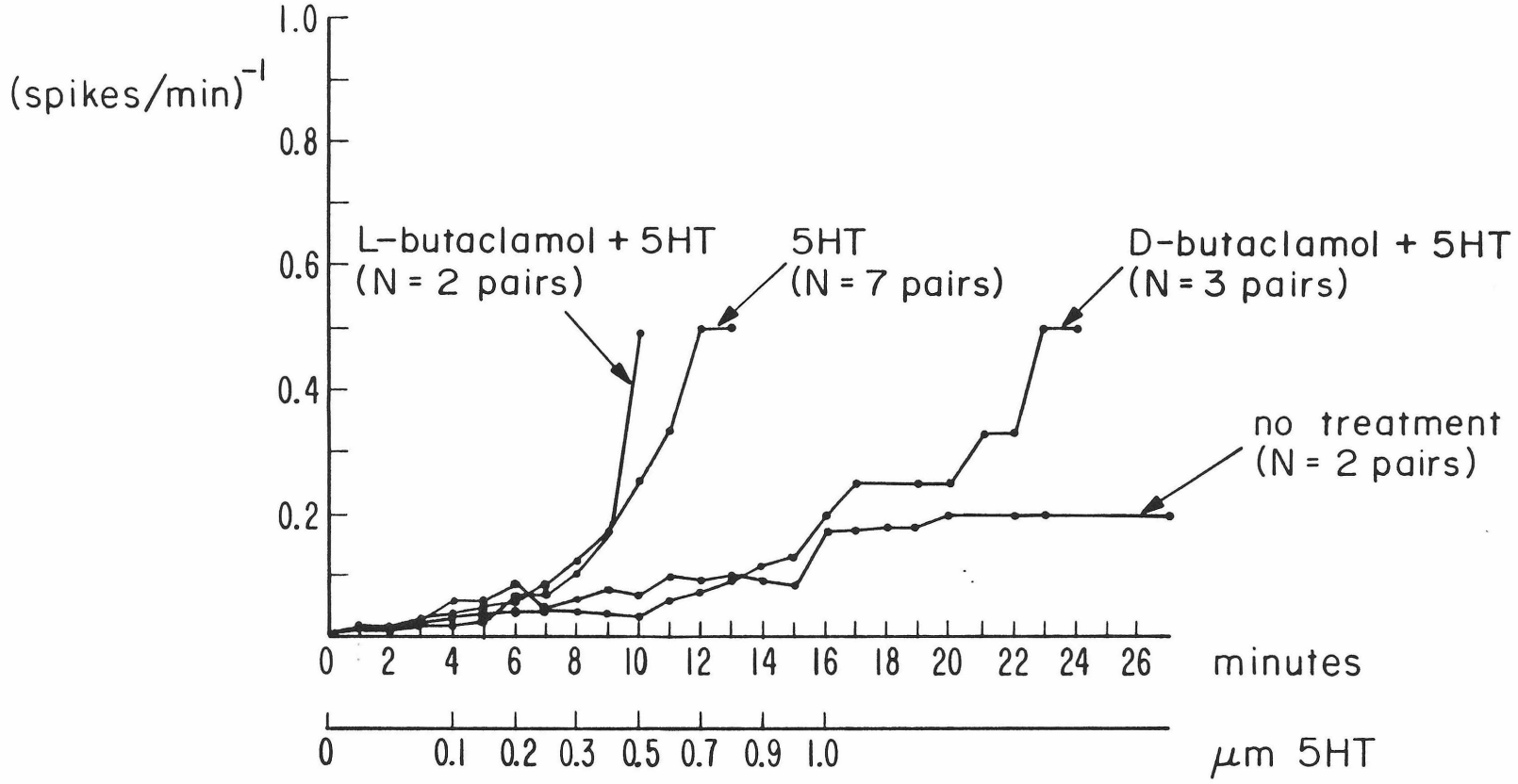
(spikes/min)$^{-1}$
1.0
0.8
0.6
0.4
0.2
L-butaclamol + 5HT
(N = 2 pairs)
5HT
(N = 7 pairs)
D-butaclamol + 5HT
(N = 3 pairs)
no treatment
(N = 2 pairs)
0 2 4 6 8 10 12 14 16 18 20 22 24 26
minutes
0 0.1 0.2 0.3 0.5 0.7 0.9 1.0
μm 5HT

Fig. 2. TEA activation of afterdischarge. In a bag cell preparation pre-treated with 10^{-5} M serotonin electrical stimulation at 22 V at 6 Hz for 15 sec was incapable of inducing an afterdischarge. Bath application of TEA to a final concentration of 100 mM caused a spontaneous afterdischarge within 20 sec.

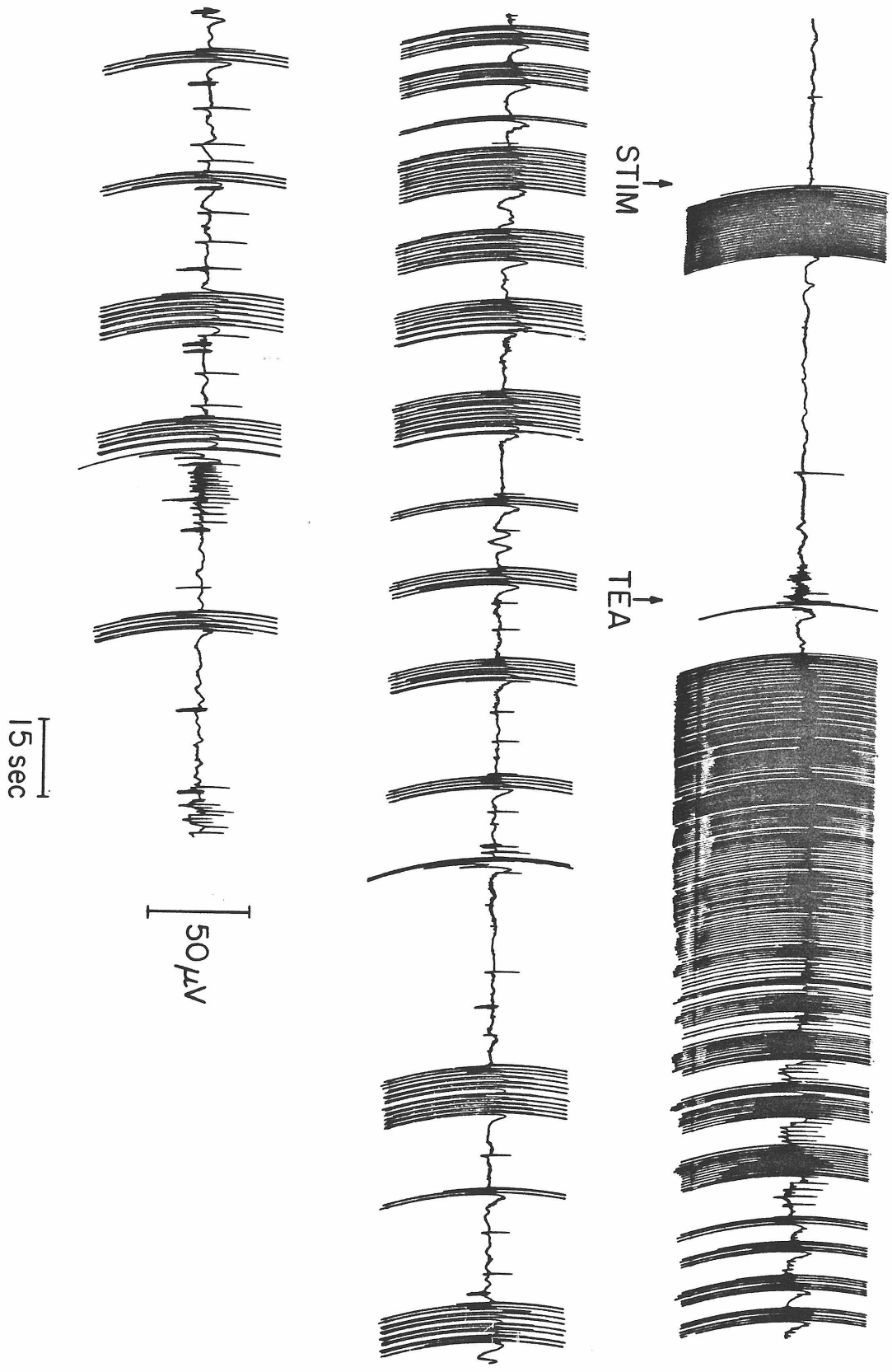
STIM
TEA
50 μV
15 sec

Fig. 3. Serotonergic actions on spike waveform. Intracellular recordings were obtained from electrically-stimulated afterdischarge. At time points of 4 min and 6 min into the afterdischarge aliquots of either serotonin (5HT) in filtered seawater or filtered seawater alone (FSW) were bath applied. The data are plotted from the chart record over the appropriate time interval and show the average value $\pm$ S.E.M.

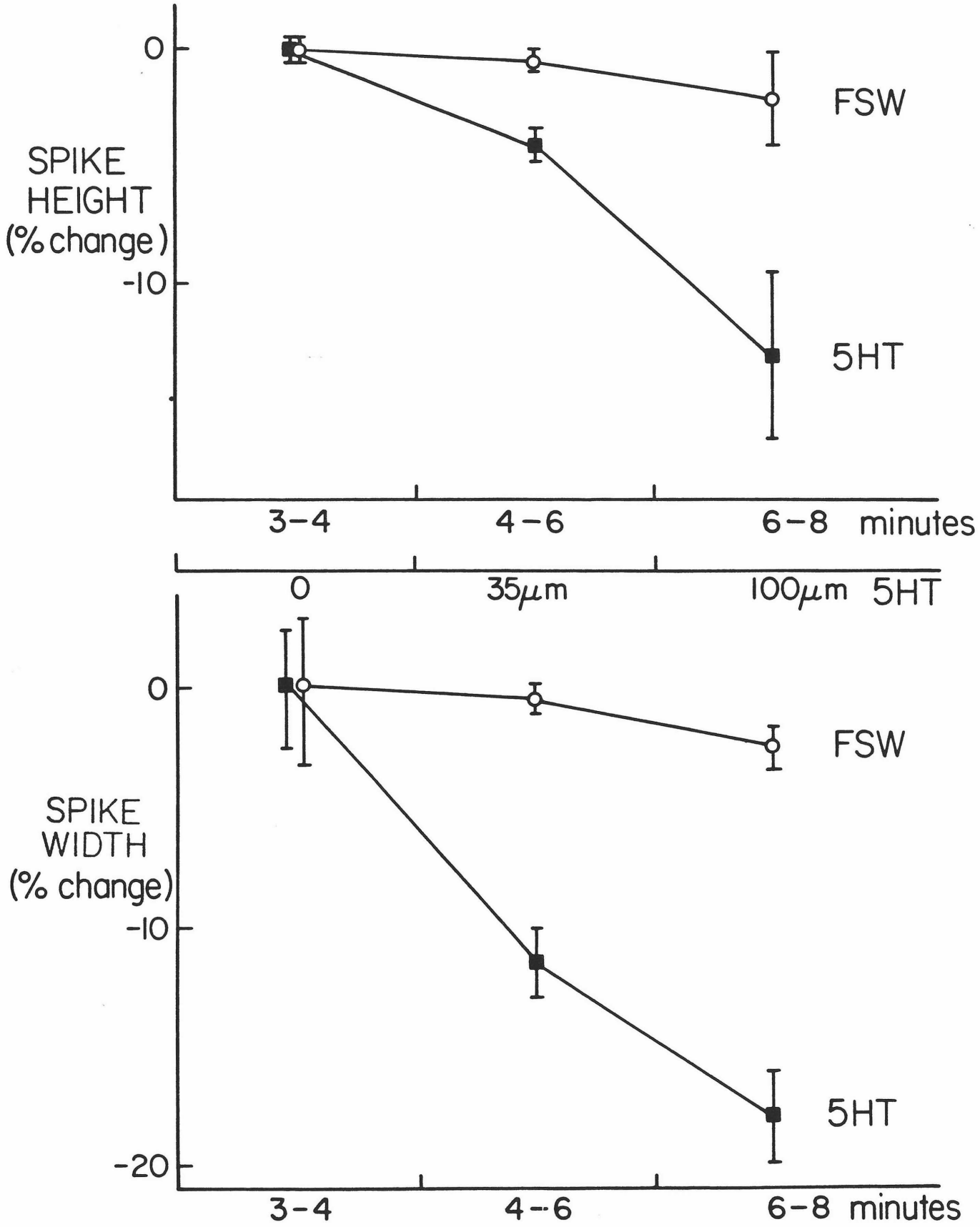
0
SPIKE
HEIGHT
(% change)
-10
FSW
5HT
3-4
4-6
6-8 minutes
0
35μm
100μm 5HT
0
SPIKE
WIDTH
(% change)
-10
-20
FSW
5HT
3-4
4-6
6-8 minutes

Fig. 4. Serotonin actions on mean spike latency. Depolarizing current pulses (300 msec, 2-10 nA) were applied to elicit bag cell spikes. After the response of the cell to current had stabilized, 50 μM serotonin was added (time 0) and the increase in spike latency monitored and plotted as a percent change. The graph shows the spike latency change as a function of time before and after serotonin addition. The graph represents the mean (±S.E.M.) observed in a pooled sample of 7 out of 8 experiments. (a) shows oscilloscope tracings of the latency shift observed in a representative experiment. The lower traces show current pulses and the upper show the evoked voltage responses. (b) is the restoration of spike generating properties at higher DC stimulus strength in the same experiment as (a), 6 min after serotonin addition.

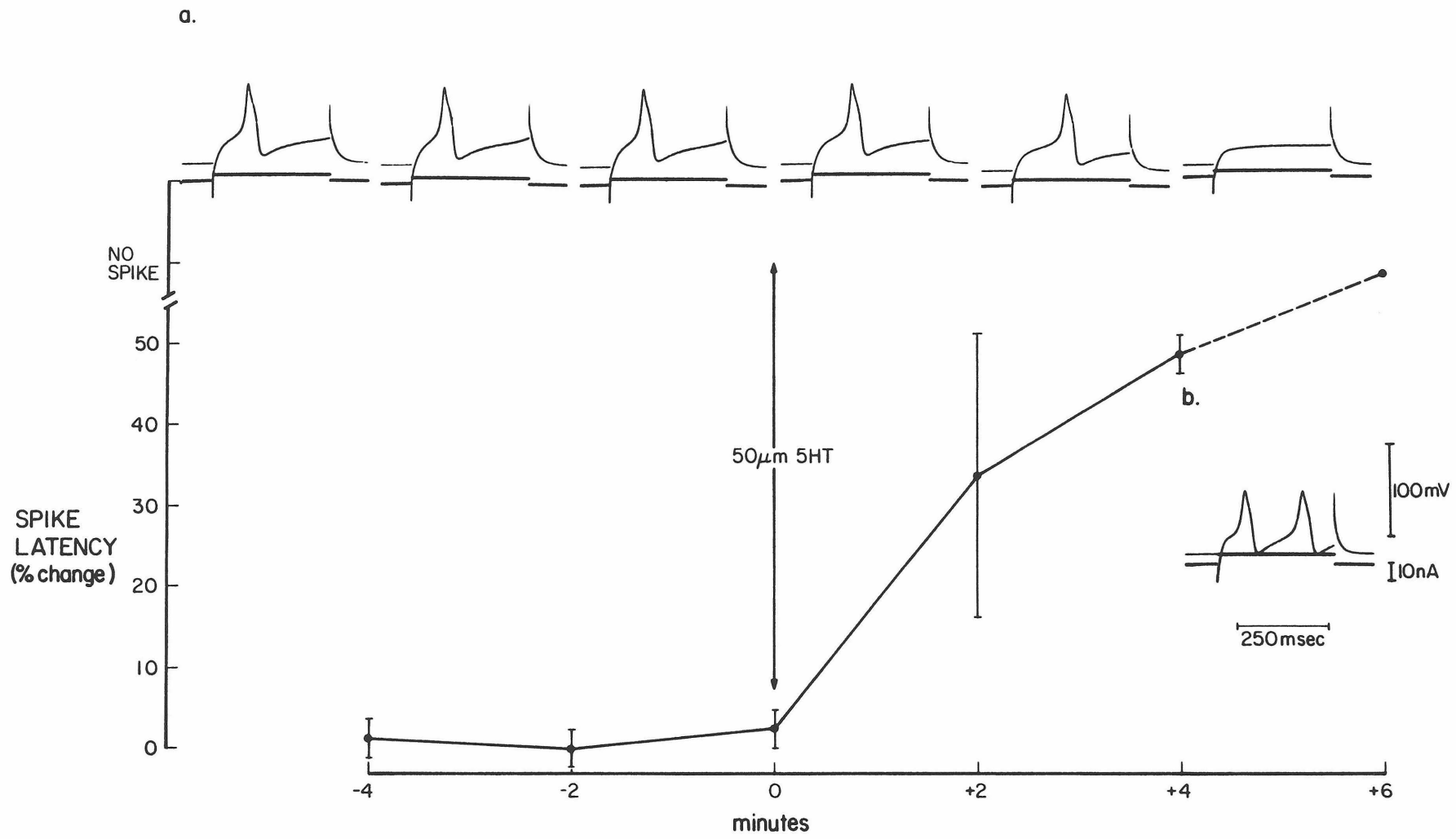
a.
NO SPIKE
50
40
30
20
10
0
SPIKE LATENCY (% change)
50μm 5HT
b.
100mV
10nA
250msec
-4
-2
0
+2
+4
+6
minutes

Fig. 5. Serotonergic inhibition of atrial peptide induced egg-laying. Serotonin and tissue homogenates or pure peptide "B" were coinjected in a sodium phosphate carrier buffer into egg-laying competent animals at 19° ± 0.5°C. Egg-laying was determined to have occurred when the egg strand appeared within a 3 hr time period following injection.

AGh = atrial gland homogenate, BCh = bag cell homogenate.

Peptide "B" = purified atrial gland peptide "B".

The amount of tissue homogenate/peptide "B" injected was equivalent to that isolated from one animal.

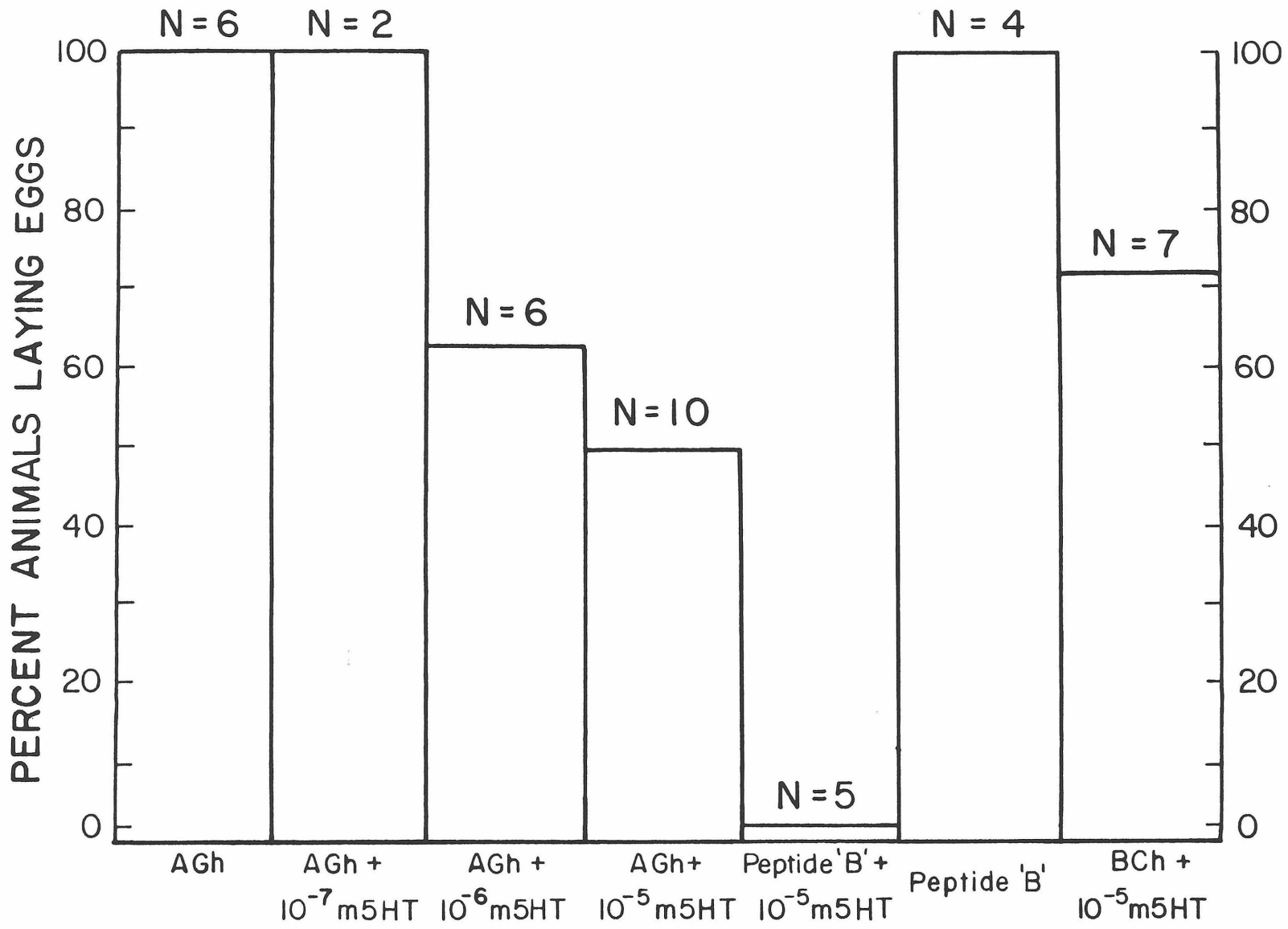
N = 6
N = 2
N = 6
N = 10
N = 5
N = 4
N = 7
PERCENT ANIMALS LAYING EGGS
100
80
60
40
20
0
AGh
AGh + 10-7 m5HT
AGh + 10-6 m5HT
AGh+ 10-5 m5HT
Peptide 'B' + 10-5 m5HT
Peptide 'B'
BCh + 10-5 m5HT

TABLE 1

Serotonin effects in Aplysia:

Preparation:	Effective dose of serotonin:	Synapse described:	Reference:
R15 burster	1×10^{-7} M	No	Drummond et al. (1980)
Cerebral, buccal and abdominal ganglion	10^{-6}-10^{-5} M	No	Gerschenfeld and Paupardin-Tritsch (1974)
Accessory radula closer muscle	10^{-8} M	Yes	Weiss et al. (1975)
L7 sensory neuron	10^{-4} M	Yes	Klein and Kandel (1978)
LB and LC neurons	Iontophoretic application	No	Pellmar and Carpenter (1980)

APPENDIX I

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Neurobiology

Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture

(*Aplysia*/protein phosphorylation)

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Contributed by Paul Greengard, September 15, 1980

ABSTRACT We have found that the calcium action potentials of bag cell neurons from the abdominal ganglion of *Aplysia* may be enhanced by intracellular microinjection of the catalytic subunit of cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37). The catalytic subunit was purified from bovine heart and shown to be effective in stimulating the phosphorylation of bag cell proteins in homogenates at concentrations of 10–50 nM. Intracellular injection into isolated bag cell neurons maintained in primary culture was through pressure applied to microelectrodes filled at the tip with catalytic subunit (5–22 μM). In 11 of 16 injected cells, both the slope of the rising phase and the height of the action potentials evoked by a constant depolarizing current were markedly enhanced relative to the pre-injection control (mean increases, 73% and 35%, respectively). This effect could occur with no change in resting potential or in the latency of the action potential from the onset of the depolarizing pulse. The effect was observed with enzyme dissolved in three different salt solutions (Na phosphate, K phosphate, or KCl). In two experiments, tetrodotoxin (50 μM) added to the extracellular medium had no effect on the enhanced action potentials. Subsequent addition of the calcium antagonist Co^{2+}, however, diminished or abolished the spikes. In more than half of the experiments, the injection of catalytic subunit was accompanied by an increase in the input resistance of the cells as measured by applying small hyperpolarizing current pulses. In three experiments, subthreshold oscillations in membrane potential resulted from the injections. Control injections (24 cells), carried out either with carrier medium alone or with heat-inactivated enzyme preparations, did not produce spike enhancement, increased input resistance, or oscillations. Our data suggest that the stimulation of intracellular protein phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase enhances the excitability of bag cell neurons by modifying calcium and potassium channels or currents.

For many neurons, including the bag cell neurons in the abdominal ganglion of *Aplysia*, brief electrical stimulation or exposure to transmitter substances or to cyclic AMP analogues produces long-lasting changes in electrical excitability (1–4). These same agents can also produce changes in the phosphorylation state of neuronal proteins, supporting the hypothesis that an alteration in phosphorylation state may underlie certain changes in electrical activity (5, 6). In this report we describe the effects of a cyclic AMP-dependent protein kinase on the electrical properties of bag cell neurons. These neurons respond to brief electrical stimulation or to cyclic AMP analogues by generating a long-lasting afterdischarge, after which they become relatively refractory to further stimulation. Because the bag cells form an electrically coupled network in the intact abdominal ganglion (7, 8), intracellular injection experiments could be difficult to interpret. We therefore used isolated bag cell neurons in primary culture (7, 9) for this study. The action potentials in these neurons are predominantly due to activation of a calcium channel (10).

Cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) is a tetrameric enzyme consisting of two cyclic AMP-binding subunits and two catalytic subunits (11). On binding to cyclic AMP, the tetramer dissociates, releasing the catalytic subunits which are then able to transfer the terminal phosphate of ATP to serine or threonine residues on substrate proteins. We present evidence that the catalytic subunit of cyclic AMP-dependent protein kinase (PKC) purified from bovine heart increases the phosphorylation of bag cell proteins and enhances the calcium action potentials.

In the companion paper, Castellucci *et al.* (12) describe effects of intracellularly injected PKC on the duration of calcium action potentials and on transmitter release in sensory cells of the abdominal ganglion incubated in the presence of tetraethylammonium ion.

MATERIALS AND METHODS

Culture of Bag Cell Neurons. *A. californica* was kept at 14°C, and all electrophysiological experiments were carried out at this temperature. Abdominal ganglia were dissected out and incubated at 22°C for 6 hr in filtered seawater containing 1.25% neutral protease (7, 9). The connective tissue capsules were removed and the bag cells were disaggregated by using a pasteur pipette. The cells were seeded into 35-mm Falcon tissue culture dishes containing L-15 medium (13) (GIBCO) made up in artificial seawater. The cells rapidly attached to the bottom of the culture dishes and within 2–3 days had developed elaborate neuritic branches. Electrophysiological studies were usually done within 1 week of seeding.

Intracellular Injection and Electrophysiological Recording. Glass microelectrodes were pulled on a Brown–Flaming electrode puller. The tips were then brushed against a ground glass plate to produce final tip diameters of $\approx$1.0 μm. When filled with enzyme or control solutions, these electrodes typically had resistances of 30–60 MΩ. Pressure injection (14) was used for moving material out of the electrodes. They were mounted in an electrode holder connected to a cylinder of N_2 gas so that pulses of pressure (4–40 psi; 10 sec–4 min) could be applied under the control of a solenoid-operated three-way valve (15). Cultured bag cell neurons were penetrated, under visual control, on a Leitz Diavert microscope. Recording of

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Abbreviations: $PhMeSO_2F$, phenylmethylsulfonyl fluoride; R-II, regulatory subunit of cyclic AMP-dependent protein kinase (type II); PKC, catalytic subunit of cyclic AMP-dependent protein kinase; PEM, phosphate/EDTA/2-mercaptoethanol buffer.

membrane potential was through an M701-WPI electrometer, and the intensity of current applied to depolarize or hyperpolarize the cell was monitored by using both the current monitor circuit of the electrometer (shown on figures) and an independent virtual ground circuit. When cells were impaled with a single electrode, the input resistance of the cells was estimated by measuring the maximal voltage displacements to a range of small hyperpolarizing currents after correction for bridge imbalance. In some experiments the cells were also penetrated with a second microelectrode filled with 2 M potassium citrate (resistance, 30–100 MΩ) and used for voltage recording only.

Preparation of the PKC (Type II) from Bovine Heart. The procedure used was an adaptation of the combined methods of Beavo *et al.* (16) and Corbin *et al.* (17). All steps were carried out at 4°C unless indicated otherwise. Bovine heart (4 kg) was homogenized in 3 vol (vol/wt) of PEM buffer (10 mM potassium phosphate/1 mM EDTA/15 mM 2-mercaptoethanol, pH 6.8) to which had been added 100 mM NaCl and 50 μM phenylmethylsulfonyl fluoride ($PhMeSO_2F$). The homogenate was centrifuged (11,000 × *g*, 30 min), and the supernatant was filtered through glass wool and stirred in 4 liters of packed DEAE-cellulose (Whatman DE-52) for 15 min. The resin was washed on a large Buchner funnel with 16 liters of homogenization buffer, resuspended in 4 liters of PEM to which 300 mM NaCl had been added, and stirred for 15 min. The filtrate was removed and the washing step repeated. The filtrate from both steps (8 liters) was combined, solid ammonium sulfate was added (314 g/liter), and the solution was stirred for 1 hr and centrifuged (11,000 × *g*, 30 min). The pellet was resuspended in 200 ml of PEM and dialyzed overnight against three 4-liter changes of the same buffer. The dialyzed sample was centrifuged (10,000 × *g*, 30 min) and the supernatant was adjusted to pH 6.1 with 1 M acetic acid.

This supernatant was then subjected to serial adsorption of unwanted protein to CM-Sephadex C-50 (three times, 50 ml of resin) equilibrated in PEM at pH 6.1. The filtrate was saved each time, and its pH was adjusted back to pH 6.8 with 1 M NaOH prior to subjecting it to four cycles of adsorption to CM-Sephadex C-50 equilibrated in PEM (pH 6.8). The filtrate was saved and applied, in series at 10–20 ml/hr, first to a cyclic AMP-Sepharose affinity column (0.5 × 4 cm) (17) equilibrated with PEM and then, by means of continuous tubing, to a column of CM-Sephadex C-50 (1 × 4 cm) equilibrated in the same buffer. At this stage the PKC was bound to the CM-Sephadex column and the regulatory subunit (R-II) was bound to the affinity column. The PKC was eluted by using a linear gradient (60 ml) of 10–300 mM potassium phosphate (pH 6.8). The position of elution was identified by assaying for histone kinase activity (18). The active fractions were pooled, dialyzed against PEM, and concentrated to about 0.3 mg/ml by reapplying the sample to a CM-Sephadex column (0.5 × 2 cm) equilibrated in PEM. The enzyme was eluted with a step of 300 mM potassium phosphate (pH 6.8). R-II was removed from the cyclic AMP-Sepharose affinity column at 24°C. The column was washed with: 5 ml of PEM at pH 6.8; 5 ml of 10 mM AMP in PEM; 5 ml of PEM; and 5 ml of 10 mM cyclic AMP (pH 6.8) seven times. The elution position of R-II was identified by using $NaDodSO_4$/polyacrylamide gel electrophoresis.

The R-II and PKC were then recombined in a 55:40 weight ratio in the presence of 10 mM $MgCl_2$ and 0.1 μM ATP. After 30 min, the holoenzyme was dialyzed three times against 4 liters of PEM. The protein was then applied to a DEAE-cellulose column (1 × 3 cm) equilibrated in PEM. The column was washed with: 7 ml of 80 mM NaCl/PEM four times; 5 ml of 40 mM NaCl/PEM; and 5 ml of 40 mM NaCl/100 μM cyclic AMP/PEM three times. The pure PKC was eluted at this stage. The fractions were pooled, concentrated by vacuum dialysis to 0.22–1.13 mg/ml, and finally dialyzed against 300 mM sodium or potassium phosphate (pH 6.8) with 5–15 mM 2-mercaptoethanol. This purified enzyme preparation was used to fill the tips of the injection microelectrodes. For some experiments, 200 μl of the enzyme was further dialyzed against 250 ml of 0.3 M KCl/15 mM 2-mercaptoethanol for 24 hr prior to intracellular injection. In some preliminary experiments, 10% glycerol was also present in the injection solution. For control experiments with heat-inactivated enzyme, solution containing active enzyme was heated in a water bath at 80°C in a sealed vial for 20 min.

For the assay of PKC activity in bag cell homogenates, intact bag cell clusters and surrounding connective tissue were dissected away from the abdominal ganglion. Four clusters were homogenized in 100 μl of ice-cold 50 mM sodium acetate, pH 6.5/10 mM $MgCl_2$/1 mM Zn acetate/1 mM theophylline. The reaction was initiated by adding 50 μl of homogenate to plastic tubes containing 20 μl of 70 μM [γ-^{32}P]ATP (25 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels; ICN) and PKC at varying concentrations at 24°C. After 2 min, the reaction was terminated by addition of 75 μl of 10% (wt/vol) glycerol/2% (vol/vol) $NaDodSO_4$/5% (vol/vol) 2-mercaptoethanol/0.001% bromophenol blue followed by heating at 90°C for 15 min. The solubilized proteins were separated by 0.5% $NaDodSO_4$/10% polyacrylamide slab gel electrophoresis with 0.05 M Tris/0.6 M glycine, pH 8.5, as buffer. The gels were stained and subjected to autoradiography. The stimulation of ^{32}P incorporation into individual phosphoprotein bands was quantitated by scanning of the autoradiograms on a densitometer.

RESULTS

Activity of the Catalytic Subunit. Addition of the PKC from bovine heart to homogenates of bag cells containing [γ-^{32}P]ATP enhanced the incorporation of ^{32}P into all observed phosphoprotein bands in a dose-dependent manner (Fig. 1). The range of PKC concentrations used was about 1/2000th to 1/400th the concentrations of the most concentrated solutions used to fill the tips of the microelectrodes (1.13 mg/ml).

Injection of PKC into Bag Cell Neurons. After penetration of a bag cell neuron with either one or two microelectrodes, a series of 10 suprathreshold depolarizing current pulses (250-msec pulses, 0.05–0.25 nA, 0.83 pulse/sec) was applied. The usual response to this type of stimulation was a progressive increase in the height and width of the action potentials elicited by each pulse (Fig. 2*B*, cells 1 and 2). In some cases, however, adaptation occurred such that later pulses in the train failed to

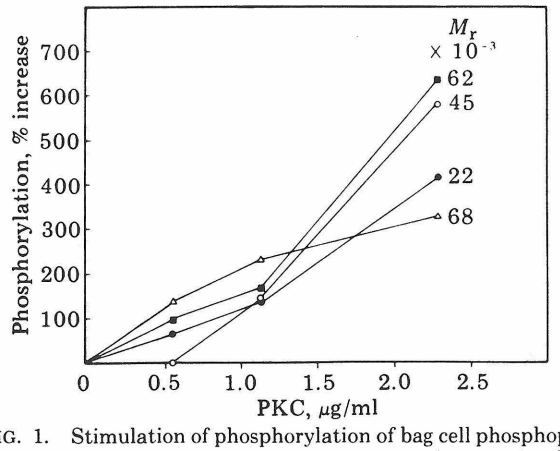

FIG. 1. Stimulation of phosphorylation of bag cell phosphoproteins by PKC from bovine heart. The percentage increase in phosphorylation was quantitated by densitometric scanning of autoradiograms and is shown for four representative bag cell phosphoproteins of M_r 22,000, 45,000, 62,000, and 68,000. The PKC concentrations shown are equivalent to ≈12 to 54 nM.

produce full action potentials (Fig. 2*B*, cells 3 and 4). A series of small hyperpolarizing current pulses (0.01–0.15 nA, 1–4 sec) was also delivered to determine the input resistance of the cell. The series of depolarizing and hyperpolarizing current pulses was repeated at 5-min intervals. When the response to both types of stimulus had remained stable for at least 20 min, a pulse of pressure (2–10 psi, 5 sec) was applied to the enzyme-containing microelectrode. If no change in the response of the cell to the depolarizing and hyperpolarizing pulses was observed, the pressure pulse was repeated at higher pressure and for longer times (up to 45 psi, 4 min) until a change was seen.

In 11 of 16 cells injected with the PKC, the first detectable change in electrical response was an increase in the height and in the slope of the rising phase of the action potentials evoked by a constant depolarizing current pulse. This can be seen in Fig. 2*A* by examining the tracings of the first spikes evoked by the repetitive pulses. The mean (±SEM) increase in the amplitude of these first action potentials for the 11 cells was 35 ± 10.1% (measured from overshoot to undershoot) and the increase in the slope of the rising phase of the action potentials was 72.9 ± 17.3%. Fig. 2*B* shows that the subsequent action potentials evoked by the later pulses also were greater in amplitude and, in some cases, significantly broader than their control counterparts. The change in the shape of the spikes could occur with no detectable change in either the resting potential of the cell or the latency of the spike from the onset of the depolarizing pulse. The effect was observed with enzyme that was dissolved in each of the three salt solutions used as carrier [Na phosphate, three experiments (Fig. 2, cell 2); K phosphate, six experiments (Fig. 2, cells 1 and 3); KCl, two experiments (Fig. 2, cell 4)]. After injection of PKC, the effect of enhanced spike electrogenesis generally remained stable for the remainder of the experiment (up to 60 min).

To determine if the enhanced spikes that follow injection of PKC were due to an enhancement of sodium or calcium components, we added tetrodotoxin (50 μM) to the extracellular medium after intracellular injection in two experiments. In both cases, tetrodotoxin had no effect on the enhanced spikes, suggesting that they were carried by Ca^{2+}. Subsequent addition of the calcium antagonist $CoCl_2$ (12 mM) to the extracellular medium substantially diminished the evoked action potentials in one case and totally abolished them in the other.

The change in the configuration of evoked spikes was often accompanied by an increase in the input resistance of the cells (Fig. 3). Input resistance was monitored in 9 of the 11 positively responding cells. Five had input resistances that were significantly increased over the pre-injection control values (mean increase, 65%). The remaining cells' resistances remained unchanged or diminished slightly compared with control values. The mean increase for the nine cells was 32 ± 16% (SEM).

In three of the cells injected with PKC, subthreshold oscillations in membrane potential developed after injection and persisted for the remainder of the experiment. An example of such subthreshold oscillations is shown in Fig. 4. In one of the three cells, the oscillations reached spike threshold and resulted in irregular repetitive firing for 9 min after the injection (not shown). The subthreshold oscillations were membrane potential-dependent, being increased both in frequency and amplitude by subthreshold depolarizing current pulses and diminished or abolished by hyperpolarizing pulses (Fig. 4*B*).

Control Injections. Control data for the effects of injected PKC were obtained by injecting either heat-inactivated enzyme preparations [six experiments (Fig. 5, cell 1)] or carrier alone [0.3 M Na phosphate/10 mM 2-mercaptoethanol, 3 experiments (Fig. 5, cell 2); 0.3 M K phosphate/15 mM 2-mercaptoethanol, 11 experiments (Fig. 5, cell 3); 0.3 M KCl/15 mM 2-mercaptoethanol, 4 experiments (Fig. 5, cell 4)]. Of these 24 injected cells, only 1 gave a small increase in spike height after intracellular injection [7.5% when injected with K phosphate-based carrier medium (Fig. 5, cell 3)]. The other cells showed no change in their electrical properties until, after repeated injections, there was some loss of resting potential and spike height associated with a decrease in input resistance. The five cells that were injected with active PKC but failed to respond positively also showed these changes which are a nonspecific effect of excessive intracellular pressure. After some of these control injections the width of the spikes observed in response to a train of depolarizing current pulses was increased, compared with

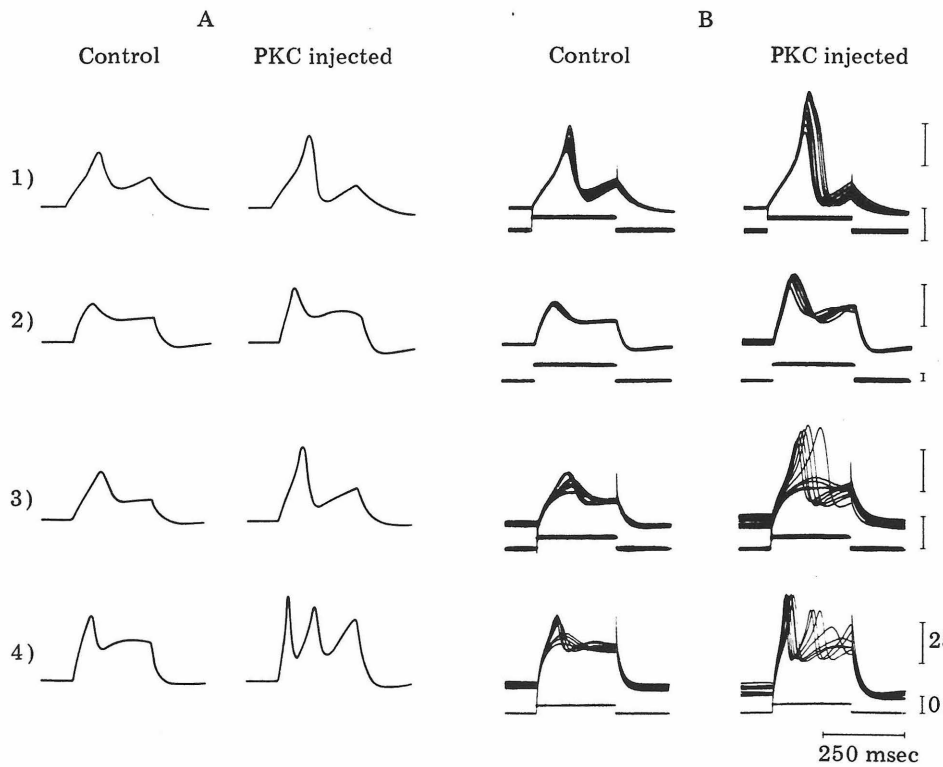

FIG. 2. Effect of intracellularly injected PKC on action potentials of bag cell neurons. (*A*) Control shows tracings of first action potential evoked by a train of depolarizing current pulses prior to injection of PKC. The second column shows the response of the cells to the same current pulses after injection. (*B*) Superimposed oscilloscope tracings of the response to multiple depolarizing current stimuli at a frequency of 0.83/sec before and after injection of PKC, for the same four cells as in *A*. Lower traces show the applied transmembrane current and upper traces show the action potential responses. The solutions at the electrode tips contained PKC (0.5–1.13 mg/ml), 2-mercaptoethanol (10–15 mM), and 0.3 M K phosphate (cells 1 and 3), 0.3 M Na phosphate (cell 2), or 0.3 M KCl (cell 4). The injection variables for cells 1–4 were: 7 psi, 40 sec; 2 psi, 12 sec; 43 psi, 240 sec; and 11 psi, 60 sec.

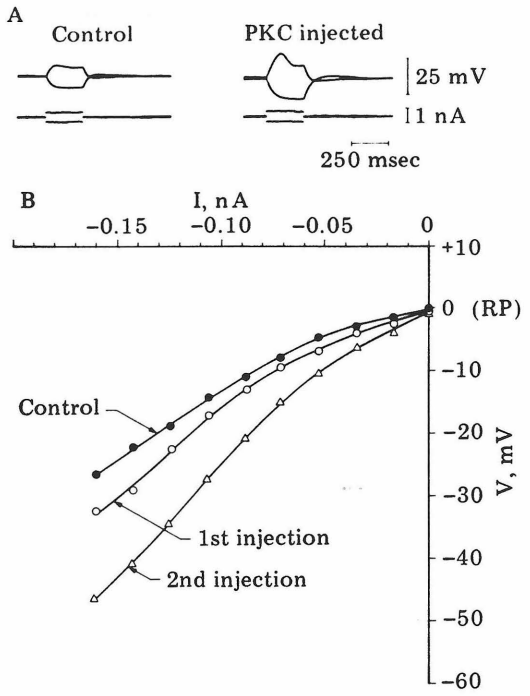

FIG. 3. Increased input resistance in a bag cell neuron after injection of PKC. (*A*) Voltage responses (upper traces) to constant depolarizing and hyperpolarizing current pulses (lower traces) before and after injection. The injection electrode contained PKC (0.54 mg/ml) in 0.3 M Na phosphate/10 mM 2-mercaptoethanol; injection was at 2 psi for 12 sec. (*B*) Current–voltage relationships for another bag cell neuron. The voltage axis is relative to the resting potential (RP) of the neuron. The electrode tip contained PKC (1.13 mg/ml) in 0.3 M K phosphate/15 mM 2-mercaptoethanol. The first PKC injection (18 psi, 10 sec) produced an increase in resistance with no change in resting potential. A second, larger, injection (43 psi, 240 sec) further increased input resistance. This second injection was associated with a small depolarization (4 mV) from the preinjection resting potential of 37 mV.

controls, even though their amplitude was not enhanced. This change was always accompanied by a large depolarization (≥10 mV) and shortening of the latency from the onset of the current pulse to the onset of the action potential. In Fig. 5, cell 2 shows

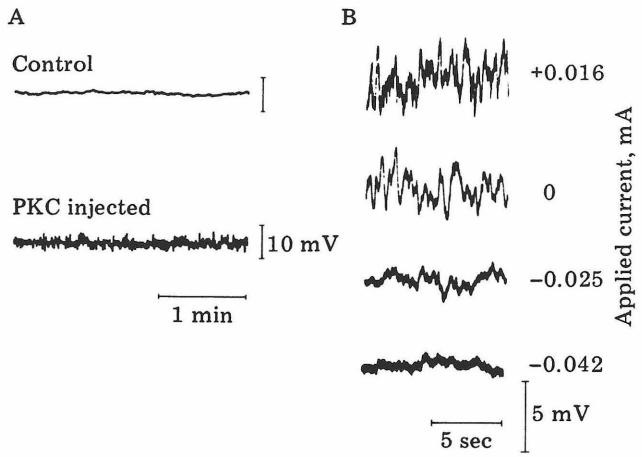

FIG. 4. Emergence of subthreshold oscillations in membrane potential of a bag cell neuron after injection of PKC. (*A*) Slow-speed chart tracings of membrane potential before and after injection (11 psi, 60 sec). The electrode tip contained PKC at 1.13 mg/ml in 0.3 M KCl/15 mM 2-mercaptoethanol. (*B*) Oscilloscope tracings of the induced subthreshold oscillations in the same cell as in *A*. Depolarizing current (top trace) increased frequency and amplitude; hyperpolarizing current (bottom two traces) diminished oscillations.

the most extreme example of this effect which was most likely due to inactivation of delayed outward currents by the injection-induced depolarization (19). In no control experiments was a sustained increase in input resistance observed after injection. The mean (±SEM) pre-injection resting potential of the control group of injected cells was 34.7 ± 1.9 mV compared with 35.3 ± 2.3 mV for the experimental group. These resting potentials are lower than those of bag cells in the intact abdominal ganglion or of isolated bag cells used in other studies (9, 20) and may result from penetration by the larger tips of pressure-injection microelectrodes.

DISCUSSION

We have shown that, in bag cell neurons, presumed calcium spikes may be enhanced by intracellular injection of PKC. Although we have shown that bovine PKC enhances phosphorylation of *Aplysia* bag cell proteins *in vitro*, we can make no statement about the degree of intracellular phosphorylation

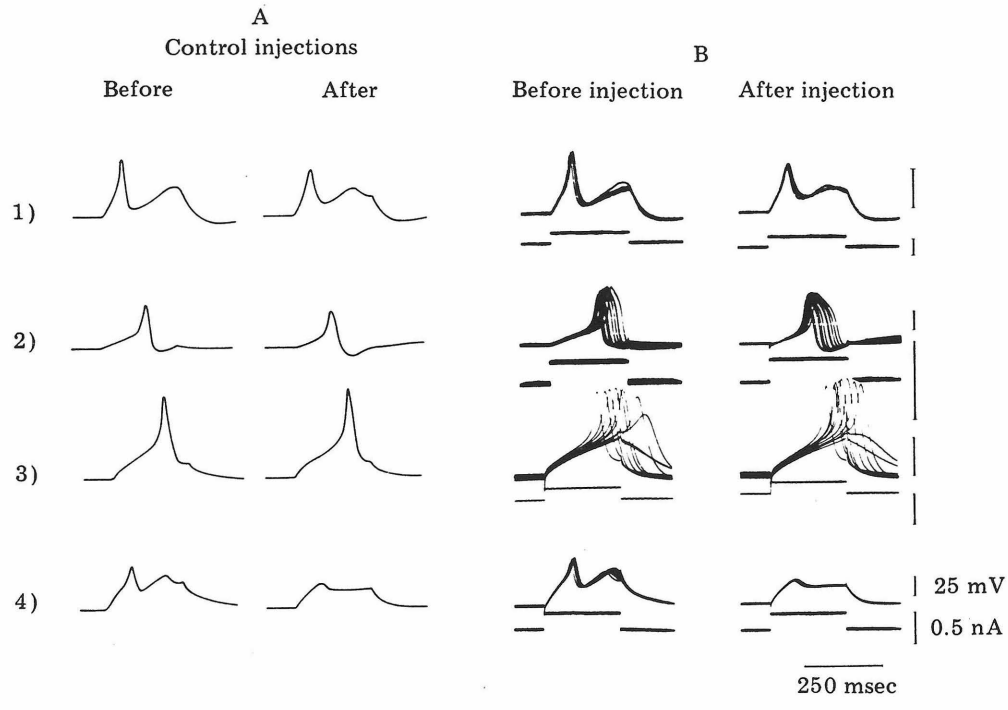

FIG. 5. The effects of control injections of heat-inactivated enzyme solutions and carrier solutions on action potentials of bag cell neurons. (*A*) Tracings of first action potential evoked by a train of depolarizing current pulses before and after injection. (*B*) Superimposed oscilloscope tracings of the response to multiple depolarizing current stimuli at a frequency of 0.83/sec before and after injection for the same four cells as in *A*. Lower traces give the applied transmembrane current; upper traces show the action potential responses. Cell 1 was injected (6 psi, 3 min) with heat-inactivated PKC (0.54 mg/ml) in 0.3 M Na phosphate/10 mM 2-mercaptoethanol. Cells 2, 3, and 4 were injected with 0.3 M Na phosphate (5 psi, 4.5 min), 0.3 M K phosphate (40 psi, 12 min), and 0.3 M KCl (6 psi, 8 min), respectively, with 10–15 mM 2-mercaptoethanol present in each case.

that was induced in the injection experiments or about its spatial extent. The effect of PKC could be a direct effect on components of the calcium or potassium channels or could be secondary to some less-specific metabolic effect of kinase injection—for example, calcium currents could be enhanced by a kinase-induced decrease in intracellular pH (21). In addition, the state of excitability of neurons in primary culture may be subject to metabolic influences that do not play a role in naturally occurring excitability changes of the same neurons *in vivo*. Nevertheless, the fact that changes similar to those that we have described for kinase injection do occur in the natural activity of these cells (see below) suggests that changes in phosphorylation state do control electrical excitability.

The bag cell neurons in the abdominal ganglion of *Aplysia* generate a long-lasting afterdischarge (≈30 min) in response to brief synaptic stimulation or to cyclic AMP analogs (1, 3, 22). The shape and amplitude of the action potentials change dramatically during the course of the afterdischarge, with the maximal height and width of the intracellularly recorded action potentials being observed 2 min after the onset of the afterdischarge (10). This maximum is also correlated with a peak in cyclic AMP levels within the bag cell cluster (3). Although bag cells show frequency-dependent broadening and augmentation of spike height with repetitive depolarizing current pulses (≈1/sec), this effect occurs too quickly to account for the slow increase in spike height and width over the first 2 min of afterdischarge, during which the cells discharge at a relatively fast rate (2–4 spikes/sec). The data of this paper suggest that the enhanced spikes during afterdischarge may result from the increased phosphorylation of certain bag cell proteins.

An increase in input resistance, the onset of subthreshold oscillations in membrane potential, and a long-lasting repetitive discharge are readily observed in isolated cultured bag cell neurons after the extracellular addition of a membrane-permeant cyclic AMP analogue (20, 22, 23). Subthreshold oscillations and repetitive discharge were observed in only some of the present experiments with injected kinase. Whether this is due to insufficient phosphorylation of specific substrate proteins or to factors unrelated to phosphorylation remains to be determined. An alternative approach to resolve this would be to attempt to block the response to cyclic AMP with protein kinase inhibitor (24) as is being done for other *Aplysia* neurons (25). The increase in membrane resistance in response to cyclic AMP analogues or intracellular protein kinase injection could be due to modification of the potassium channels by protein phosphorylation. We suggest (10) that phosphorylation increases the open time of the voltage-dependent calcium channel and decreases that of a potassium channel.

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APPENDIX II

An early sodium and a late calcium phase in the afterdischarge of peptide-secreting neurons of Aplysia

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Key words: bag cells, afterdischarge, secretion, Aplysia, refractoriness, sodium, calcium

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SUMMARY

Following brief electrical stimulation of a pleuroabdominal connective nerve, the clusters of peptidergic bag cell neurons of Aplysia generate a long-lasting (~30 min) synchronous afterdischarge. In a normal seawater medium, the afterdischarge comprises an early phase of rapid firing (2-6 Hz) lasting for less than one minute, followed by a second, prolonged phase of lower firing rate (< 0.5 Hz). We have found that the transition from the first, rapid-firing phase to the prolonged second phase of afterdischarge is associated with an increase both in the width and height of intracellularly recorded action potentials. In addition, we find that in the presence of the potassium channel blocker, TEA, such biphasic afterdischarges may be triggered by depolarizing current that evokes action potentials in a single bag cell neuron in a cluster.

Afterdischarges resembling either the first or the second phase of a normal afterdischarge may be produced by using media deficient in calcium or sodium, respectively, in combination with potassium channel blockers. Brief stimulation in media deficient in calcium give rise to afterdischarges of high firing frequency (2-6 Hz) lasting for less than one minute (mean duration = 41 sec) while, in a sodium-free medium, longer afterdischarges (mean duration = 18 min) of lower firing rate (<0.3 Hz) were generated.

At the end of afterdischarge in a normal seawater medium, containing both sodium and calcium ions, the bag cell neurons become refractory to stimulation. Thus, further stimulation either fails to induce afterdischarge or results in afterdischarges that are attenuated both in frequency of firing and in duration compared with the first afterdischarge. In these experiments we found that the brief afterdischarges, observed in low-calcium media, which resemble the first phase of a normal afterdischarge, did not induce subsequent refractoriness while afterdischarges evoked in the normal calcium, sodium-free medium did result in refractoriness to further afterdischarge.

Our data indicate that both phases of a bag cell afterdischarge are due to intrinsic bag cell mechanisms and suggest that the firing pattern in the first phase is largely sodium dependent and that of the second, slow phase is calcium dependent. Moreover, our data indicate that it is the second, calcium-dependent phase that induces the refractoriness that follows bag cell afterdischarge.

INTRODUCTION

In many vertebrate and invertebrate peptidergic cells, a brief stimulus brings about the onset of a prolonged period of repetitive electrical activity[5,8,19-22]. In cases where electrical recordings from single cells in situ have been made, the firing rate has sometimes been observed to undergo two distinct phases following stimulation, an early response of rapid firing lasting about one minute followed by a second period of slower firing lasting for many minutes. Clear examples of such biphasic responses, with a long-lasting slow component, are to be found in the insulin secreting β-cells of the pancreatic islet[21] and in the model neuropeptidergic systems of the caudo-dorsal cells of Lymnea stagnalis[5] and the bag cell neurons of Aplysia[19]. In this paper we have further examined the characteristics of these two phases of firing in the bag cell neurons.

The bag cell neurons comprise two clusters of 200-400 electrically-coupled cells[3,15], anatomically isolated from other neurons, and located at the junctions of the pleuroabdominal connectives and the abdominal ganglion[9]. In the absence of stimulation, these neurons show no spontaneous electrical activity. In response to certain peptides purified from the reproductive tract[11] or in response to brief electrical stimulation of a pathway from the head ganglia, the bag cell neurons depolarize and generate an afterdischarge which lasts about 30 min. This afterdischarge results in the release of the peptide ELH (egg laying hormone)[23] as well as other peptides and serves to trigger egg-laying behavior. At the end of an afterdischarge, the bag cell neurons become refractory in that further stimulation generally fails to evoke a second afterdischarge although intense electrical stimulation can sometimes produce a much shorter afterdischarge[17,19].

In this paper we show that both the initial high-firing rate phase and the second slower phase of the afterdischarge are intrinsic to the bag cell neurons

themselves and may, in the presence of TEA (tetraethylammonium ions), be triggered solely by transmembrane depolarization of a single bag cell neuron. We further show that the transition from the first to the second phase is accompanied by a characteristic increase in the width and height of action potentials and that each phase may be pharmacologically dissected from the other by using media deficient in either sodium or calcium. Finally we demonstrate that the second, calcium-dependent, phase induces a subsequent refractoriness.

METHODS

Aplysia californica was collected locally (Southern California coast) and kept in a seawater system at 14°C. All electrophysiological experiments were also carried out at this temperature. The pleuroabdominal connectives that join the abdominal ganglion to the head ganglia were cut close to the pleural ganglia. Abdominal ganglia were then dissected out with the entire length of these connectives. For experiments in which extracellular recordings only were made of bag cell afterdischarges, the ganglia were then placed directly in a recording chamber containing either Millipore-filtered seawater (FSW), artificial medium (460 mM NaCl/10.4 mM KCl/11.0 mM $CaCl_2$/55 mM $MgCl_2$/10 mM Tris-HCl pH 7.8) or similar media deficient in sodium or calcium (see below). A recording suction electrode was placed over the rostral-ventral part of each bag cell cluster. Because the bag cell neurons discharge synchronously and are anatomically isolated from other neuronal types, this positioning of the suction electrodes registers large bag cell compound action potentials uncontaminated by other units[6,7]. A stimulating suction electrode was placed at the pleural end of one of the pleuroabdominal connective nerves.

For intracellular experiments, the connective tissue overlying the bag cells was first softened to allow microelectrode penetration. Ganglia were incubated

for 2 hr in seawater containing 2 mg/ml of collagenase (Worthington Type III) and 0.5 mg/ml of elastase (Sigma Type I). After this treatment they were rinsed several times with filtered seawater before being pinned to the base of the recording chamber. Such enzyme treated ganglia show all the normal aspects of afterdischarge. Glass microelectrodes were filled with 2 M potassium citrate and typically had resistances of 30-100 MΩ. Recordings were made through an M4-A WPI electrometer and firing frequency and action potential height and width were recorded both on oscilloscope and chart records. The height of action potentials was measured from the point of inflection at the base of the rising phase of the action potential to its peak. The width of action potentials was measured at half maximal amplitude. Intracellular stimulation was achieved by passing current through the recording electrode after balancing the bridge circuit. When the mean duration of afterdischarges is given in this paper, it is followed by the standard error or the mean.

RESULTS

Intracellular Stimulation of Afterdischarge

As described by others, intracellular stimulation of a single bag cell neuron of Aplysia californica in a normal seawater medium does not induce the bag cell cluster to afterdischarge nor does an intracellularly stimulated action potential propagate to other neurons within the cluster[6,19]. Thus intracellular stimulation fails to induce extracellularly recorded compound action potentials over the bag cell cluster or over the proximal part of the pleuroabdominal nerve that is rich in bag cell processes[4,9,15]. Since the bag cell processes are electrically coupled as evidenced by morphological[15] and electrophysiological[3,15] evidence, we wondered to what extent we could enhance propagation through the network by suppressing potassium conductance. We used TEA which is known to suppress voltage-dependent and calcium-activated potassium channels[12]. This would be expected to increase

the effective length constant and thus presumably to enhance electrical coupling. Another well known effect of TEA is to enhance the duration of sodium and calcium spikes.

We have found that after addition of TEA (25-100 mM) to a normal seawater medium, action potentials produced by intracellular stimulation of a single bag cell neuron frequently propagate through the entire bag cell network, as judged by extracellular recordings. One or more such stimuli may induce the characteristic long-lasting afterdischarge of the bag cell cluster. Figure 1A shows an intracellular recording from a bag cell neuron in the presence of TEA (100 mM). At the start of the trace, two depolarizing current pulses were applied to the cell triggering an afterdischarge in which the cells fire at a relatively high frequency for about one minute followed by a second period of slower firing. The time course of firing frequency and also of spike height and spike width for the onset of an intracellularly stimulated afterdischarge in TEA (30 mM) in another experiment is shown in Fig. 1B. In all cases if intracellular stimulation was not applied to single bag cell neurons following the addition of TEA, afterdischarges would ensue spontaneously[13] after a mean delay of 52 min ($\pm$ 27 min SEM, N=5). Furthermore, the duration of afterdischarges in TEA, whether evoked intracellularly or spontaneously, was significantly longer than those in normal seawater medium (mean duration in TEA = 102.0 $\pm$ 25.5 min, N=6; in FSW = 29.8 $\pm$ 4.3 min, N=19).

Two Phases of Afterdischarge

Brief (5-15 sec, 1-6 Hz) extracellular stimulation of the bag cell cluster or of a pleuroabdominal connective nerve in normal seawater medium results in the depolarization of bag cells and the onset of afterdischarge. An example of the time course of firing frequency and also of spike width and height in a normal seawater medium, at the onset of afterdischarge, is shown in Fig. 2D. The frequency of firing reaches a maximum ($\sim$2.5-6 Hz) within one minute of the end of stimulation

and thereafter declines rapidly to a lower level ($<\sim$0.8 Hz) within 3-4 min after which it declines only slowly over the remainder of the afterdischarge (mean duration = 29.8 min). The firing rate towards the end of an afterdischarge may, however, be as low as 0.06 Hz.

While it has been reported that during stimulation to evoke afterdischarge the bag cell spikes potentiate[6,7], in the past no mention has been made of changes in the shape of the intracellular soma spike during the afterdischarge itself. Within the first 10 sec of the afterdischarge, an increase in spike height and width may be observed (i.e., the jump from 23 to 44 msec width and 40 to 50 mV height in Fig. 2D). The subsequent shape of spikes during the first minute of high frequency discharge undergoes only small changes. At the end of one minute, however, as the frequency of firing of the first phase declines, the width and height of the action potentials begin to increase markedly, reaching a maximum value at 2 min. Thereafter the spikes usually decreased slightly in height and width but, as shown in Fig. 2B and 2D, reached a steady value that was greater than that of the first high-frequency firing phase. This sustained enhancement of spike electrogenesis was observed in five of seven experiments.

Two phases of afterdischarge, an early phase of rapid firing and a second prolonged phase of slower firing with enhanced spike width and height, could also be observed in afterdischarges induced in normal seawater media containing TEA (25-100 mM) (Figs. 1, 4).

The Different Nature of the Two Phases of Spike Broadening

The increase in spike width and height that occurs in the first 10 sec of an afterdischarge closely resembles the frequency-dependent spike broadening which occurs with repetitive intracellular or extracellular stimulation of bag cell neurons and which is illustrated in Fig. 3. This phenomenon has been examined previously both in bag cells in the intact cluster[1] and in isolated bag cell neurons

in culture[16]. The first few action potentials, evoked at frequencies greater than 0.2 Hz, progressively increase in width, and usually in height. As shown in Fig. 3, however, this effect rapidly reaches a plateau, usually within 5-10 sec, after which no further change in spike shape occurs. Moreover, this effect, which is observed in many types of neurons[2], is a monotonic function of frequency, i.e., repetition of action potentials at low frequencies (<0.2 Hz) gives little or no change with repetition, frequencies up to 1 Hz give progressively greater enhancement of spikes while frequencies above 1 Hz give maximal enhancement[2,16]. The increase in spike amplitude and width that occurs after one minute of afterdischarge, as the firing frequency falls to give the second prolonged slow phase of afterdischarge, therefore reflects the emergence of a second mechanism to enhance the action potentials during the second phase.

Afterdischarge in Low-Calcium Media

Low-calcium media were prepared by replacing the normal calcium concentration of seawater media (11.0 mM) with one of the following: i) 0.5 mM Ca^{2+} with elevated Mg^{2+} (125 mM), ii) 0.5 mM Ca^{2+} + 20 mM Co^{2+}, or iii) 0-Ca^{2+} + 2 mM EGTA. Similar results were obtained with each of these calcium-deficient media. Although action potentials could usually be evoked by stimulation of the pleuroabdominal connectives, even in some cases the very distal end of the connectives, only 2 of 7 bag cell clusters generated an afterdischarge (duration < 1 min) in response to the stimulation. After addition of TEA (50-100 mM) however, afterdischarges could always be obtained, often following a single stimulus pulse. The mean duration of these afterdischarges appeared to include only the first phase of a normal afterdischarge (41.1 $\pm$ 11.9 sec, $\pm$ SEM, N=9) (Fig. 4, center). The firing rate was high (2-6 Hz) throughout the afterdischarge as in the first phase of a normal afterdischarge.

Afterdischarges in Sodium-Free Medium

Sodium-free medium was made by replacing sodium in normal seawater medium by 460 mM Tris-HCl pH 7.8. Although action potentials could be evoked by intracellular stimulation, direct extracellular stimulation of bag cell neurites in the proximal pleuroabdominal connective failed to evoke either intracellularly or extracellularly recorded action potentials at the somata, indicating that propagation of impulses through the bag cell neurites requires Na^+. Both propagation and the ability to afterdischarge could be restored by addition of 50-100 mM TEA with a second potassium channel blocker 4-AP (4-aminopyridine) (10 mM), although in one experiment TEA alone produced full restoration (Fig. 4, lowest trace). The mean duration of these afterdischarges was 18.0 $\pm$ 2.9 min (N=4). The firing rate during these afterdischarges was low (0.3-0.03 Hz) and spikes were broad (400-1200 msec). The afterdischarges started slowly, reaching the maximum firing rate only after 2 min following stimulation (Figs. 4, 5).

Refractoriness Following Afterdischarge

At the end of a bag cell afterdischarge in normal seawater medium, stimulation frequently fails to provoke a second afterdischarge[17,19] and action potentials evoked by stimulation frequently fail to invade the somata[6,7]. Intense repetitive stimulation (up to 20 V, 2.5 msec, 6 Hz, 15 sec) may, however, provoke shorter afterdischarges of lower firing frequency (Fig. 5, top line) in which both the initial high-frequency phase and the slower second phase of the first afterdischarge are attenuated. In low-calcium media with TEA, the high frequency "first-phase" afterdischarges produced little or no subsequent refractoriness and consecutive multiple high frequency afterdischarges could readily be evoked. In sodium-free media, with potassium channel blockers, the afterdischarges that were evoked, however, did result in subsequent refractoriness. In three of four cases, no further afterdischarge could be induced following the end of the first afterdischarge (Fig. 5, bottom line) and in one case only a short (5-1/2 min) discharge was obtained.

DISCUSSION

It is known, from direction of propagation studies, that action potentials during afterdischarge originate in the distal tips of bag cell neurites[7], most of which extend up the pleuroabdominal connective nerve[4,9,15]. That the mechanism of afterdischarge resides in these neuritic tips is also supported by the fact that neurites in the connectives, isolated from their somata, are readily capable of producing normal afterdischarge on stimulation[17].

We have shown that, in the presence of TEA, bag cell afterdischarge may be evoked by intracellular stimulation of a single bag cell neuron. We believe that a likely explanation for the intracellularly triggered afterdischarges is that TEA allows action potentials at the soma to propagate to the distal tips of the neurites where afterdischarge is normally generated. In agreement with such a hypothesis is the observation that intracellular stimulation, even in the presence of TEA, fails to induce afterdischarge in intact clusters that have been surgically isolated from their neurites in the surrounding connective tissue or in single, primary cultured, cells (unpublished observations). We cannot, however, exclude the possibility that TEA also acts on the, as yet, uncharacterized synaptic input to the neurites of bag cells, thereby modifying their excitability to intracellular stimulation as well as inducing spontaneous afterdischarge.

Our data have clearly shown that there exist two phases to the bag cell afterdischarge, each of which has its characteristic frequency and duration of firing and spike shape. We have shown that the early high frequency phase may be produced in isolation in Ca^{2+}-deficient media, while afterdischarge resembling the second slow phase may be isolated pharmacologically in Na^{+}-free media. This suggests that the firing pattern of the first phase depends primarily on sodium conductance mechanisms while that of the second phase is largely determined by calcium conductances. Moreover, in a normal afterdischarge a transition between these two mechanisms occurs.

We have previously provided evidence that cyclic AMP plays an important role in the genesis of bag cell afterdischarge. Cyclic AMP analogues initiate afterdischarges in intact bag cell cluters[17], in isolated neurites in connectives[17], and in isolated bag cell neurons[16]. In the isolated neurons, the firing rate induced by cyclic AMP analogues is low ($\leq$0.5 Hz) and thus comparable to that of the second phase of an afterdischarge of an intact cluster[16]. Cyclic AMP levels in a bag cell cluster substantially increase at the onset of afterdischarge reaching a maximum at 2 min[17]. In isolated bag cell neurons, cyclic AMP analogues or intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase, also enhance both the height and width of calcium action potentials[18] in a manner similar to that which occurs after the first phase of a bag cell afterdischarge. These data raise the possibility that the transition from the first to the second phase is brought about by cyclic AMP-dependent protein phosphorylation. In support of this idea we have determined that the phosphorylation state of at least two phosphoproteins is enhanced during a bag cell afterdischarge[14].

Finally we have observed that afterdischarge induced in media containing calcium induce a subsequent refractoriness such that either no afterdischarge or only severely attenuated afterdischarges may be evoked following the end of the first afterdischarge. Refractoriness of the isolated first phase is not observed, however, in low-calcium media. This suggests that calcium stimulated intracellular events may play a role in the refractory period that follows the second phase of a normal afterdischarge.

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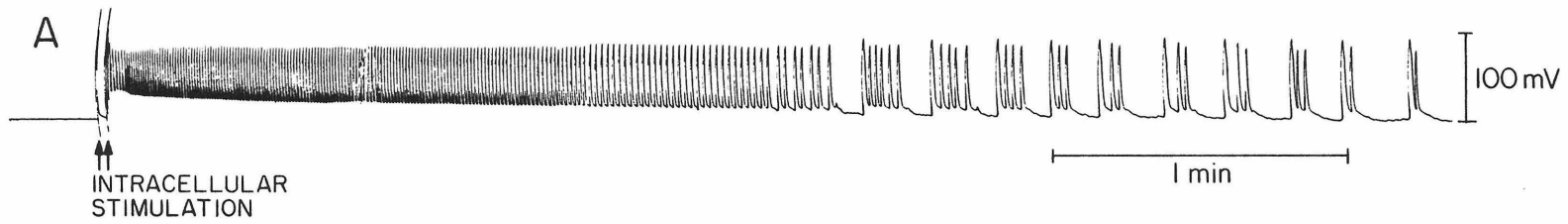
FIGURE LEGENDS

Fig. 1. A: An intracellular record of the onset of bag cell afterdischarge in the presence of TEA (100 mM). At the points marked by the arrows, two depolarizing current pulses (250 msec) were applied through the recording microelectrode, each triggering an action potential, and resulting in the onset of afterdischarge. The transition from an early phase of rapid firing to a second phase of lower firing rate with enhanced action potentials is clearly visible. B: A plot of the firing rate, spike width and spike height at the onset of another bag cell afterdischarge triggered by intracellular stimulation in TEA (30 mM) in a normal seawater medium.


A
100 mV
INTRACELLULAR
STIMULATION
1 min

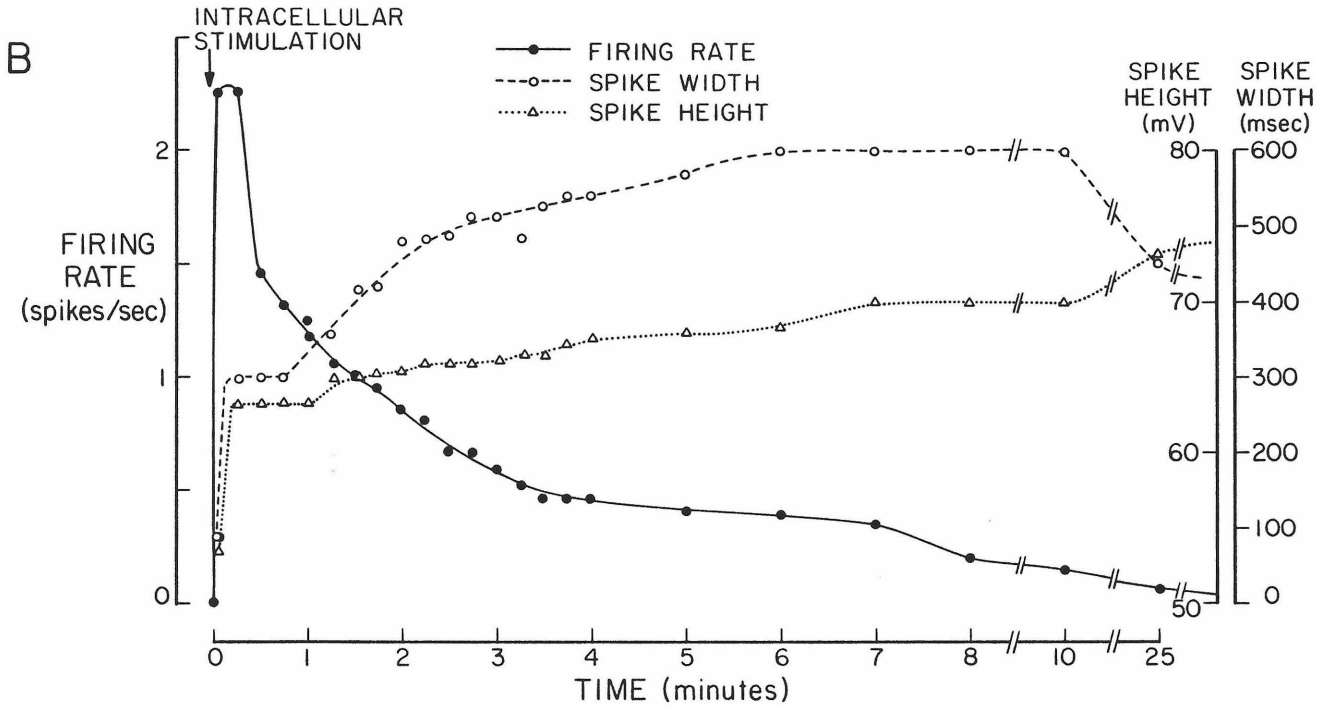

B
INTRACELLULAR
STIMULATION
FIRING RATE
SPIKE WIDTH
SPIKE HEIGHT
SPIKE
HEIGHT
(mV)
SPIKE
WIDTH
(msec)
FIRING
RATE
(spikes/sec)
2
1
0
80
70
60
50
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
10
25
TIME (minutes)

Fig. 2. A, B, C: Intracellularly recorded bag cell action potentials. Trace A shows spikes evoked by extracellular stimulation (20 V, 2.5 msec) of a pleuroabdominal connective nerve at the onset of a bag cell afterdischarge. Trace B shows the shape of the enhanced action potentials 10 min after the onset of afterdischarge. Trace C shows an action potential evoked after the end of afterdischarge. D: A plot of the firing rate, spike width and spike height at the onset of a bag cell afterdischarge triggered by extracellular stimulation (20 V, 2.5 msec, 1 Hz, 10 sec) of a pleuroabdominal connective nerve in a normal seawater medium.

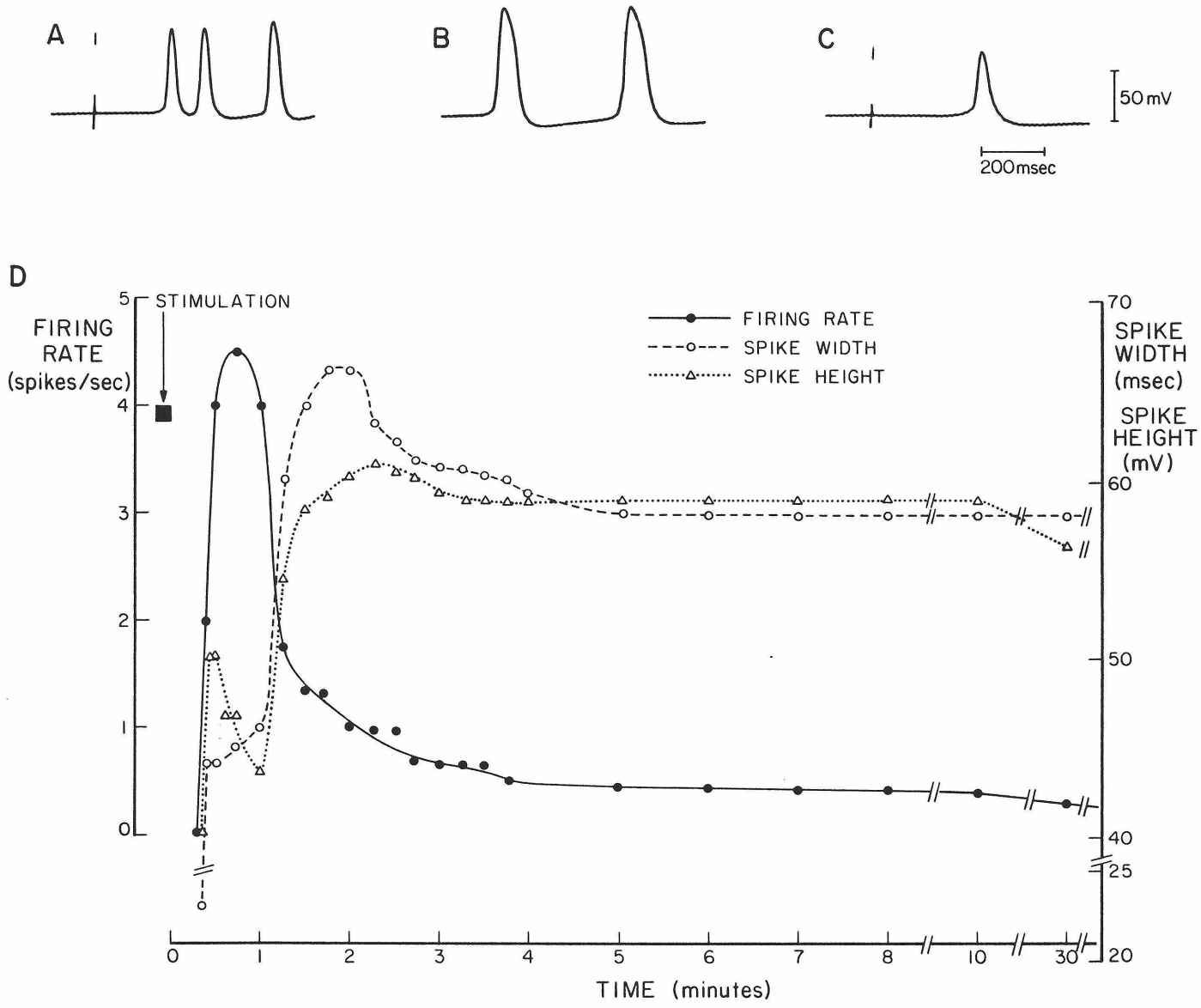

A
B
C
50 mV
200 msec
D
STIMULATION
FIRING RATE
(spikes/sec)
FIRING RATE
SPIKE WIDTH
SPIKE HEIGHT
SPIKE WIDTH (msec)
SPIKE HEIGHT (mV)
TIME (minutes)
0
1
2
3
4
5
6
7
8
10
30
20
25
40
50
60
70

Fig. 3. Frequency-dependent spike broadening with intracellular stimulation of a bag cell. Depolarizing current pulses (200 msec, 12.5 nA) were applied at a frequency of 0.83 Hz. The oscilloscope trace (inset) shows the superimposed responses of the cell to the first five pulses in a train. The graphs show the height (--Δ--) and width (--O--) of successive evoked spikes in a train of 10 pulses.

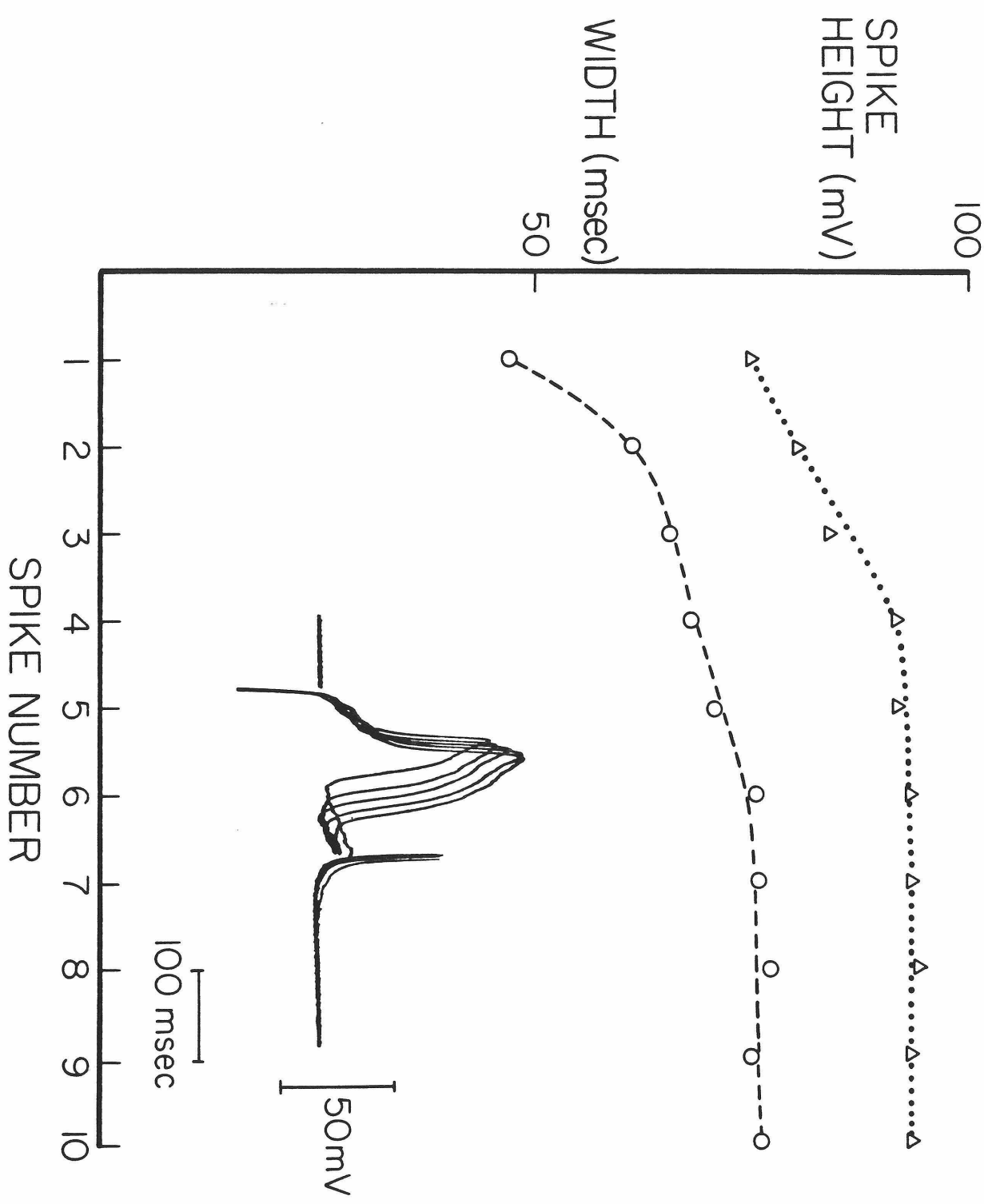

SPIKE HEIGHT (mV)
100
WIDTH (msec)
50
1
2
3
4
5
6
7
8
9
10
SPIKE NUMBER
100 msec
50mV

Fig. 4. Extracellular recordings of the onset of afterdischarge (at arrows) in bag cell clusters in three different media each containing 100 mM TEA. The top trace (FSW) shows an afterdischarge in a normal seawater medium. The center trace (0-Ca^{2+}) shows a record of afterdischarge in a medium containing 0.5 mM Ca^{2+} and 20 mM Co^{2+} in place of the normal calcium concentration. The lowest trace (0-Na^{+}) shows afterdischarge evoked in a medium of normal (11 mM) calcium concentration but with sodium replaced by Tris-HCl (460 mM, pH 7.8).

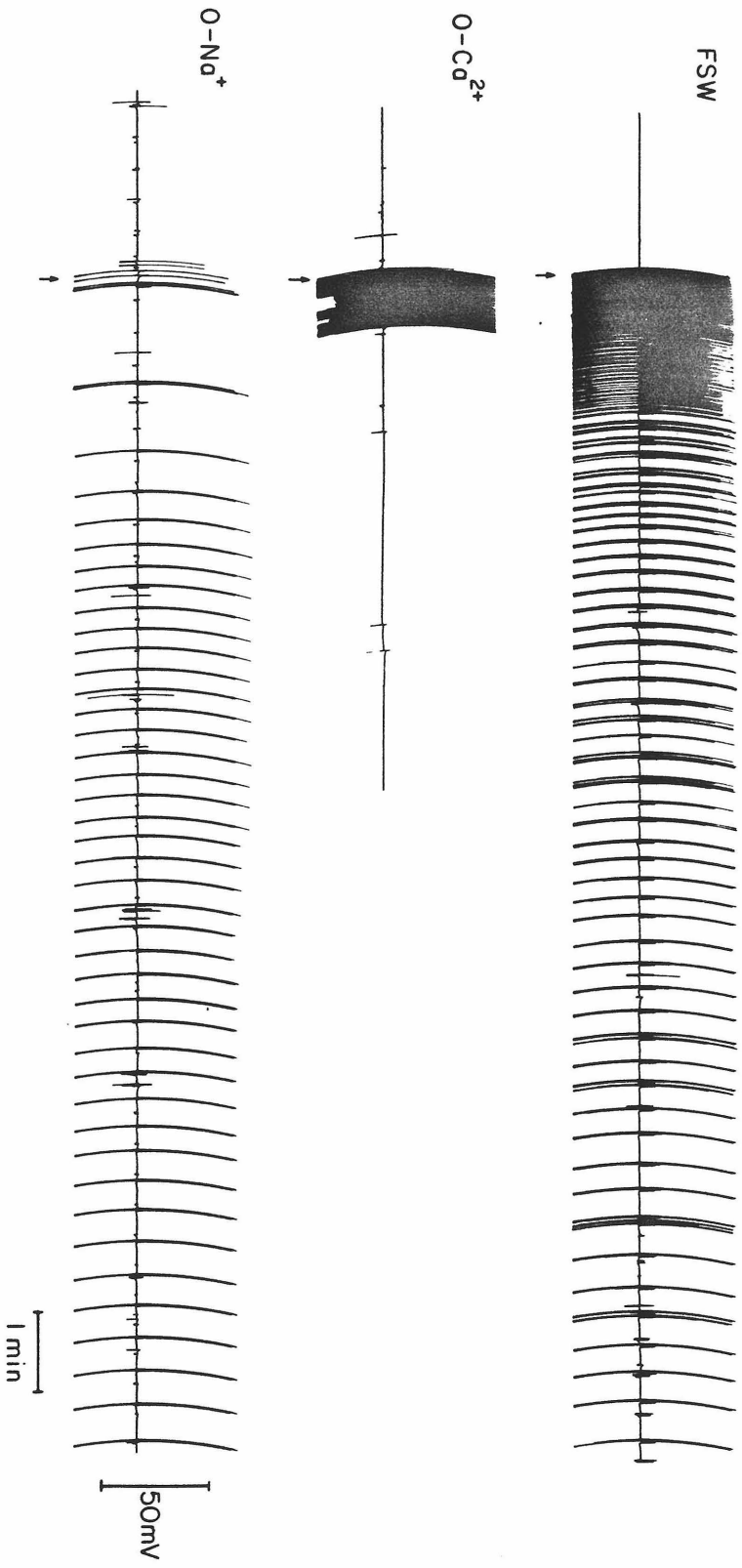
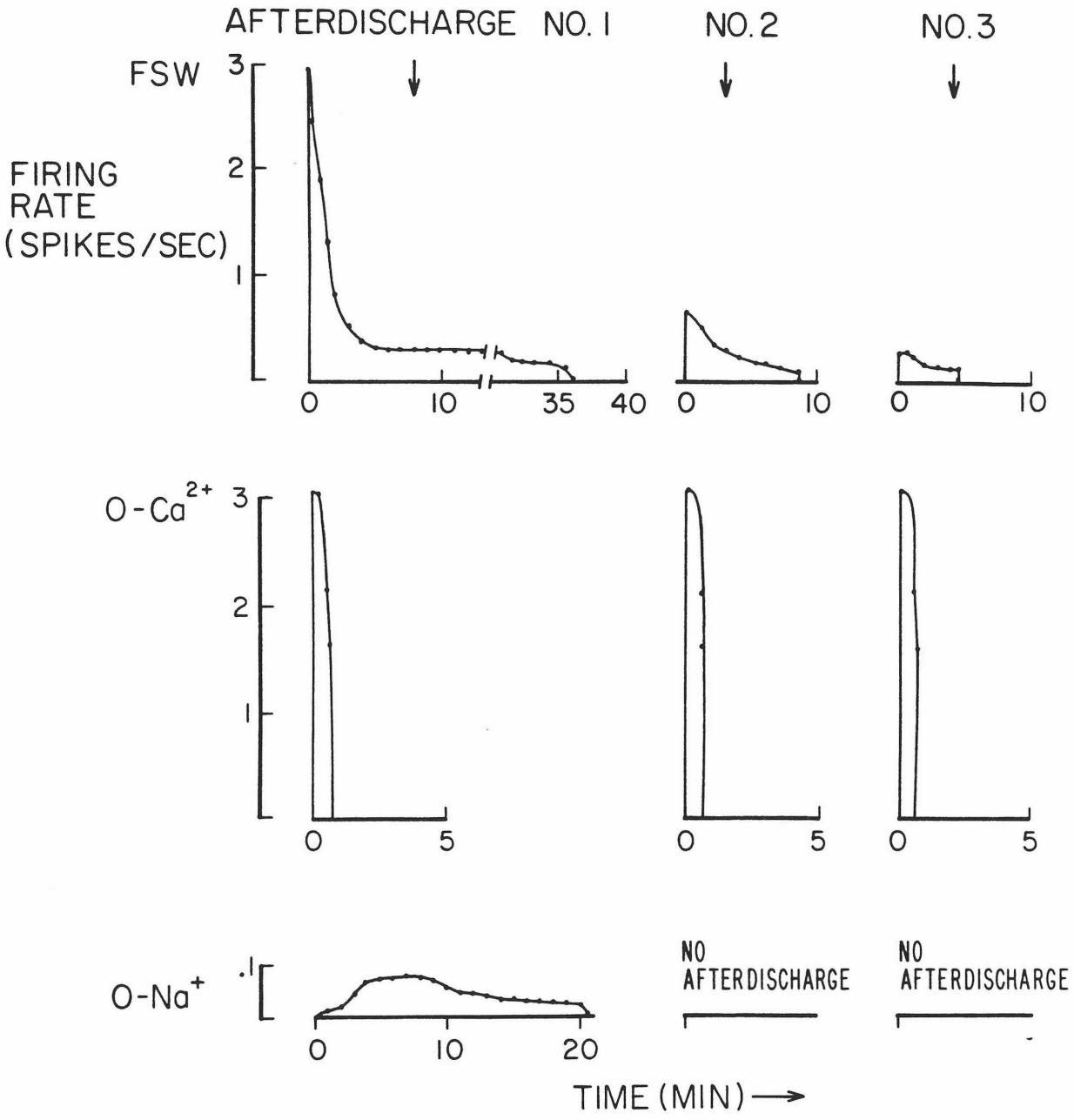

FSW
O-Ca2+
O-Na+
1 min
50mV

Fig. 5. The onset of refractoriness following bag cell afterdischarge in three media of differing ionic composition. All graphs show the firing rate of bag cells following brief (20 V, 2.5 msec, 6 Hz, <15 sec) stimulation of a pleuroabdominal connective nerve. In all cases, afterdischarge No. 1 indicates the first afterdischarge that was triggered while No. 2 and No. 3 show successive responses to stimulation after the end of the first afterdischarge. No longer than 10 min elapsed between the end of one afterdischarge and the subsequent stimulus. The top row (FSW) shows responses in a normal seawater medium. The second row (0-Ca^{2+}) shows responses in a medium containing 0.5 mM Ca^{2+} and 20 mM Co^{2+} in place of the normal calcium concentration and with 100 mM TEA present. The bottom row (0-Na^{+}) shows responses in a medium of normal calcium concentration (11 mM) but with sodium replaced by Tris-HCl (460 mM, pH 7.8) and with 100 mM TEA.


AFTERDISCHARGE NO. 1
NO. 2
NO. 3
FSW
FIRING RATE (SPIKES/SEC)
3
2
1
0
10
35
40
0
10
0
10
O-Ca2+
3
2
1
0
5
0
5
0
5
O-Na+
.1
0
10
20
NO AFTERDISCHARGE
NO AFTERDISCHARGE
TIME (MIN) ⟶