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Nonsynaptic Characteristics of Neurotransmission Mediated by Egg-laying Hormone in the Abdominal Ganglion of *Aplysia*[1]

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Abstract

The bag cell neurons of the marine mollusk *Aplysia* are a putative multitransmitter system which utilizes two or more neuropeptides that are enzymatically cleaved from a common precursor protein. It has been proposed that one of the neuropeptides, egg-laying hormone (ELH), acts nonsynaptically as a neurotransmitter in the abdominal ganglion by diffusing long distances to target neurons compared to conventional transmitters acting at synapses. To test this idea further, we investigated the physiological properties of neurotransmission mediated by ELH. We found that ELH acts directly to duplicate two types of responses produced by a burst discharge of the bag cells: prolonged excitation of LB and LC cells, and the previously described effect of ELH, burst augmentation of cell R15. Analysis of perfusate collected after electrical stimulation of the bag cells showed that the peptide is released in sufficient quantity to diffuse long distances within the ganglion without being completely inactivated. To mimic the way the peptide is thought to be released physiologically, ELH was arterially perfused into the ganglion. The response normally produced by bag cell activity was duplicated by 0.5 to 1.0 μM concentrations of ELH and showed no rapid desensitization. ELH had no effect on cells that are unaffected by bag cell activity and no effect on cells that are inhibited (LUQ cells) or transiently excited (cells L1 and R1) by bag cell activity. Acidic peptide, another peptide encoded on the ELH precursor protein, was found to be synthesized and released by the bag cells, but it had no effect on the cells we tested. We conclude that the combined properties of ELH neurotransmission resemble the properties of transmission at autonomic nerve endings on cardiac and smooth muscle rather than those of conventional synaptic transmission. ELH released from bag cells is dispersed throughout the interstitial and vascular spaces of the ganglion to produce responses in the cells that have receptors for the peptide. The results also suggest that ELH mediates only a subset of the responses induced by bag cell activity; they are consistent with data indicating that the other responses are mediated by other bag cell peptides derived from the same precursor protein as ELH.

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Much of what is known about communication between neurons has come from detailed studies of a small number of experimental preparations. Perhaps most notable among these is the skeletal neuromuscular junction of vertebrates which has served as a model for understanding synaptic transmission in the CNS (Katz, 1966). Recently it has been proposed that some CNS transmitters may act by diffusing over longer distances to their targets than is the case for transmitters acting at synapses (Branton et al., 1978a; Jan et al., 1980). For this mode of communication the release of transmitter from postganglionic autonomic nerve endings may serve as a more appropriate model.

The properties of neurotransmission mediated by acetylcholine (ACh) at parasympathetic nerve endings on cardiac muscle (Loffelholz, 1981) differ in several ways from those of ACh at the neuromuscular junction. First, the response produced in cardiac muscle has a longer latency and duration than does the response produced at the neuromuscular junction. This is due in part to the longer distance that transmitter must diffuse through interstitial and sinus spaces to reach target cells (Glitsch and Pott, 1978; Hill-Smith and Purves, 1978). Second, ACh released from nerve endings in the heart can be detected in perfusate collected after nerve stimulation (Loewi, 1921), whereas at the neuromuscular junction, ACh is completely inactivated after release (Dale et al., 1936; Krnjevic and Mitchell, 1961). Thus, to diffuse a longer distance to its targets without being totally inactivated, more transmitter must be released or inactivation must be less effective, or both. Third, the concentration of transmitter required to induce a response is much lower for the heart, where the neurally induced response is mimicked by ACh at 0.1 to 1 μM concentration (Glitsch and Pott, 1978), than for the neuromuscular junction, where the concentration of a single quantum of ACh at postjunctional receptors is 300 μM (Hartzell et al., 1975). Fourth, the response of heart muscle to application of ACh does not desensitize for several tens of minutes (Glitsch and Pott, 1978), whereas at the neuromuscular junction it desensitizes within seconds (Katz and Thesleff, 1957). This lack of rapid desensitization may be important for chemical messengers that exert their effects for prolonged periods.

Chemical messengers that diffuse long distances between neurons have been termed locally acting hormones (Branton et al., 1978a) or nonsynaptic transmitters (Dismukes, 1979; Mayeri and Rothman, 1982; Jan and Jan, 1983). This mode of action seems an attractive possibility for some of the neuroactive peptides recently discovered in the CNS because many of the peptides have well defined roles as peripheral hormones and, like their actions on peripheral tissue, they have long-lasting actions on central neurons. However, as in studies of conventional transmitters at synapses, unequivocally establishing that transmitters can function by diffusing long distances in the CNS requires detailed cellular studies.

It has been suggested that egg-laying hormone (ELH) and perhaps other bag cell neuropeptides are dispersed over long distances to

act on target neurons in the abdominal ganglion of *Aplysia* (Branton et al., 1978a, b; Mayeri et al., 1979a, b; Mayeri and Rothman, 1982). ELH, a 36-amino acid peptide, is released from the bag cells to act as a transmitter within the abdominal ganglion (Branton et al., 1978b) and also as a neurohormone that enters the general circulation to act on other parts of the CNS (Stuart and Strumwasser, 1980) and on gonadal tissue to induce egg laying (Rothman et al., 1983a). A 20-min repetitive discharge of impulse activity in the bag cells, triggered by brief electrical stimulation, produces several types of long-lasting responses in neurons located in the abdominal ganglion (Mayeri et al., 1979a, b). The responses have slow onsets and prolonged durations (up to 3hr), and they include several types of excitation and inhibition. The bag cells contain ELH, α-bag cell peptide (α-BCP), and other neuropeptides that are encoded on a common precursor molecule (Scheller et al., 1983a; Rothman et al., 1983b). It was previously shown that application of ELH duplicates at least one type of bag cell-induced response, burst augmentation of cell R15 (Branton et al., 1978b). α-BCP is a putative transmitter for bag cell-induced inhibition, including inhibition of left upper quadrant (LUQ) neurons, L2 to L4 and L6, and for autoexcitation of the bag cells (Rothman et al., 1983b; Sigvardt et al., 1983; Sigvardt et al., submitted for publication).

In this paper we investigate further the physiological role of ELH. We show that ELH is released in sufficient quantities to act by diffusion over long distances, that it is effective at lower concentrations than are synaptic transmitters, and that there is little or no rapid desensitization of the responses in target cells. When perfused into the ganglion at physiological concentrations it acts selectively, affecting a subgroup of the neurons that respond to bag cell activity, without affecting other neurons. The results suggest that ELH diffuses long distances to act on target neurons.

Preliminary reports of part of this work have been published (Rothman et al., 1979, 1981; Mayeri and Rothman, 1982).

Materials and Methods

Purification of ELH and acidic peptide. Large (1 to 3 kg), sexually mature *Aplysia californica* were obtained from either Pacific Biomarine (Venice, CA) or Sea Life Supply (Sand City, CA) or were collected from Monterey Bay, California. Abdominal ganglia were dissected from the animals and incubated in a solution of 50% isotonic $MgCl_2$ and 50% *Aplysia* blood which had been passed through a glass fiber filter (Millipore AP 25 prefilter). Bag cell clusters were surgically removed from the ganglion and stored at −80°C until homogenized. Four clusters were labeled with [^{3}H]leucine, [^{3}H]lysine, and [^{3}H]arginine (Amersham Corp., Arlington heights, IL) in a 12-hr pulse, 12-hr chase paradigm (cf. Arch, 1972). One hundred frozen clusters plus the radiolabeled ones were homogenized and subjected to a three-step purification procedure as described previously (Rothman et al., 1983b). Briefly, the clusters were homogenized in a 1.0 M acetic acid solution containing 0.1% β-mercaptoethanol and several protease inhibitors. After centrifugation the supernatant was applied to a Sephadex G50 (Superfine) column (2.5 × 30 cm) equilibrated at 4°C with 1.0 M acetic acid and eluted at a rate of 5 ml/cm^2 hr. Fractions of 4 ml each were collected and the A_{280} of each fraction was determined on a Gilford model 240 spectrophotometer. Fifty microliters of each fraction were dissolved in 5 ml of Aquasol (New England Nuclear, Boston, MA) and counted on a Beckman LS-3133P liquid scintillation counter at 35% efficiency.

Pooled fractions were further purified on a C_{18} reversed phase high performance liquid chromatography (HPLC) column (Whatman Partisil 10-ODS or Supelco DB-C18, 4.6 × 250 mm). The protein in column eluates was monitored by a fluorescamine detection system (Stein, 1981).

HPLC analysis and purification of releasate. To purify ELH and acidic peptide (AP) released from abdominal ganglia, a ganglion was placed in a small chamber used for serial perfusion experiments (see below) and perfused with artificial seawater (ASW) containing the protease inhibitors, leupeptin and antipain (200 μg/ml each). Releasate consisted of perfusate collected for two contiguous 30-min periods beginning at the onset of a bag cell burst discharge that was initiated by brief electrical stimulation of one of the bag cell clusters. Perfusate collected during the 30-min period preceding the bag cell burst discharge served as a control. Releasate was collected from seven bag cell burst discharges, which had been initiated in four ganglia, and combined to a total volume of 14 ml. Releasate or control perfusate (1 ml each) was applied to a Supelco C_{18} DB column (4.6 × 250 mm) equilibrated with pyridine acetate (pH 4.0) and run at a rate of 0.6 ml/min. The column was then washed for 30 min and the peptides eluted with a linear gradient of *n*-propanol (0 to 30% in 120 min).

Electrophysiology and arterial perfusion of peptides. More than 120 ganglia from animals weighing 300 to 2000 gm were used in the electrophysiological experiments. For each experiment, an isolated abdominal ganglion was pinned in a 1-ml-capacity chamber lined with silicone elastomer (Fig. 1). The preparation was steadily superfused with bathing medium at a rate of 7 ml/hr. Experiments were performed at room temperature (19 to 23°C), which varied less than 1° during a single experiment. Intracellular recordings were made from up to four cells at a time using conventional methods, as described previously (Mayeri et al., 1979a, b), and were stored on magnetic tape. The bag cells were continuously monitored during all experiments by placing an extracellular recording electrode on one of the bag cell clusters or by recording intracellularly from a single bag cell.

Bathing medium consisted of 50% filtered *Aplysia* blood and 50% SW. The medium was buffered with 10 mM HEPES (pH 7.6). Low Ca^{2+}, high Mg^{2+}, 1 mM Mn^{2+} medium contained 410 mM NaCl, 28 mM Na_2SO_4, 10 mM KCl, 0.3 mM $CaCl_2$, 75 mM $MgCl_2$, 1 mM $MnCl_2$, and 10 mM HEPES (pH 7.6).

ELH and other substances were assayed by perfusion into the caudal artery of the abdominal ganglion (Fig. 1). the artery was cannulated with a length of polyethylene tubing (Intramedic PE 50) attached by a knot of surgical suture. Experimental solutions were perfused at a rate of 3 μl/min by means of a micrometer syringe (Gilmont Instruments, Inc., Great Neck, NY) connected to the cannula. Patency of the cannulation and ganglionic vascular system was tested by perfusing 1 mM cytochrome *c* (Sigma Chemical Co., St. Louis, MO), which has a red color. Cytochrome *c* was seen to exit from the vascular and interstitial spaces of the ganglion after arterial perfusion of 0.1 to 0.2 μl of solution in a 500-gm animal. If one assumes that the actual volume of the spaces is 2 to 3 times this amount, with an application of ≥5 μl of test solution one could expect the final concentration in the ganglion to be near that of the test solution.

ELH and other purified peptides to be assayed for activity were aliquoted into microcentrifuge tubes and lyophilized. Immediately before perfusion, the peptides were dissolved in filtered (0.22-μm pore size, Millipore) superfusion medium. In most experiments the medium also contained 250 μg/ml, each, of the protease inhibitors, bacitracin, ovomucoid, and lima bean trypsin inhibitor. However, for all of the physiological experiments, similar ($N = 3$) results were obtained in the absence of the three inhibitors. Focal pressure application of peptides via a micropipette placed near the cell soma was performed as described by Branton et al. (1978a).

Serial perfusion of ganglia. An abdominal ganglion from which bag cell releasate was to be collected was placed in a sealed chamber of about 200 μl dead volume. The ganglion was cannulated by means of a polyethylene tube inserted tightly through a small hole in the chamber wall. A wire, insulated except at the tip, was placed on each bag cell cluster. The two wires served as both stimulating and recording electrodes for the bag cells. A third wire attached to the chamber acted as ground. Electrical activity was monitored by a differential AC preamplifier. Bag cell burst discharges were triggered by a train of constant-current electrical pulses (0.2-msec pulses, 5/sec for 2 sec; 1 to 10 mA) delivered through the two-bag cell electrodes. The ganglion was simultaneously perfused and superfused at a rate of 7 μl/min each by means of a peristaltic pump. Bathing medium flowed out of the chamber under positive pressure and into a polyethylene tube leading to the caudal artery of an assay ganglion located in a second chamber (see Fig. 6*A*). Intracellular recordings were made from neurons in the assay ganglion. The dead volume of the polyethylene tube was approximately 120 μl. An extracellular electrode placed on one of the assay ganglion bag cell clusters showed that the bag cells did not discharge during the experiments.

Results

Purification of ELH and AP. ELH is the putative transmitter for one of the five types of responses induced by a burst discharge of the bag cells. To study the role of ELH further and identify transmitters mediating the other types of responses, ELH and other bag cell peptides were purified from bag cell extracts. For these studies it was important to obtain peptides of high purity and be able to apply them at known concentrations.

Bag cell extract was fractionated on a Sephadex G50 column equilibrated with 1.0 M acetic acid (Fig. 2*A*). Lyophilized fractions from the column were redissolved in perfusion medium and assayed for ELH-like activity by arterial perfusion into the abdominal ganglion. Most of the neuronal activity later identified as ELH was contained

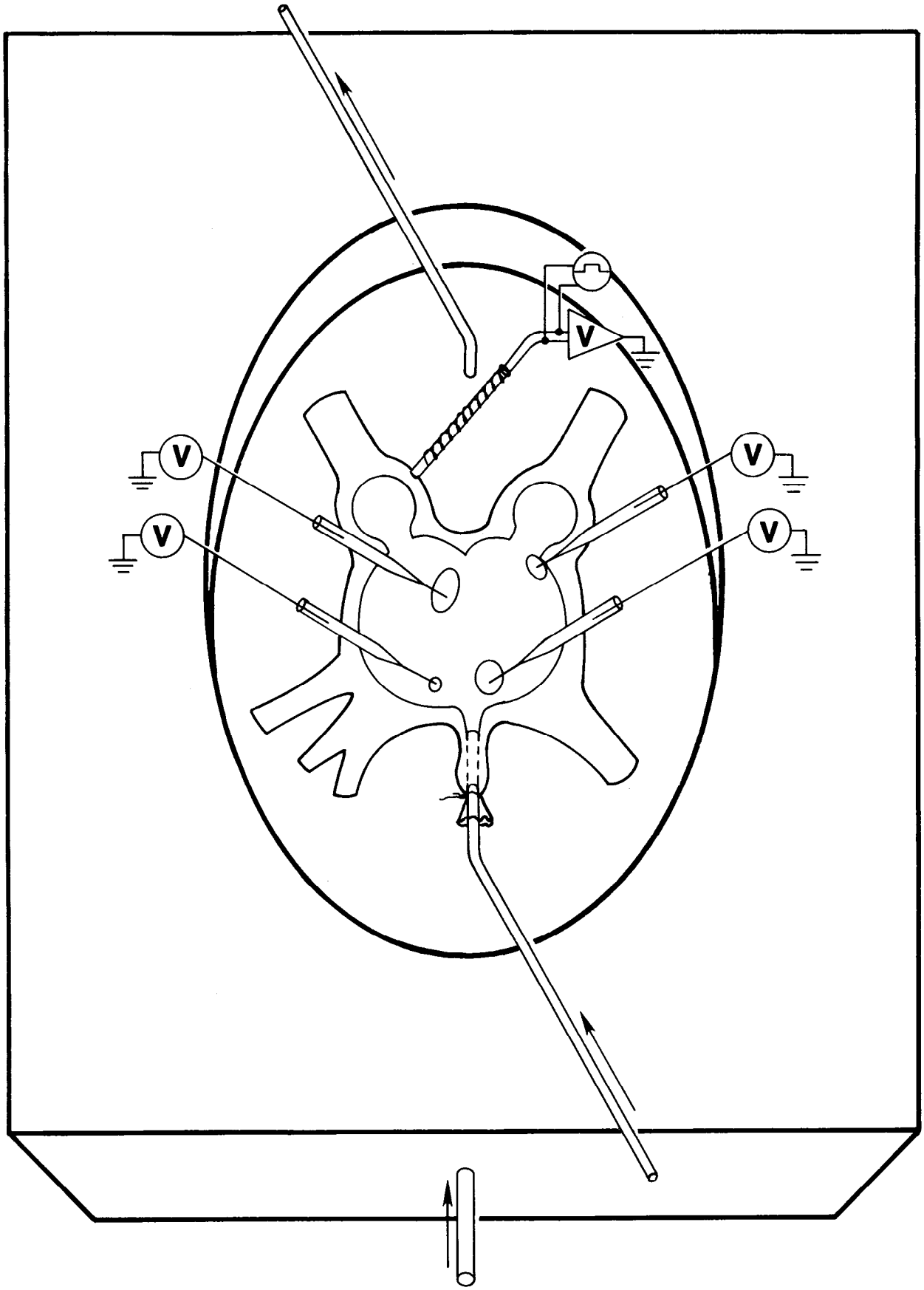

Figure 1. Schematic diagram showing the method of arterial perfusion of peptides into the isolated abdominal ganglion. The abdominal ganglion was pinned in a small chamber and superfused at a constant rate with medium that entered and exited at opposite ends of the chamber. Peptide solutions were perfused into the vascular spaces of the ganglion via a polyethylene tube inserted into an artery located at the caudal end of the ganglion. Intracellular recordings (*V*) were made from up to four cells at a time. An extracellular electrode was placed on one of the two bag cell clusters to record and/or stimulate bag cell spike activity.

in the peak of ^3H radioactivity and A_{280} occurring at K_{av} = 0.25 to 0.50 (M_r = 2,000 to 12,000). Fractions containing this medium molecular weight material were pooled and further purified by HPLC (Fig. 2*B*). ELH (peak 2) from the HPLC eluate was identified by neuronal assay and repurified to apparent homogeneity in a subsequent HPLC run under isocratic conditions (Fig. 2*C*). Amino acid composition analysis, kindly provided by Dr. J. Ramachandran, confirmed that the material was ELH as described by Chiu et al. (1979). Both peak 2 and the ELH repurified in Figure 2*C* were used in experiments described below.

The material in peak 1 of Figure 2*B* was identified by microsequencing (Scheller et al., 1983a) as AP, a 27-amino acid peptide that is encoded on the ELH/bag cell peptide precursor. Peak 1 (like the ELH peak) incorporated the ^3H radioactivity, indicating that AP is synthesized by the bag cells. This AP may differ from the one originally purified from the bag cells by Arch et al. (1976), since it does not contain a methionine residue (see Rothman et al., 1985).

Prolonged excitation of left lower quadrants (LLQs) by ELH. ELH and other substances to be tested were perfused through the vascular spaces of the abdominal ganglion, whereas intracellular recordings were made from as many as four neurons simultaneously. The arterial perfusion method proved to be a sensitive and efficient means of investigating the neuronal effects of peptides because as little as 5 μl of test solution was sufficient to bathe the vascular and interstitial spaces of the ganglion at a concentration approximating that of the test solution. With this assay we could consistently detect 10^{-13} mol of ELH.

We confirmed that ELH, either arterially perfused or applied directly to somata, mimicked the augmentation of bursting pacemaker activity in cell R15 (Branton et al., 1978b). In a survey of other ganglion neurons we found that ELH also mimicked a second bag cell-induced response: prolonged excitation of cells of the LB and LC clusters, which are located in the LLQ of the ganglion (Mayeri et al., 1979b). These LLQ cells are normally either silent or fire at low rates in the isolated ganglion. As shown in Figure 3, arterial perfusion of ELH at 1 μM concentration caused an increase in firing rate of LLQs which persisted for an hour or more. The response was usually accompanied by a membrane depolarization of 2 to 3 mV. After correcting for arterial dead space, we calculated that the latency to response onset, measured as the time to first spike, was in the range of 0.5 to 2 min (N = 8), which is similar to that seen in prolonged excitatory responses following initiation of bag cell activity (Mayeri et al., 1979a). As with burst augmentation of R15, the response did not require the continued presence of ELH, since there was little change in response when the preparation was perfused with control solutions after ELH administration (see Fig. 11). Both prolonged excitation and burst augmentation could be elicited by perfusion of the ELH that had been purified to apparent homogeneity. This indicates that the two types of responses are produced by a single peptide.

Almost all of the cells located in the caudomedial region of the LLQ of the ganglion responded to ELH. These cells were members of the LB and LC clusters, although on occasion (N = 3) some LD cells were also responsive. In cells that were responsive, stimulation of a bag cell discharge (after the response to applied ELH had ended) also produced prolonged excitation, as expected.

All other peaks and troughs of medium molecular weight material (Fig. 2*B*) and low molecular weight material (Rothman et al., 1983b) purified by HPLC were assayed on LLQs, LUQ cells (L2 to L4 and L6, which are inhibited by bag cell activity), R15, and various other ganglion neurons at concentrations ≤250 μg/ml. ELH was the only factor that duplicated the prolonged excitation or burst augmentation produced by bag cell activity. Also, AP, the other identified peptide purified from medium molecular weight material, had no effect on ganglion neurons or on the bag cells themselves.

Direct action of ELH. To test for direct action of ELH on LLQ cells and R15, the ganglion was bathed in low calcium, high magnesium, 1 mM Mn^{2+} medium until the amplitude of synaptic transmission, as judged by the large cholinergic excitatory postsynaptic potential (EPSP) in R15 from Interneuron XII (Koester et al., 1974), was decreased to less than 1% of its original amplitude. With synaptic transmission blocked, a 1- to 2-min application of ELH produced a slow depolarization and repetitive spike activity in LLQ cells characteristic of prolonged excitation (Fig. 4). The amplitude and time course of these spikes indicated they had failed to actively invade the cell soma. The low amplitude of the spikes was presumably due to reduction of the Ca^{2+} currents by the low Ca^{2+} medium. Responses lasted about 20 min rather than the usual ≥1 hr. Although the basis for the shorter response duration is unknown, it is conceivable that the normal response is dependent on an influx of Ca^{2+}.

Application of ELH to isolated groups of LLQ cells (not shown) provided further evidence that ELH acts directly. Clumps of 5 to 10 cells together with a small amount of neuropil containing their axons were surgically isolated from the rest of the ganglion (N = 4). Somatic application of ELH (70 μM) produced a normal prolonged excitatory response. Before the application there was spontaneous low amplitude (<0.5 mV) excitatory postsynaptic activity in some LLQ cells, which indicated that not all of the synaptic inputs to these cells had been abolished. Nevertheless, the rate of this spontaneous activity was unchanged after ELH application.

In the low Ca^{2+}, high Mg^{2+}, 1 mM Mn^{2+} medium, bursting pacemaker activity in cell R15 was augmented by somatic application (N = 4) of ELH (Fig. 5), indicating that ELH also acts directly on this cell. Burst intensity and mean spike rate increased as expected, and the response far outlasted the period of ELH application although, as with LLQ cells, response duration was shorter than for ELH applied in normal bathing medium (see Branton et al., 1978b).

Release of sufficient amount of transmitter for a nonsynaptic action. If transmitter is released by bag cells in sufficient quantities to diffuse into the interstitial and vascular spaces of the ganglion to act on target neurons, then one might expect to stimulate a bag cell burst discharge in one ganglion and show that the releasate has effects on ELH target neurons in a second, assay ganglion. To test this possibility, a tandem ganglion experiment was conducted as depicted in Figure 6*A*. An abdominal ganglion, termed the source ganglion, was placed in a small, sealed chamber, its artery was cannulated, and it was continuously perfused and superfused at a rate of 7 μl/min each. Two extracellular electrodes, one placed on each bag cell cluster, were used to stimulate and record bag cell activity. The perfusate from this ganglion was led directly to the artery of a second, assay ganglion, located in the usual recording chamber.

Figure 6*B* shows simultaneous recordings from a bag cell cluster in the source ganglion and from an LC cell and cell L4 in the assay ganglion. A bag cell burst discharge was triggered in the source ganglion by a 2-sec train of electrical stimulation (Fig. 6*B*, *arrow*). After a delay for transit time between the two chambers, transmitter released during the bag cell burst discharge in the source ganglion produced a prolonged excitatory response in the LC cell of the assay ganglion. This response lasted for more than 1 hr and was apparently identical to that produced by a bag cell discharge in a single ganglion or by arterial perfusion of ELH. In contrast to the LC cell and other LLQ cells, there was no inhibitory response (N = 4) to the releasate in L4 and other cells which normally respond with prolonged inhibition to bag cell activity. Instead, L4 was excited slightly by the releasate. It is possible that this excitation is due to ELH, which excites L2 and L4 at high concentrations (see below). There was also no effect of the releasate on cells L1 and R1, which are transiently excited by bag cell activity. Experiments described elsewhere have shown that these two types of responses, prolonged inhibition and transient excitation, respectively, are mimicked by releasate, provided that appropriate protease inhibitors are added to the perfusion medium (Sigvardt et al., 1983).

We also tested for nonsynaptic release of transmitter mediating burst augmentation in R15. For this experiment, an abdominal ganglion was bathed in 30 μl of medium while a bag cell burst

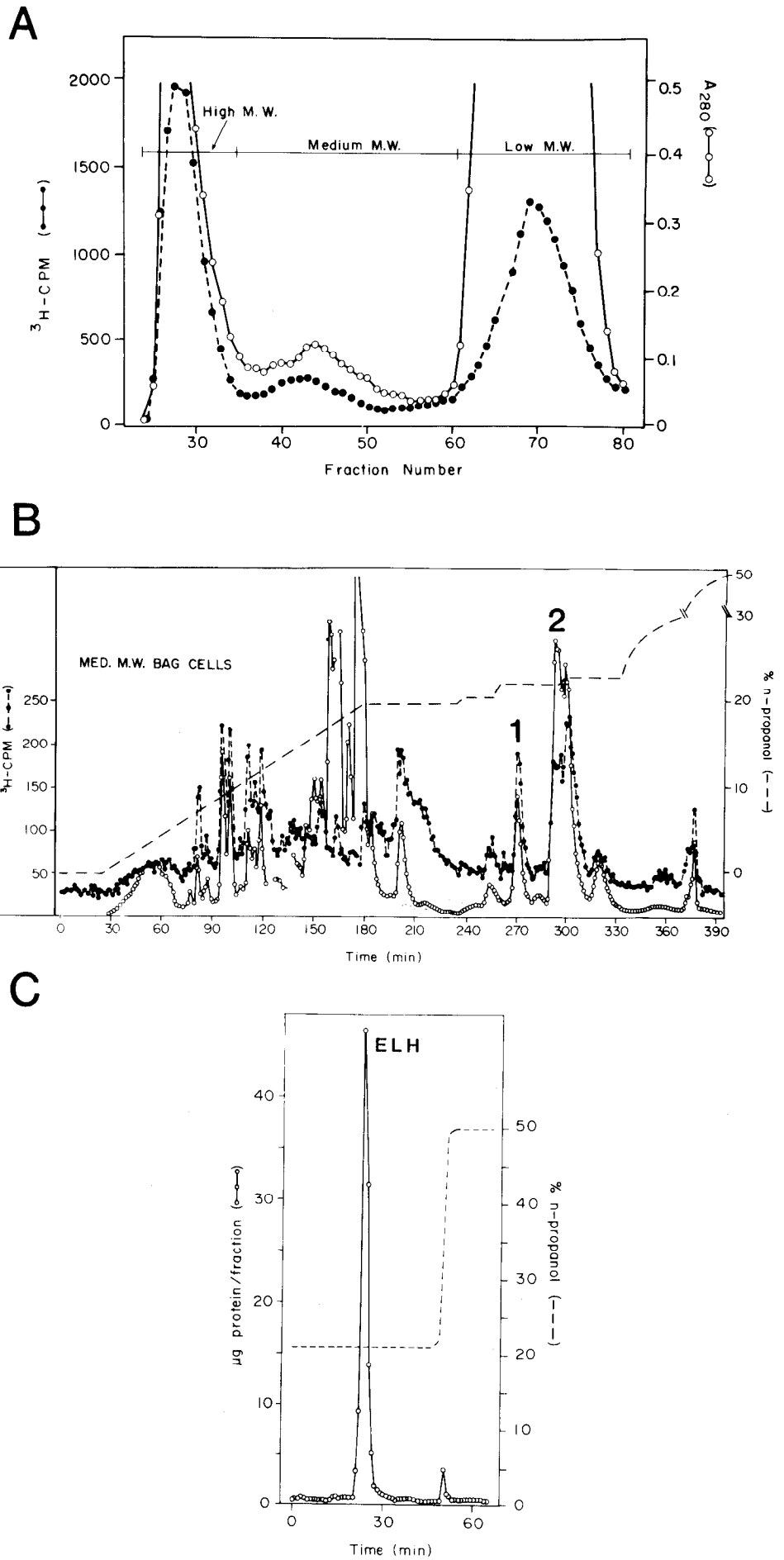

Figure 2. Purification of ELH and acidic peptide. *A*, Fractionation of bag cell extract by gel filtration chromatography. An acetic acid extract of 100 bag cell clusters was applied to a Sephadex G50 column equilibrated at 4°C with 1.0 M acetic acid and eluted at a rate of 5 ml/cm^2 hr. Four of the bag cell clusters were pulse-chase radiolabeled with [^{3}H] arginine, [^{3}H]lysine, and [^{3}H]leucine. The indicated fractions were separately pooled into high, medium, and low molecular weight material. *B*, Purification of medium molecular weight material on HPLC. Pooled medium molecular weight fractions from *A* (75 ml) were applied to a C_{18} reverse phase HPLC column (Whatman Partisil 10 ODS) equilibrated in pyridine acetate at pH 4.0 and run at a rate of 2.0 ml/min. After washing the column for 100 min, material was eluted with a gradient of *n*-propranol. Acidic bag cell peptide (67 μg) eluted as peak 1 and ELH (333 μg) eluted as peak 2. *C*, Repurification of ELH to apparent homogeneity. One hundred eleven micrograms of peak 2 material (see *B*) were applied to a Supelco DB-C_{18} column equilibrated with pyridine acetate, pH 5.5 and run at a rate of 0.6 ml/min. The column was washed for 30 min and the buffer was raised to 21% *n*-propranolol (v/v) at time 0 min. A symmetric peak containing 100 μg of ELH eluted at 23 min. A small peak containing 4 μg of material eluted after the propanol concentration was raised to 50%.

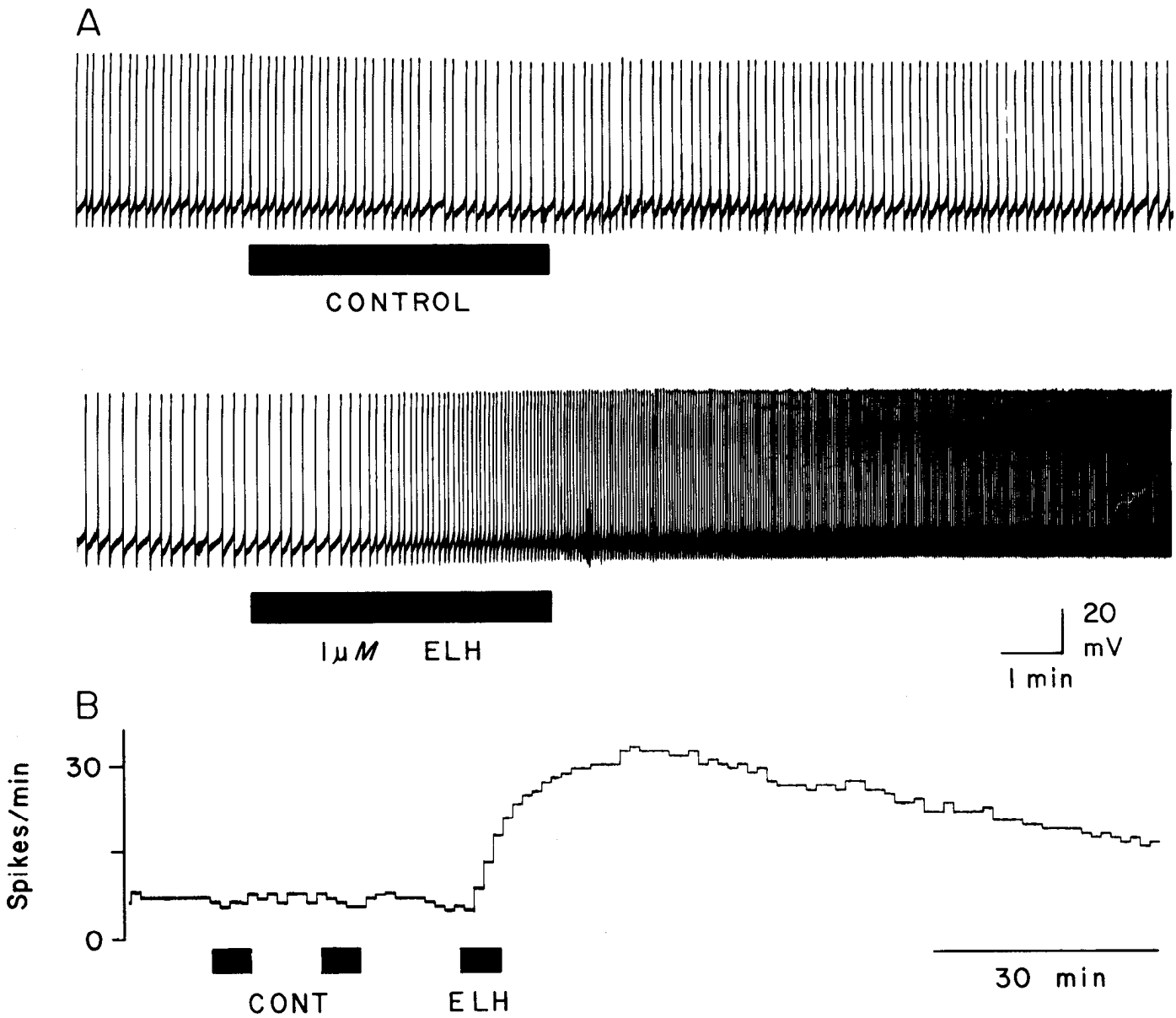

Figure 3. Arterial perfusion of ELH mimics the prolonged excitatory response produced by the bag cells in LB and LC cells (LLQ cells). *A*, Continuous intracellular recording from an LB cell. *Upper trace:* A control solution containing protease inhibitors was perfused at a rate of 3.3 μl/min (*bar*). *Lower trace:* ELH (1 μM) plus protease inhibitors was perfused. *B*, Histogram of the time course of the response shown in *A*. The *bar* indicating perfusion has not been adjusted for arterial dead space, which was approximately 1.5 μl in this example.

discharge was triggered by electrical stimulation. Twenty minutes later, 5 μl of the bathing medium were focally applied to R15 in an assay ganglion by a micropipette placed near the soma. This resulted in a normal burst augmentation response, characterized by an increase in bursting pacemaker potential, peak spike rate, and mean spike rate (Fig. 7). In this type of experiment, essentially identical results were obtained whether whole ganglia ($N = 4$) or isolated bag cell clusters ($N = 2$) were used as the source of released transmitter. Unstimulated control bathing media did not produce responses.

Identification of ELH and AP in the releasate. To identify the active factor in the releasate, individual ganglia were placed in the small sealed chamber used for the above experiments and were perfused and superfused with ASW containing the protease inhibitors leupeptin and antipain. Perfusate was collected at 30-min intervals before and after an electrically triggered bag cell burst discharge. Perfusates from four ganglia yielding a total of seven bag cell burst discharges were pooled and aliquots were analyzed by HPLC using a linear propanol gradient. Several peaks were present in the releasate collected from the ganglia after the bag cell burst discharge had been stimulated but were not present in control perfusion medium collected before bag cell stimulation (Fig. 8). Two of these peaks were identified as ELH and AP, respectively, by co-migration on HPLC with the peptides that had been purified from bag cell extracts (not shown). (The order of elution of ELH and AP was reversed compared to the data shown in Fig. 2 because of small differences in the properties of the C_{18} columns used in the two experiments.) The remaining pooled releasate was subjected to preparative HPLC to yield 1 μg of ELH, which, as expected, produced prolonged excitation of LLQ cells.

Concentration-response relationships. If one assumes that ELH concentration in the interstitial space surrounding target neurons remains fairly constant for several minutes after onset of a bag cell burst discharge, then one can obtain an estimate of the effective concentration of the peptide by comparing the amplitude of the neurally induced response to that produced by known concentrations of arterially perfused ELH. As shown in Figure 9, ELH was

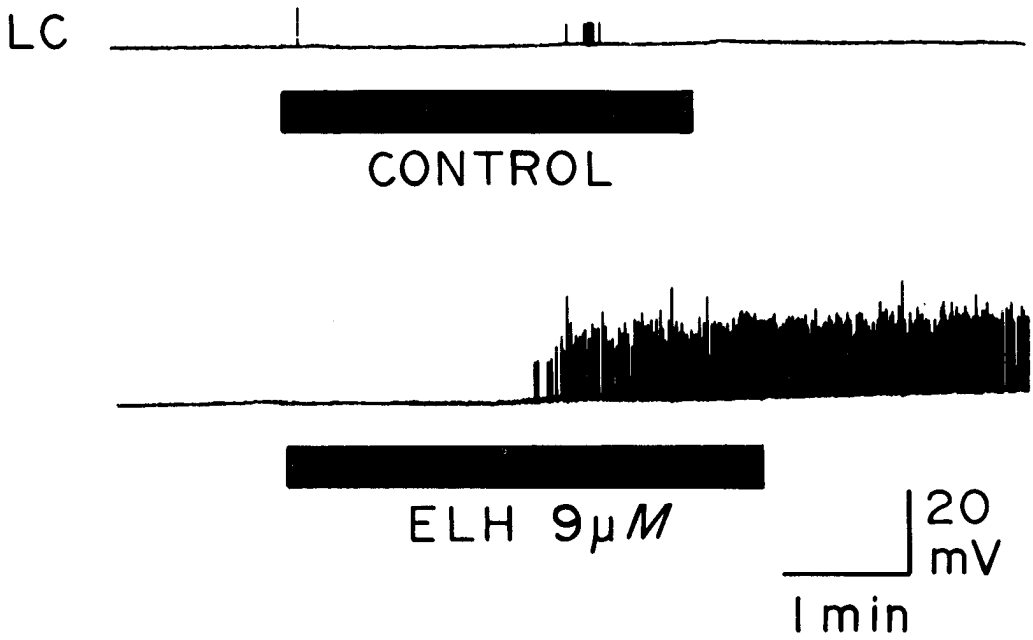

Figure 4. Direct action of ELH on an LC cell. An abdominal ganglion was superfused with a low Ca^{2+}, high Mg^{2+} ASW solution. *Upper trace*, Arterial perfusion of this solution plus protease inhibitors had little effect on LC activity. *Lower trace*, Twenty minutes later, ELH (9 μM) dissolved in the same solution was arterially perfused (*bar*). The LC cell began spiking about 2 min later and the response lasted > 20 min. Spike amplitudes were attenuated by failure of spikes to invade the cell body.

arterially perfused approximately every 10 min at increasing concentrations while recording from three LLQ cells. Increasing concentrations of ELH produced greater responses. The threshold for prolonged excitation, as measured by the onset of spike activity in previously silent cells was 10 nM ($N = 8$ cells in four experiments).

In a separate set of experiments a bag cell discharge was electrically triggered under conditions nearly identical to those of perfusion of ELH. The firing rate of LLQ cells increased from 7 ± 12 to 47 ± 12 (mean ± SD) spikes/min ($N = 7$ cells in four experiments). The magnitude of this change is similar to that seen after arterial perfusion of approximately 0.5 to 1.0 μM ELH (Fig. 9). This range of concentrations is thus a first approximation of the concentration of ELH in the interstitial and vascular spaces of the ganglion during the bag cell discharge.

Although ELH also produced concentration-dependent burst augmentation in R15, the apparent threshold varied and was higher (often ≥1 μM) than that of LLQ cells. The higher threshold may have been because, at the time of ELH application, R15 was already in a partially activated state (due to mechanisms other than ELH action). In particular, the bursting pacemaker activity of R15 is diurnal and increases during the day (Audesirk and Strumwasser, 1975). Greater concentrations of ELH may therefore be needed to augment this activity than to activate the LLQs, which are normally silent.

Selective action of ELH. Although the bag cells induce responses in a large proportion of ganglion neurons, many other neurons are unresponsive. If after release from the bag cells, ELH diffuses throughout the ganglion, one would expect that it acts selectively by affecting only those neurons that have receptors for ELH. Correspondingly, one would predict that when arterially perfused, ELH would produce responses only in those neurons or a subgroup of the neurons that are also affected by bag cell activity. Previous studies have shown that an identified cluster of neurons, the RB cells, shows no direct response to bag cell activity (Mayeri et al., 1979b); unlike LLQs and LUQs, the mean spike rate of RBs is unaffected by bag cell activity, although the pattern of firing is altered slightly as a result of bag cell action on L10 and the consequent increase in the frequency and amplitude of EPSPs produced by L10 in the RBs. To test for selective action we recorded from two RB cells and one LLQ cell. When arterially perfused at concentrations ranging from 5 to 50 μM, ELH did not change the mean spike rate of the RB cells compared to control perfusions (Fig. 10). By contrast, LLQ cells showed the appropriate prolonged excitatory response to ELH. There was, however, an increase in the frequency and amplitude of L10 EPSPs and a slight alteration in the pattern of firing of the RBs, which we attribute to an ELH action on L10. Similar results were obtained with other cells that are known to be unresponsive to bag cell activity (Mayeri et al., 1979a, b), including L11, L7, and unidentified cells. In many instances ($N = 9$) a bag cell burst was stimulated after application of the peptide, and in every case it was found that cells unresponsive to bag cell activity were unresponsive to ELH.

Lack of rapid desensitization. There was no sign of rapid desensitization (seconds to minutes) of ELH-induced responses, even when ELH was applied at very high concentration. As shown in Figure 11, perfusion of ELH at 310 μM concentration for 3.5 min resulted in large, sustained responses in R15 and an LLQ cell. The responses persisted even after the peptide was washed out of the vascular spaces by arterial perfusion of control medium (Fig. 11*B*).

ELH does not mediate all bag cell-induced responses. ELH did not mimic three types of responses produced by bag cell activity: prolonged inhibition of cells L2 to L4 and L6, and certain other neurons, transient excitation of L1 and R1, and depolarization of the bag cells that occurs during the bag cell discharge. ELH had no apparent effect on these cells at physiological concentrations and higher ($\leq 10^{-4}$ M) (Figs. 11 and 12).

As shown in Figure 12, ELH had no effect on cells L1 and R1. However, one of the bag cell factors we purified from low molecular weight material mimicked the transient excitatory response in L1. This factor has the amino acid composition of β-BCP which is encoded on the ELH/BCP precursor. Arterial perfusion of 34 μM ELH to the same cell had no effect, although the LC cell responded normally to the ELH (Fig. 12, *upper right traces*).

ELH had no effect on the membrane potential of bag cells at concentrations ranging from 10^{-9} to 10^{-4} M. This is in contrast to α-BCP, the putative autotransmitter, which depolarizes bag cells at 1 μM concentration (Rothman et al., 1983b). In many experiments, however, the excitability of the bag cells seemed reduced after arterial perfusion of ELH; stronger electrical stimulation was needed to evoke a bag cell burst discharge and the burst discharge had a shorter than usual duration. It is therefore possible that ELH has a modulatory effect on bag cells which decreases excitability without affecting membrane potential.

At very high concentrations, ELH had a slight excitatory effect on cells L2 and L4 (Fig. 11*B*). In Figure 11, 340 μM ELH produced a 25% increase in L2 spike rate compared to a much larger (3-fold) increase in R15 spike rate. In a fourth, unidentified cell (Fig. 11*B*,

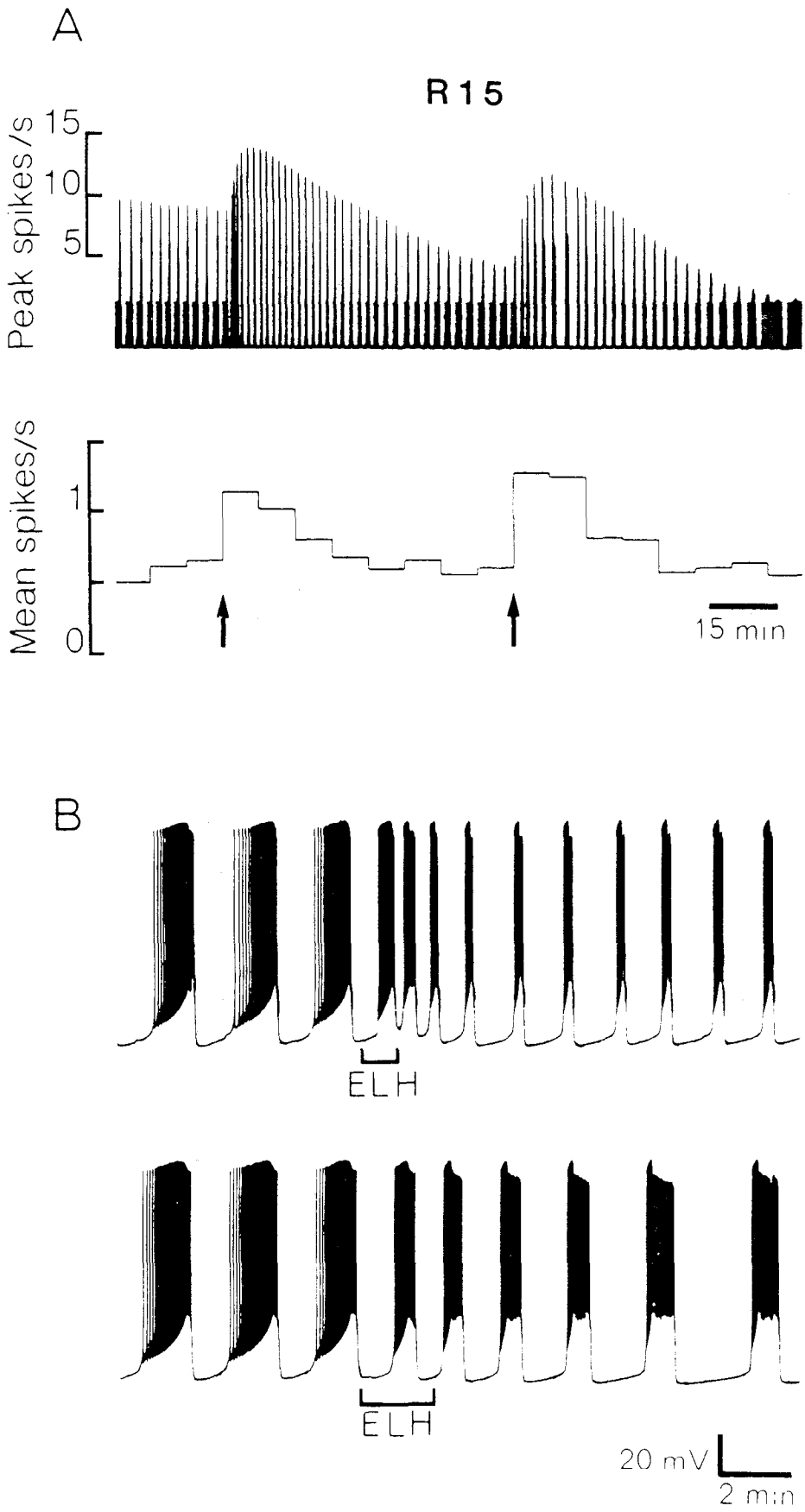

Figure 5. Direct action of ELH on R15. An abdominal ganglion was maintained in a low Ca^{2+}, high Mg^{2+} ASW solution. *A*, ELH (1.2 μl at a concentration of 70 μM) was applied via a micropipette to the soma of cell R15 at two times (*arrows*). In both instances the cell responded with an increase in burst intensity (maximal spike rate during each burst) and mean spike rate (spikes per second averaged over several burst cycles). Bathing medium applied in a similar fashion as a control produced no response (not shown). *B*, Intracellular recordings from cell R15 during responses shown in *A*.

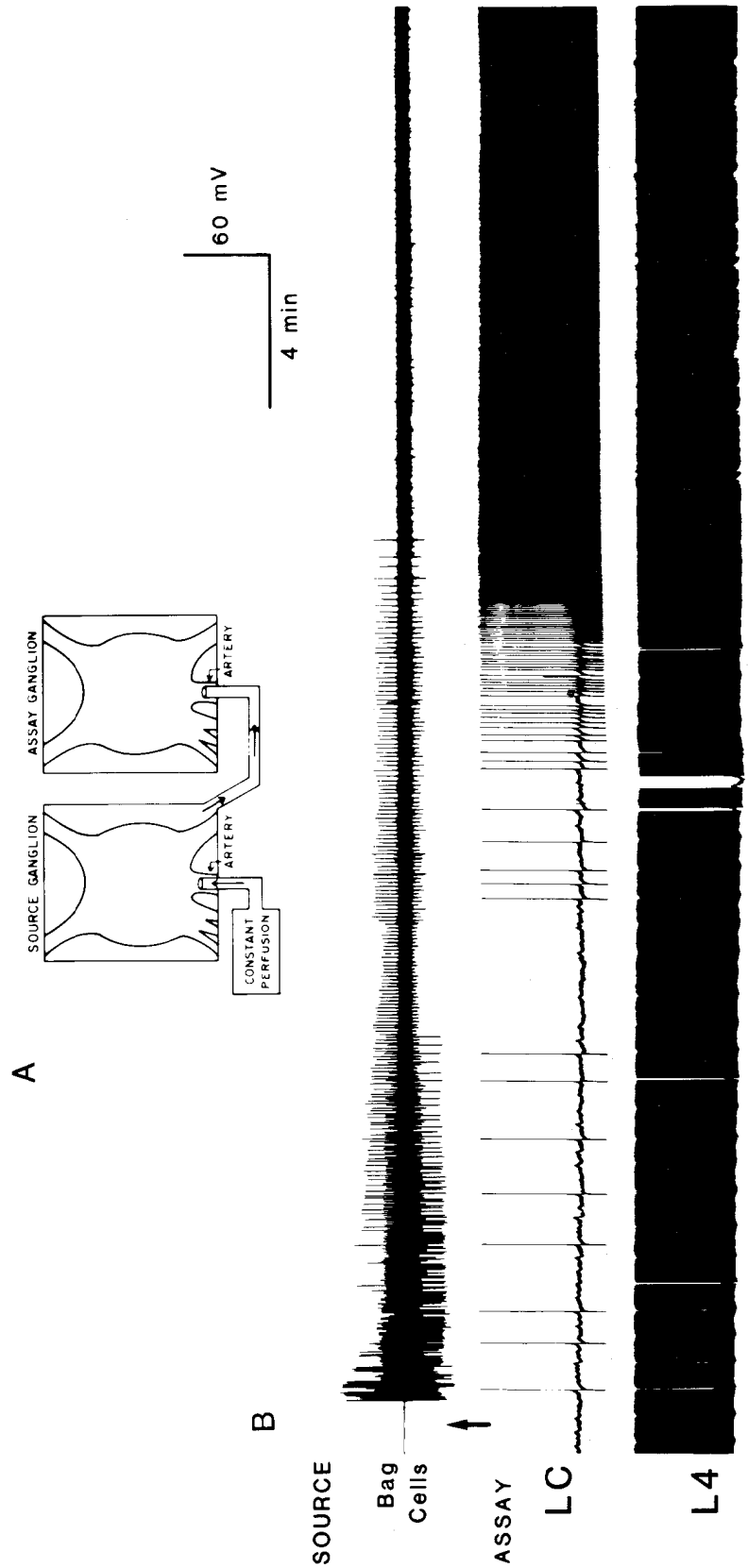

Figure 6. Releasate containing bag cell transmitter produces prolonged excitation in an LLQ cell of a second, assay ganglion. *A*, Experimental arrangement for serial perfusion of two ganglia. An abdominal ganglion was sealed in a small chamber containing two electrodes for stimulating and recording from the bag cells. The ganglion was simultaneously perfused and superfused. The combined perfusate/superfusate was fed directly into the caudal artery of an assay ganglion located in a separate chamber. *B*, Simultaneous recordings from a bag cell cluster in the source ganglion (an extracellular recording) and two cells in the assay ganglion (intracellular recordings). An electrically triggered burst discharge in the bag cells of the source ganglion produced prolonged excitation of the LC cell in the assay ganglion. Cell L4, which is normally inhibited by bag cells, was unaffected. The 60-mV *calibration bar* is for the intracellular recordings only.

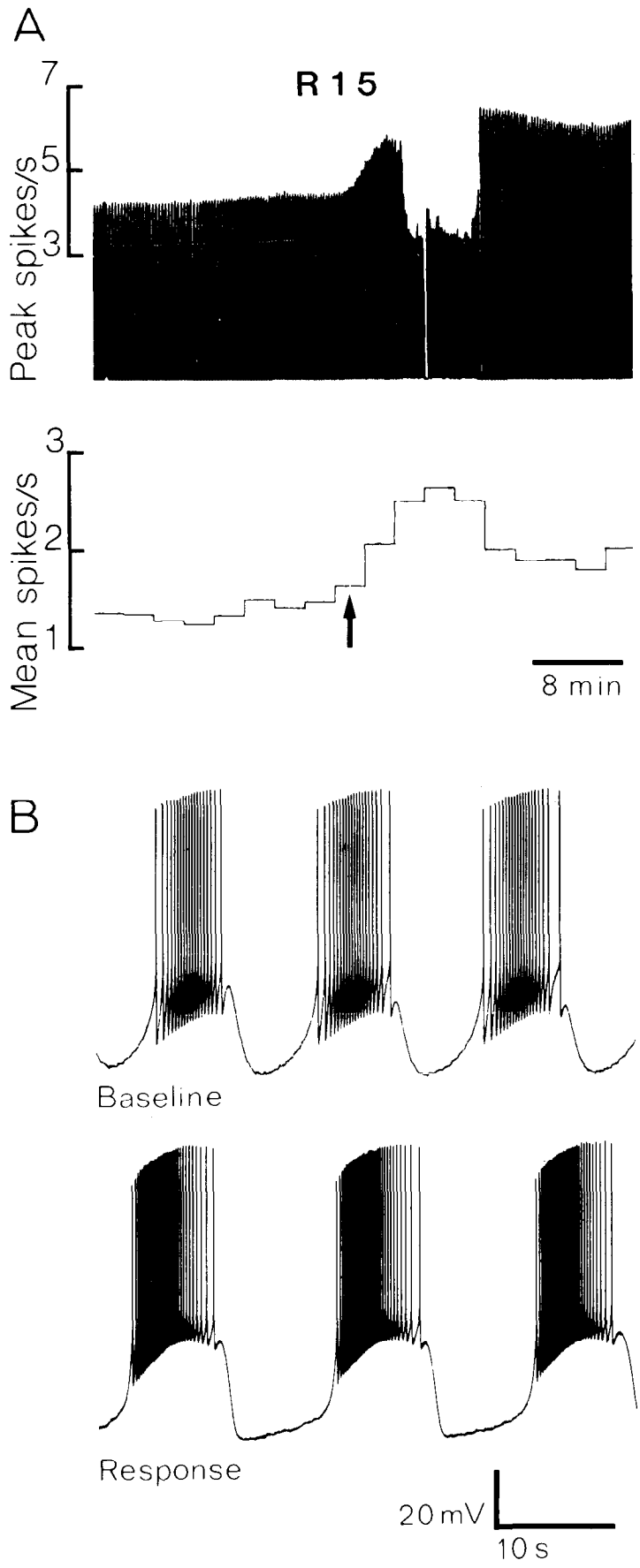

Figure 7. Burst augmentation produced by releasate from bag cells. A bag cell burst discharge was electrically triggered in a ganglion immersed in 30 μl of bathing medium. Twenty minutes later, 5 μl of the bathing medium were applied to the soma of R15 in another ganglion. *A*, Peak spike rate and mean spike rate of R15 versus time. Bathing medium (releasate) was applied at the *arrow*. The transient drop in peak spike rate is due to a period of sustained depolarization in which there was tonic firing, which is characteristic of strong R15 responses (see Fig. 11 and Mayeri et al., 1979a). *B*, Intracellular recordings from R15 1 min before (*Base line*) and 12 min after (*Response*) application of the bathing medium. The response consists of augmented burst activity resulting from an increase in the amplitude of the bursting pacemaker potential underlying each burst.

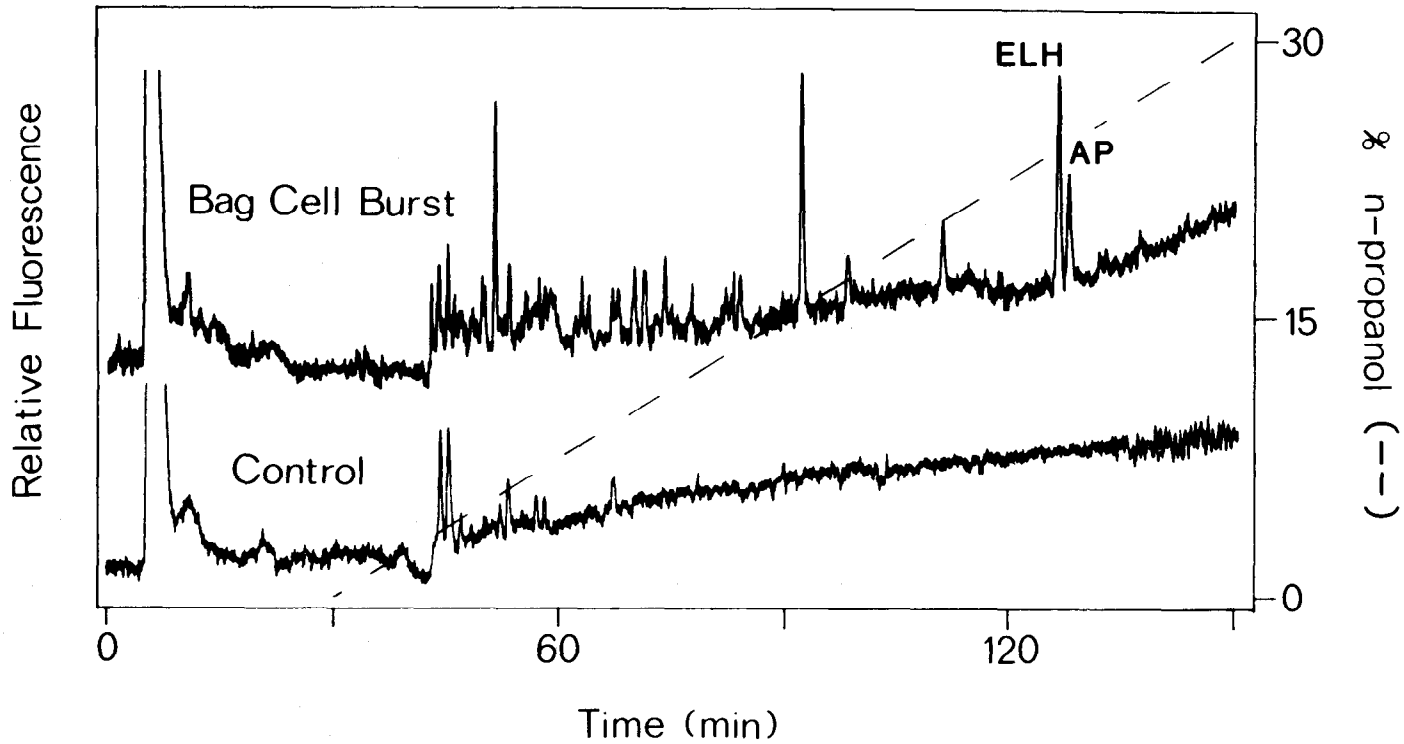

Figure 8. Identification of ELH and acidic peptide in releasate from bag cells. HPLC of releasate (*upper trace*) collected following an electrically stimulated bag cell burst discharge is compared to control perfusate (*lower trace*) collected before the bag cell burst discharge. Four prominent peaks of material were present in the releasate. The ELH and AP peaks were identified on the basis of co-migration with peptides purified and identified from extracts (not shown). Releasate or perfusate (1 ml each) was applied to a Supelco C_{18} DB column equilibrated with pyridine acetate (pH 4.0) and run with a linear *n*-propanol gradient (0 to 30%) in 120 min.

third trace) there was no change in resting membrane potential, but ELH induced an increase in the rate of periodic bursts of spike activity due to bursts of EPSPs from Interneuron II. In semi-intact animals this bag cell-induced increase in interneuron II activity results in an increase in spontaneous gill withdrawal activity during egg laying (Brownell and Schaefer, 1982). ELH also did not alter the inhibition produced in LUQ cells by application of the putative transmitter for these cells, α-BCP, when the two peptides were applied together (not shown). It is conceivable that there are low affinity ELH receptors that are responsible for excitation of L2 and L4 at high concentration because (*1*) ELH had no effect on L3 and L6 and many other cells at high concentration, and (*2*) AP and many other peptides purified with ELH had no effect on L2 and L4 at millimolar concentration.

Selective action of ELH was not due to a barrier to diffusion within the ganglion which might have prevented ELH or other substances from reaching target sites. This conclusion is supported by two types of experiments. First, in many instances, when arterial perfusion of ELH failed to produce a response in a given neuron, another peptide known to produce a response in the cell (usually α-BCP or the neuropeptide FMRF-amide) was arterially perfused and invariably produced the expected response. Second, in some experiments the sheath overlying the dorsal surface of the ganglion was surgically removed and ELH was focally applied to individual somata. For LLQs, R15, LUQs, L1, R1, RBs, bag cells, and many others, results identical to those of the arterial perfusion experiments were obtained.

Discussion

This study provides additional evidence that ELH is a neurotransmitter in the abdominal ganglion and that, compared to conventional transmitters acting at synapses, it diffuses long distances to its targets. The results also show that ELH can account for two, but not all five, bag cell-induced responses. Our findings are consistent with recent evidence that α-BCP mediates bag cell-induced inhibition of ganglion neurons and autoexcitation of the bag cells (Rothman et al., 1983b; Sigvardt et al., 1983; Sigvardt et al., submitted for publication), and they raise the possibility that β-BCP may mediate bag cell-induced transient excitation. Evidence that ELH and other BCPs are neurotransmitters has recently been reviewed (Scheller et al., 1983b; Rothman et al., 1985; Mayeri and Rothman, 1985).

Role of ELH as a neurotransmitter

Both morphological and physiological data provide indications that ELH acts by diffusing long distances. Morphological studies have shown that the bag cells, approximately 400 cells in each of the two clusters, send out tens of thousands of axonal branches, which are immunoreactive for ELH. Some of the branches extend into the sheath overlying other ganglion neurons and into the septa that subdivide the ganglion into quadrants (Chiu and Strumwasser, 1981; Haskins et al., 1981; Hopkins et al., 1982). The axonal processes do not make synapses with other neurons, but they have varicosities spaced along them at regular intervals and the varicosities are packed with dense-core vesicles (Frazier et al., 1967; Haskins et al., 1981). Similar varicosities are found along postganglionic autonomic nerve endings (McMahon and Kuffler, 1971), where they are presumed sites of transmitter release. The bag cell varicosities may therefore represent sites of nonsynaptic release of ELH and other bag cell transmitters.

The physiological characteristics of ELH neurotransmission are listed in Table I, where they are compared to postganglionic autonomic neurotransmission and conventional synaptic neurotransmission. Most of the characteristics of ELH resemble those of ACh on cardiac muscle, rather than ACh at the neuromuscular junction. The first three characteristics of ELH neurotransmission were shown in previous studies (Branton et al., 1978a, b; Mayeri et al., 1979a, b; see also Mayeri, 1979; Mayeri and Rothman, 1982). Compared to conventional postsynaptic responses, the bag cell-induced responses have long latencies and durations. There is also a lack of

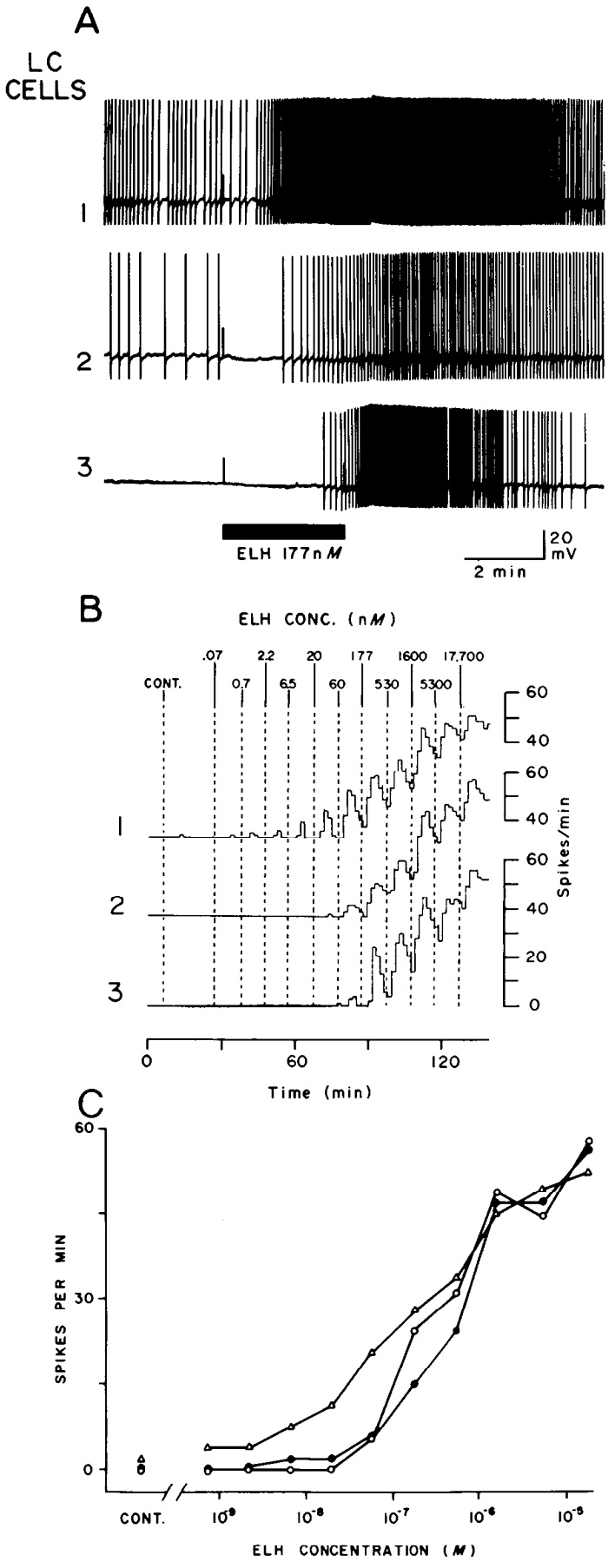

Figure 9. Response of LC cells to increasing concentrations of ELH. Three LC cells were monitored simultaneously while control or ELH solutions were arterially perfused approximately every 10 min. *A*, Intracellular records from the three cells responding to 177 nM ELH (*bar*). The activity present in the *top two traces* prior to the ELH application was a result of a previous ELH application at a lower concentration. *B*, Spike rate histograms for the entire experiment. Threshold for spike activity of LC cell 1 was 6.5 nM. Spiking threshold for the other two cells was about 10 times higher. *C*, Peak spike rate for each of the three cells versus ELH concentration.

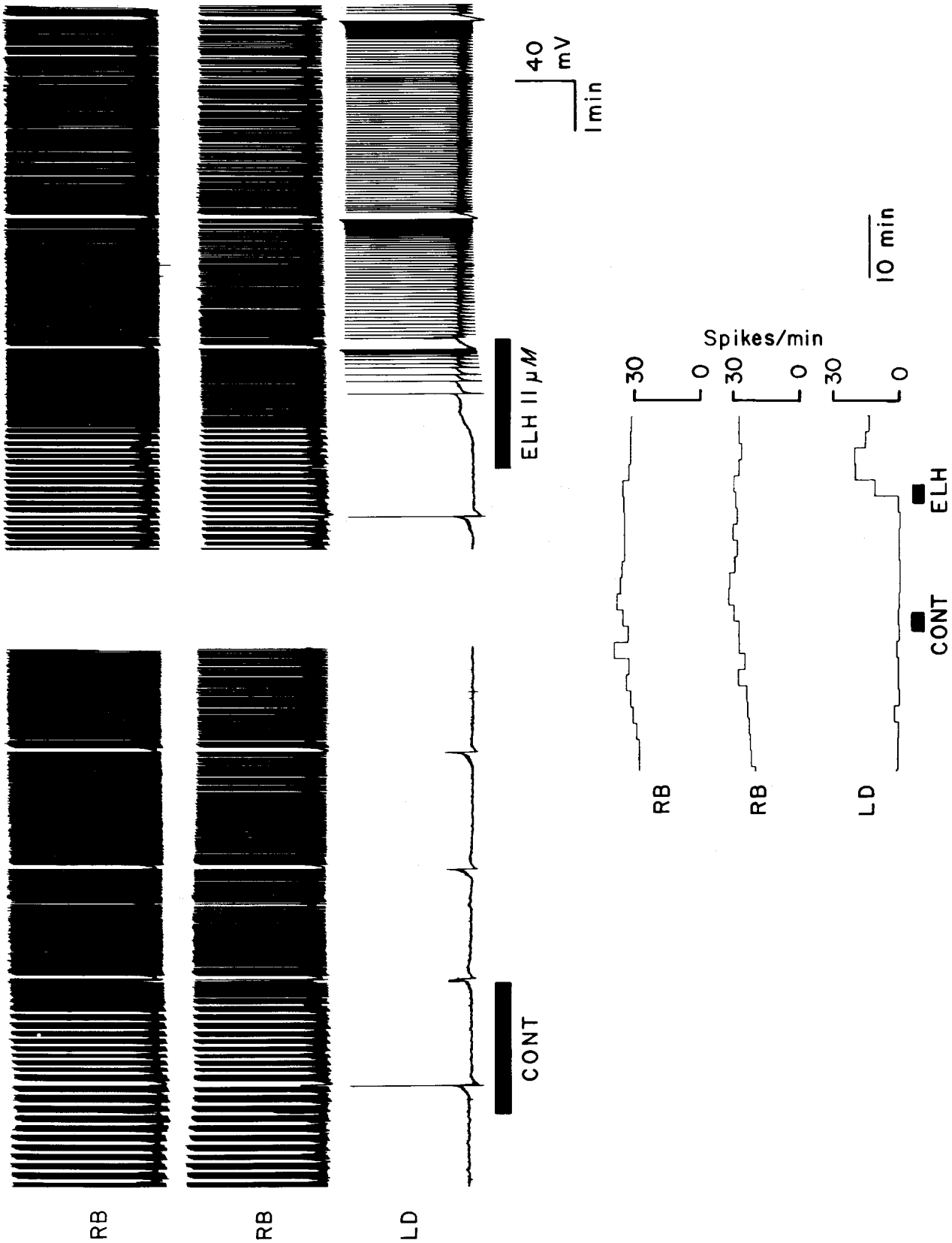

Figure 10. Selective action of ELH. The *upper three traces* are simultaneous recordings from two cells of the RB cluster and an LD cell. *Upper left traces*, Arterial perfusion of control solution had no effect on RB cells other than to slightly alter the spontaneous firing pattern. *Upper right traces*, Arterial perfusion of ELH had no effect on the RB cells, but it produced prolonged excitation of the LD cell. It is known that a bag cell burst discharge similarly has no effect on RB cells but produces prolonged excitation of LLQ cells such as the LD cell. *Bottom traces*, Spike rate of the three cells during the same experiment.

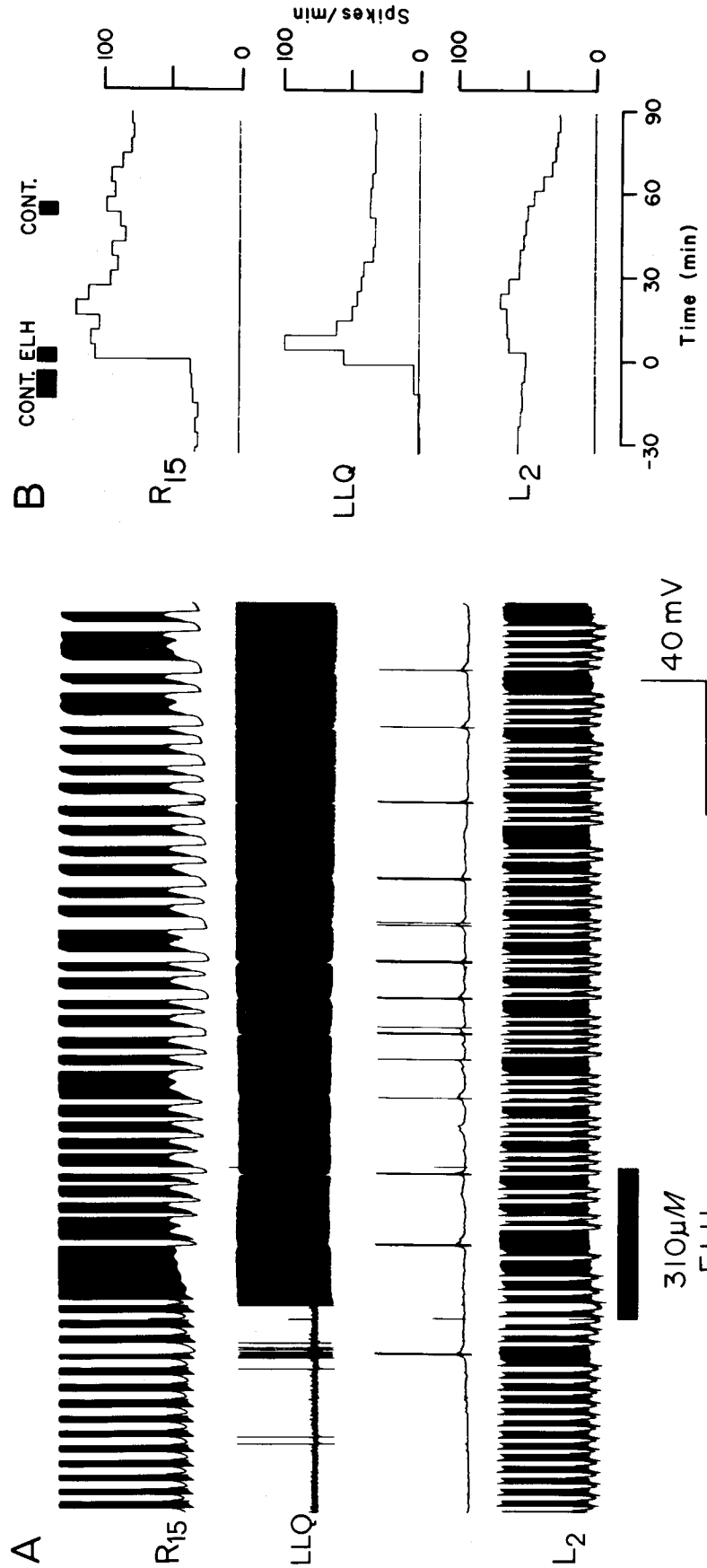

Figure 11. ELH does not mimic prolonged inhibition. *A*, Simultaneous recordings from four neurons during arterial perfusion of a high concentration of ELH (*bar*, 310 μM at 2.3 μl/min). Cell L2, which is known to be inhibited by bag cell activity, was not inhibited by ELH. R15 and an LLQ cell show larger responses than normally occur following a bag cell discharge. An unidentified cell located on the left side of the ganglion (*third trace*) exhibited no change in membrane potential with ELH perfusion, but it showed an increase in the rate of periodically occurring clusters of spikes that were driven by EPSPs from interneuron II. *B*, Spike rates of three of the cells shown in *A*.

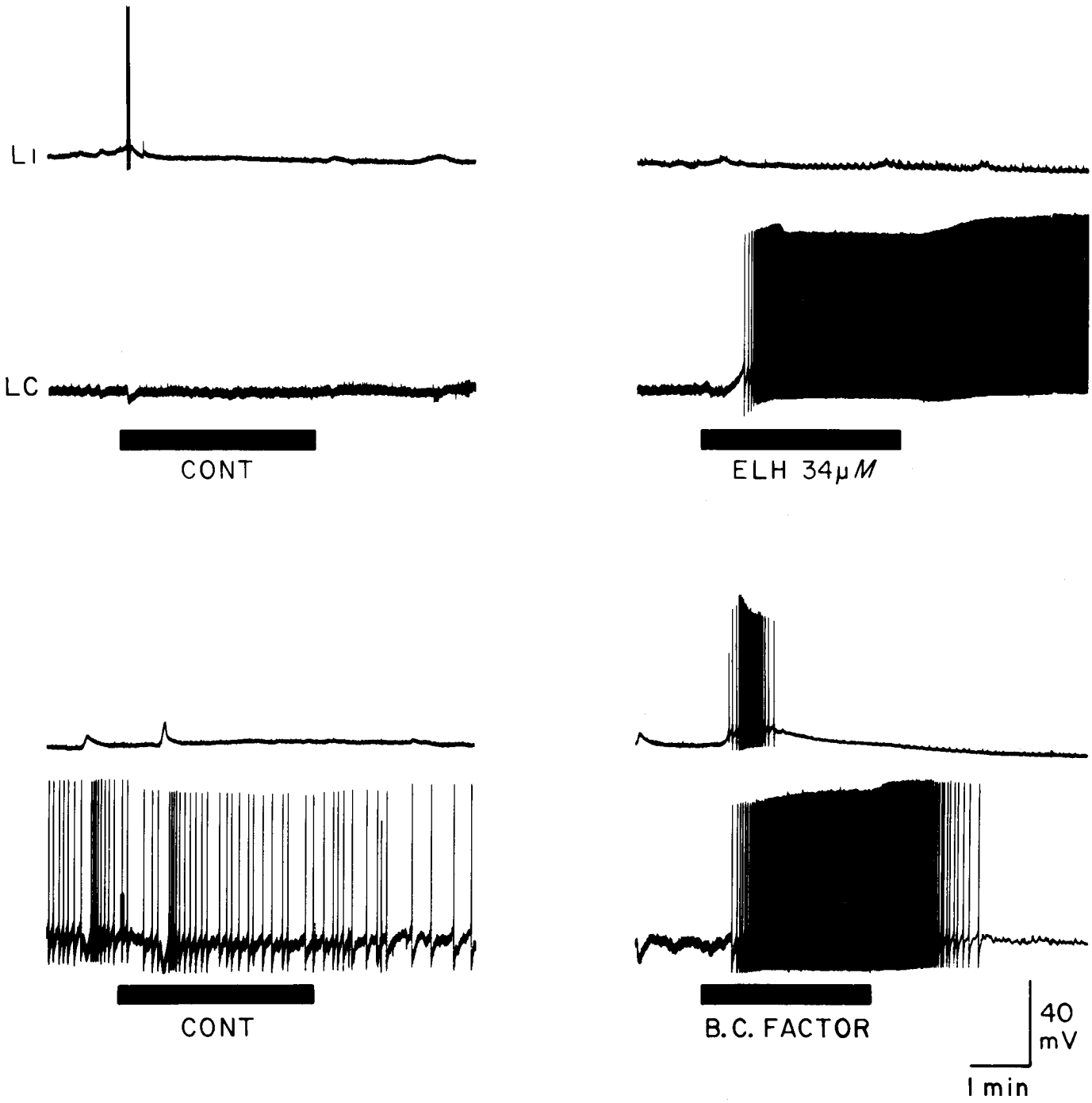

Figure 12. ELH does not mimic transient excitation. Simultaneous recordings from L1 and an LC cell. *Upper left*, Perfusion of a control solution had no effect. *Upper right*, A high concentration of ELH had no effect on L1, although a small EPSP was activated. L1 is known to be depolarized 5 to 10 mV for 10 to 15 min by a bag cell burst discharge. ELH produced prolonged excitation of the LC cell, as expected. *Lower left*, Perfusion of another control solution had no effect on L1 or the LC cell, which was still active due to the prior ELH application. Lower right: Perfusion of a purified bag cell factor (*B. C. FACTOR*) mimicked the transient excitation in L1 and excited the LC cell. The bag cell factor had an amino acid composition equivalent to β-BCP.

individual deflections in target neurons corresponding to individual bag cell spikes. Rather, a repetitive discharge of the bag cells is needed to result in sufficient release of transmitter for a discernable response to occur. Unlike the neuromuscular junction, where the excitatory junctional potential is duplicated by electrophoresis of ACh from a micropipette for 0.5 msec (Hartzell et al., 1975), for ELH a 1- to 2-min somatic application is needed to duplicate the magnitude and time course of the neurally evoked response in R15, which peaks after 10 to 15 min and lasts >1 hr (Branton et al., 1978a, b).

The four additional features described here, overflow, low effective concentration, lack of rapid desensitization, and selective action of ELH, also resemble the properties of ACh action on cardiac muscle.

Overflow of ELH. Loewi (1921) first demonstrated that transmitter (ACh) is released from autonomic nerve endings in the heart in such large quantities that there is overflow, i.e., some of the ACh escapes hydrolysis and can be detected in the releasate when assayed on a second heart. This is in contrast to the neuromuscular junction, where ACh is entirely hydrolyzed after release and cannot be detected in releasate unless anticholinesterase is present (Dale et al., 1936; Krnjevic and Mitchell, 1961). (ACh released at synapses

TABLE 1

Physiological characteristics of ELH neurotransmission compared to postganglionic autonomic and conventional synaptic neurotransmission in vertebrates

Characteristic	ELH on LLQs	ACh on Cardiac Muscle	ACh at Skeletal Neuromuscular Junction
1. Long response latency	Yes[a,b] (15–30 sec)[c]	Yes (100 msec)[d]	No (0.5 msec)[e]
2. Prolonged response duration	Yes[a,b] (>1 hr)	Yes (2–30 sec)[d]	No (30 msec)[e]
3. Repetitive discharge of releasing cells is required for discernable response in follower cells	Yes[a,b]	Usually[d,f]	No
4. Overflow	Yes	Yes[g]	No[h]
5. Low effective concentration of transmitter	Yes (0.2–1 μM)	Yes (1 μM)[d]	No (>300 μM)[i]
6. Lack of rapid desensitization	Yes	Yes[d]	No[i]
7. Selective action	Yes	No?	NA[k]
8. Transmitter functional at extrasynaptic or nonsynaptic receptors	?	Yes[f]	No[e]

[a] Branton et al. (1978a).
[b] Mayeri et al. (1979b).
[c] E. Mayeri and W. D. Branton, unpublished result.
[d] Glitsch and Pott (1978).
[e] Katz (1966).
[f] Del Castillo and Katz (1955).
[g] Loewi (1921).
[h] Dale et al. (1936).
[i] Hartzell et al. (1975).
[j] Katrz and Thesleff (1957).
[k] NA, not applicable.
[l] Hartzell (1980).

made by cell L10 in the abdominal ganglion is also entirely hydrolyzed; see Koike et al., 1974.) The finding that transmitter activity identified as undegraded ELH is present in releasate strongly indicates that transmitter is released from the bag cells in sufficient quantity to mediate the effects on target neurons by a process involving diffusion over long distances. The large amount of ELH activity in the releasate also suggests that the rate of degradation of ELH within the ganglion is low (or absent) relative to the amount released during the burst discharge. In contrast to ELH, the activities of some of the other bag cell transmitters in the releasate are destroyed unless protease inhibitors are present in the perfusion medium (Sigvardt et al., 1983). For the ELH target neurons (LLQ cells and R15) all of the transmitter activity in the releasate is attributable to ELH: no other bag cell peptides or fractions of material purified from bag cell extracts mimicked the activity of ELH.

Low effective concentration and lack of rapid desensitization. The effective concentration of ELH was estimated at 0.5 to 1 μM. The responses to ELH are unaffected by arterial perfusion of the peptide in combination with several other peptides encoded on the ELH/BCP precursor (Sigvardt et al., 1983). Thus, although we cannot entirely rule out the alternative possibility, it appears that ELH is the only bag cell transmitter mediating effects in LLQ cells and R15.

The effective concentration of ELH is approximately three orders of magnitude lower than the effective concentration of transmitter at vertebrate neuromuscular junction which was estimated by Hartzell et al. (1975) at 300 μM for a single quantum of ACh. In contrast, the effective concentration of ELH is comparable to that of ACh on vertebrate cardiac muscle (0.1 to 1 μM; Glitsch and Pott, 1978).

There was also no rapid (seconds to minutes) desensitization of the responses to ELH. This is similar to the effect of ACh on heart muscle, which is undiminished after 30 min of continuous application (Glitsch and Pott, 1978), and it contrasts with the ACh response at the neuromuscular junction which desensitizes within seconds (Katz and Thesleff, 1957). The lack of desensitization of the heart is of obvious functional importance in maintaining decreased heart rate for prolonged periods in response to sustained parasympathetic activity; similarly, lack of densensitization to ELH allows sustained increases in spike activity of target neurons for prolonged periods. Unlike ACh action on cardiac muscle, however, the response to ELH persists even with prolonged washing out of the peptide (Branton et al., 1978b). Therefore, the continued presence of the peptide near target neurons is not needed for the entire duration of the response.

Selective action. The finding that ELH acts selectively when arterially perfused into the ganglion was to be expected from earlier investigations showing that bag cell activity affects only a specific group of ganglion neurons. If ELH normally diffuses long distances to its targets, arterial perfusion of the transmitter should affect only the same group, or as it turns out, a subset of the group. This specificity is presumably because only the responsive neurons have receptors for the transmitter.

Arterial perfusion data also indicate that some of the bag cell actions, i.e., prolonged inhibition, transient excitation, and depolarization of the bag cells themselves, are not mediated by ELH. These actions must therefore be mediated by other bag cell transmitters. However, the present data do not rule out the possibility that ELH might participate with other bag cell transmitters in mediating certain types of responses. For example, although ELH applied by itself may not have an effect on a given target neuron, it might modulate the action of another transmitter.

Distribution of receptors and inactivating enzymes

Although the present data strongly suggest that ELH diffuses over long distances to its targets, more information is needed before this mode of interaction is unequivocally established. In particular, information is needed on the distribution of ELH receptors on individual target neurons and the distribution of enzymes (if any) for inactivation of ELH. On cardiac muscle ACh receptors are uniformly distributed on the plasma membranes of target cells (Hartzell, 1980), and inactivating enzymes are dispersed in the target tissue (Loffelholz, 1981). ELH receptors on target cells will presumably have a similar,

uniform distribution and the inactivating enzymes, presumably proteases, will be dispersed in vascular and interstitial spaces and/or in blood. By contrast, at the neuromuscular junction ACh receptors are restricted to the subsynaptic membrane and extrasynaptic receptors do not play a role in normal physiology of the adult (Katz, 1966). Moreover, acetylcholinesterase is also restricted to the region of the synapse.

Other considerations

It should be noted that only three of the physiological properties listed in Table I appear to be absolutely necessary for transmitters that travel long distances to their targets. These are long response latency, long response duration, and slow degradation of the transmitter. Even these properties do not depend entirely on the distance traveled by the transmitter. In the heart, for example, cellular response latency makes a greater contribution than diffusion time in determining the total latency of the ACh response (Hill-Smith and Purves, 1978). This may also be true for responses produced by ELH (W. D. Branton, L. Padgett, and E. Mayeri, unpublished observation).

Another important consideration for neural systems like the bag cells is that the distance traversed by a transmitter from point of release to target cell receptor is likely to vary from one release site to another. Thus, in cardiac muscle one can record discrete hyperpolarizing junctional potentials at locations in the muscle that are very close to the nerve terminals, and slower, more smoothly graded hyperpolarizing potentials at distant locations. These differences in the time course of responses are in part attributable to differences in diffusion distance of ACh. Most axonal processes of the bag cells are several cell diameters from LLQ and R15 target neurons, suggesting that a substantial amount of released ELH travels a comparable distance to its targets. Although we cannot rule out the possibility that a small proportion of ELH release sites are within a shorter distance, the serial perfusion experiments suggest that there is sufficient overflow of released ELH for the neural interaction to occur in the absence of close release sites.

Finally, there is a question of terminology regarding neurotransmission mediated by ELH in the abdominal ganglion and that of ACh on cardiac muscle. Although the characteristics of ELH neurotransmission are similar to those of ACh on cardiac muscle, and the latter is usually considered to be a form of synaptic transmission, it seems inappropriate to term ELH a synaptic transmitter since in its release from the bag cells ELH also serves so prominently as a neurohormone acting to induce ovulation. Whatever the terminology, there is likely to be a whole spectrum of ways in which nerve cells communicate with one other with conventional synaptic transmitters at one end of the spectrum and hormonal transmitters at the other.

Functional implications

Nonsynaptic communication provides a means for one group of neurons to communicate with others without the need for morphological contacts between them. It might also be regarded as an evolutionarily adaptive means of conserving space in the brain (see Mayeri and Rothman, 1982). For synaptic communication, space is required for the axons and dendrites that link cells to one another. For nonsynaptic communication this space is conserved because the diffusing transmitter utilizes available extracellular space to reach its targets. The savings in space is traded for a decrease in the speed of communication of the diffusing transmitter compared to that provided by axons, synapses, and dendrites. Although slower, the example of ELH indicates that nonsynaptic communication can be just as selective as synaptic communication; rather than affecting every cell it contacts, ELH affects only a subgroup of them.

The properties of nonsynaptic communication are particularly well suited for mediating long-lasting and rather complex behavioral processes such as the behavior pattern associated with egg laying in *Aplysia* (Branton et al., 1978a; Mayeri et al., 1979b; Stuart and Strumwasser, 1980; Brownell and Schaefer, 1982; Mackey and Carew, 1983). By depolarizing or hyperpolarizing specific target cells for minutes or hours, ELH and other bag cell transmitters can facilitate or inhibit reflex pathways for corresponding periods of time and thus regulate the various components of the behavior pattern.

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