

Synaptic Actions of Identified Peptidergic Neuron R15 in *Aplysia*. I. Activation of Respiratory Pumping

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The purpose of the study described in this and the following two companion papers was to determine the synaptic actions of neuron R15, an endogenously bursting neurosecretory cell in *Aplysia*, as a step toward determining its physiological function. The results described in this paper demonstrate that activity in R15 increases the frequency of bursting in the R25/L25 network that triggers respiratory pumping. This excitatory modulatory effect appears to be mediated by R15 α 1 peptide. R15 activates both strong and weak modes of respiratory pumping. In contrast, the two R20 cells, which are thought to use the neuropeptides SCP_A and SCP_B as transmitters, elicit only strong episodes of respiratory pumping. The synaptic actions of R15 also differ from those of the R20 cells in being longer lasting and in exhibiting profound desensitization. Chronic recording of R15 activity *in vivo* indicates that it does not burst spontaneously in the intact animal, so the synaptic actions of R15 are not chronically desensitized. The neuroendocrine bag cells, which initiate egg laying, had been shown by others to excite R15 and the R25/L25 network that triggers respiratory pumping. Our data indicate that the excitatory effects of the bag cells on the R25/L25 cells are mediated in part by R15.

Molluscan neurons that burst spontaneously have proven to be useful experimental preparations for the study of endogenous bursting. Despite all that has been learned about the mechanisms of burst generation and its modulation in these cells, relatively little is known about their physiological functions (Benson and Adams, 1989). Neuron R15, an endogenously bursting cell located in the abdominal ganglion of *Aplysia* (Strumwasser, 1965), is the most thoroughly studied endogenous burster in any animal (Adams and Benson, 1985). Since the first intracellular recordings from R15 over 30 years ago (Arvanitaki and Chalazonitis, 1958), it has been used as a model system to study a variety of processes, including neuropeptide synthesis and processing (Gainer et al., 1977; Buck et al., 1987; Weiss et al., 1989), synaptic plasticity (Schlapfer et al., 1974), synaptic transmission and electrical excitability (reviewed by Adams and Benson, 1985), and aging (Skinner and Peretz, 1989). Despite

this wealth of cellular and molecular studies, the physiological function of R15 remains unclear. The purpose of the study described in this and the following two companion papers was to determine the synaptic actions of R15, as a step towards understanding its physiological function.

It is thought that R15's synaptic actions are mediated by the release of neuropeptides. R15 was originally hypothesized to be peptidergic on the basis of morphological and biochemical evidence (Coggeshall, 1967; Frazier et al., 1967; Berry, 1976). Recently, Buck et al. (1987) cloned and characterized a cDNA that represents a highly abundant mRNA present in R15. The precursor of this mRNA is spliced differently in different neurons, resulting in two distinct forms of mRNA (R15-1 and R15-2). The alternative splicing affects only a limited region of the polyproteins encoded by the two forms of mRNA and can produce distinct but overlapping sets of peptides in different neurons. Buck et al. (1987) found that R15 expresses only the R15-2 version of mRNA. The peptides synthesized in R15 and their amino acid sequences were determined by Weiss et al. (1989). Three of the peptides predicted from the sequence of R15-2 mRNA were shown to be synthesized in R15 and were named R15 α 1 (38 amino acids long), R15 β (27 amino acids), and R15 γ (37 amino acids). By determining the physiological function of R15, we hope to gain insights into the functional significance of the alternative splicing in this system.

Earlier investigations have produced three lines of evidence suggesting that R15 may be involved in regulation of water balance: (1) Injection of either crude homogenates of R15 or synthetic R15 α 1 peptide causes water retention (Kupfermann and Weiss, 1976; Weiss et al., 1989). (2) R15 is inhibited when the semi-intact animal is exposed to hypotonic seawater, and excited when it is exposed to hypertonic seawater (Stinnakre and Tauc, 1969; Woodson and Schlapfer, 1979). (3) Blocking spike activity in R15 by prolonged hyperpolarization results in alterations of the solute composition of the hemolymph (Bablanian and Treisman, 1985). Although suggestive, these data supporting R15's postulated role in water balance are circumstantial.

Other evidence suggests that R15 may be involved in oviposition. The intensity of R15 bursting is enhanced by a population burst in the neuroendocrine bag cells that elicits egg-laying activity (Branton et al., 1978). We investigated the possibility that R15 might mediate various features of egg-laying behavior, such as modulation of respiratory pumping. The frequency of respiratory pumping is thought to increase during egg laying because activation of the bag cells by electrical stimulation in a reduced preparation increases the frequency of spontaneous respiratory pumping (Schaefer and Brownell, 1986).

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Respiratory pumping is a brief, stereotyped behavior, consisting of synchronous gill, siphon, parapodial, and mantle-shelf contractions, accompanied by heart inhibition and decreased vasomotor tone (Byrne and Koester, 1978; Hening, 1982). It occurs spontaneously at variable rates (1–60 events/h) and is thought to serve a respiratory function by promoting ventilation of the gill (Byrne and Koester, 1978).

The neural circuit that controls respiratory pumping includes several dozen identified cells. Many of the motoneurons that mediate respiratory pumping behavior are found in the abdominal and pedal ganglia (Koester and Kandel, 1977; Sawada et al., 1981; Hening, 1982). Two groups of interneurons in the abdominal ganglion that trigger the behavior have been identified and characterized (Byrne, 1983; Koester, 1989). These interneurons, the R25 and L25 cells, constitute two clusters of about 15 cells each. All 30 cells fire in a population burst that drives the motoneurons that generate the stereotyped motor program. The R25 and L25 cells are connected to one another by electrical and chemical synapses. The spontaneous generation of the population burst in the R25/L25 network is mediated by endogenous pacemaker activity of the R25/L25 cells and by reverberation within the network, which results from the electrical and excitatory chemical connections between the members of the network (Byrne, 1983; Koester, 1989). The spontaneous respiratory pumping bursts are usually strong and stereotyped, but respiratory pumping occasionally occurs in a weak mode that is more variable in form (Eberly et al., 1981).

In this paper we demonstrate that R15 excites the R25/L25 network. The actions of R15 on the R25/L25 cells are compared and contrasted with those of a pair of peptidergic neurons, the R20 cells, which synthesize the neuropeptides SCP_A and SCP_B (small cardiovascular peptides A and B) (Alevizos et al., 1989b). These two neural pathways switch the behavior between its strong and weak modes of operation. In the second paper we demonstrate that R15 also contracts the pleuroabdominal connectives by exciting the multimodal motoneuron L7 (Alevizos et al., 1991a). In the third paper we show that R15 triggers peristalsis of the large hermaphroditic duct, which transports the egg cordon during egg-laying behavior, and present a hypothesis concerning the role of R15 in integrating various features of egg-laying behavior (Alevizos et al., 1991b).

Some of these results have been described in a preliminary communication (Alevizos et al., 1989a).

Materials and Methods

Animals. Specimens of *Aplysia californica* weighing 15–560 gm were supplied by Marinus, Long Beach, CA; Sea Life Supply, Sand City, CA; Alacrity Marine Services, Redondo Beach, CA; and the Howard Hughes Medical Institute Marine Resources Facility at the Woods Hole Oceanographic Institute, Woods Hole, MA. Animals were kept in an aquarium containing artificial seawater (ASW; Instant Ocean) at 14–16°C until use.

Intracellular recording. Animals were anesthetized by injection of isotonic MgCl₂ (30–40% of body weight). The isolated abdominal ganglion was dissected from the animal and pinned to the silastic (Sylgard, Dow Corning) bottom of the chamber. In a few experiments, the abdominal ganglion attached to the gill by the genital and branchial nerves was excised, and the gill was attached to an isotonic movement transducer. The recording chamber was filled initially with a modified saline solution containing 192 mM Mg²⁺ (3.5 × normal) and 5 mM Ca²⁺ (0.5 × normal), prepared by mixing equal volumes of ASW and isotonic MgCl₂. ASW was used later in the experiment as the saline solution for bathing the preparation while recording.

The following “non desensitizing washing protocol” was employed in order to prevent the desensitizing effects of the synaptic actions of

R15 (see Fig. 9). Before the preparation was washed with ASW (at least 50 bath volumes), R15 was impaled and prevented from firing by hyperpolarizing current injection. After washing, R15 was kept hyperpolarized for at least 2 hr to allow any residual desensitization to decay.

In some experiments, the connective tissue capsule covering the ganglion was dissected before recording to expose the cells of interest for intracellular recording with standard microelectrodes. In other experiments, neurons were impaled by tapping the microelectrode through the sheath of the ganglion. Single-barrel glass microelectrodes (1.2–15 MΩ) filled with 3 M KCl or 2.5 M potassium acetate were used to record membrane potential and to inject current into neurons using a bridge circuit. Experiments were performed at room temperature (17–20°C). Data were tape-recorded and played back on a Gould 2400 chart recorder (full-scale cutoff frequency, 50 Hz) for analysis and photographing.

Two methods were used to test for monosynapticity of connections and to minimize polysynaptic effects of applied peptides and neurotransmitters: (1) The threshold for action potential generation was raised by bathing the ganglion in a high-divalent-cation solution containing 165 mM Mg²⁺ (3 × normal) and 30 mM Ca²⁺ (3 × normal). This solution was prepared by mixing isotonic CaCl₂, isotonic MgCl₂, and ASW. (2) A concentration of 60 μM tetrodotoxin (TTX; Calbiochem) was used to block Na⁺-dependent action potentials. This method does not completely eliminate action potentials, because some *Aplysia* neurons are able to generate Ca²⁺-dependent action potentials.

In a few experiments, the connections of the abdominal ganglion to the head ganglia by the pleuroabdominal connectives were maintained. The head ganglia were placed in a 1-ml subchamber, to which atrial gland extract (AGE) was introduced in order to excite the bag cells (Painter et al., 1988).

Pharmacology. Peptides and other neurotransmitter substances were applied to the abdominal ganglion as a concentrated bolus added to the bath close to the ganglion from a 50-μl pipette. Bath volume was 2 ml in these experiments. Peptide and neurotransmitter solutions were made fresh, from the same batch of ASW used to bathe the preparation. The following peptides were used in this work: SCP_B (Morris et al., 1982; Lloyd et al., 1987), R15α1 peptide, and R15β peptide (Weiss et al., 1989). SCP_B and R15α1 peptide were purchased from Peninsula Laboratories (Belmont, CA; R15α1 peptide is referred to as “neuron-specific peptide” by Peninsula). R15β peptide was synthesized by the Howard Hughes Medical Institute’s Protein Center Core Facility at Columbia University. Atrial gland extract (AGE) was prepared by homogenizing an atrial gland in a glass homogenizer containing 1 ml ASW. The homogenate was centrifuged for 5 min at 1200 rpm, and the supernatant was recentrifuged at the same speed for 10 min. The new supernatant was collected and stored at –13°C until used.

Extracellular recording. Specimens weighing 270–560 gm were used in chronic extracellular recording experiments. The electrode used for chronic recordings, which was made from a flexible silicon polymer (Mirror 3, Kerr Manufacturing Co., Romulus, MI), was based on a design modified from Koch and Brunner (1985). For electrode implantation, the subject was anesthetized with isotonic MgCl₂ (30% of body weight) and placed in a chamber containing a chilled solution of 1:1 ASW/isotonic MgCl₂. An incision was made in the body wall to expose the abdominal ganglion and its peripheral nerves. One of the nerves containing an axon branch of R15 was positioned on the groove of the extracellular electrode (Fig. 1) and was insulated from the hemolymph by means of a latex membrane (Carter-Wallace, New York, NY). The electrode was anchored on the pericardium by 6-0 silk suture so that its position did not change with movements of the animal. The free end of the wire was passed through the body wall of the animal, to be connected to the extracellular amplifier during the recording sessions. The incision was closed by suturing the body wall and the skin independently. In some experiments, the edges of the skin were glued with Histoacryl (B. Braun, Melsungen, GER). The animal was injected with penicillin (10,000 U/ml) and streptomycin (10 mg/ml; 50 μl/ml of body weight) and returned to the aquarium, where it was allowed 24–48 hr to recover before recording. Subjects were housed in a 7.5-liter aquarium of chilled, aerated ASW during the recording sessions, which took place 1.5–4 d following surgery. At the end of the recording period, which lasted from 1 to 4 d, the animal was killed, and the abdominal ganglion was removed, along with the extracellular electrode connected to the nerve branch. Using this reduced preparation, simultaneous recordings were made from the R15 cell body and from the chronic nerve electrode, to verify that the R15 burst activity in the nerve branch was clearly recognizable.

Only the results from animals that were judged to be healthy were tabulated. Criteria for healthiness included relaxation upon handling, normal posture and ability to attach to the substrate, normal feeding behavior and locomotion, and body weight within 20% of initial value. Of 16 animals in which electrodes were implanted, 9 animals fulfilled all of these criteria. Four additional animals were healthy, but no R15 spike could be identified in the reduced preparation in the branch from which activity was recorded *in vivo*, due to variability in R15's branching pattern. Chronic recording experiments were done from February through August.

Data presentation and statistical analysis. All data are presented as mean values \pm the standard error of mean (SEM). All statistical analyses of the results were made using a two-tailed *t* test with a cutoff of $p = 0.05$ for comparing the difference between means.

Results

The nondesensitizing washing protocol

In order to reproducibly demonstrate synaptic actions of R15, we found it necessary to block the spontaneous activity of R15 for a prolonged period. Preliminary experiments indicated that superfusing the abdominal ganglion with the synthetic R15 α 1 peptide, a putative transmitter of R15, results in a moderate increase in the rate of respiratory pumping. However, a second application of the same concentration of the peptide a few minutes later had no effect on the frequency of discharge of the R25/L25 network. This decremental effect suggested that the effect of the R15 α 1 peptide may desensitize, and led us to examine the effects of R15 and its peptide by means of a special dissection and washing protocol designed to minimize the chronic desensitization of R15's synaptic actions caused by its spontaneous bursting.

The usual dissection procedure with the *Aplysia* nervous system begins with injection of the animal with a modified saline containing $3.5\times$ -normal Mg^{2+} and $0.5\times$ -normal Ca^{2+} , which anesthetizes the animal by blocking synaptic transmission. The animal then is dissected, and the abdominal ganglion is removed and pinned to the bottom of the recording chamber in the same saline. Finally, the preparation is washed extensively with ASW and left for 30–60 min to recover from the dissection before recording is begun. Because R15 bursts endogenously, it fires during this equilibration period. This spontaneous activity may exert an excitatory modulatory effect on the frequency of bursting of the R25/L25 cells that desensitizes and cannot be observed again in the short term.

Instead of washing the preparation with ASW before the cell penetration, we started our experiments by impaling R15 while the ganglion was still in the high- Mg^{2+} /low- Ca^{2+} solution. The frequency of respiratory pumping was monitored by recording the activity of the R25/L25 cells directly, or by recording their input to LD and RD cells, which are excited by the R25/L25 cells (Frazier et al., 1967; Koester and Kandel, 1977; the R25/L25 network was referred to as "interneuron II" in these early reports). R15 was kept hyperpolarized while the preparation was washed extensively with at least 50 bath volumes of ASW. It was not released from hyperpolarization until 2 hr after the impalement, to ensure the decay of any residual desensitization. Using this "nondesensitizing washing protocol," we found that when R15 was allowed to burst spontaneously or when R15 α 1 peptide was applied to the ganglion, they had robust effects on the frequency of respiratory pumping. This washing protocol was used for all of the experiments described below.

R15 bursting increases the frequency of respiratory pumping

After R15 was released from a 2-hr hyperpolarization and allowed to burst spontaneously for 10 min, there was an increase

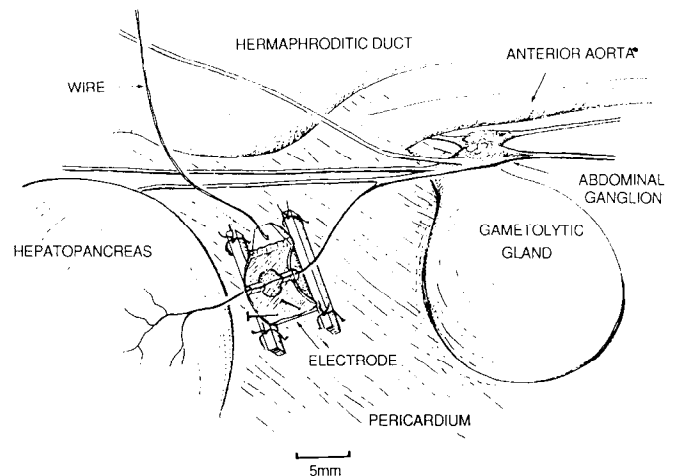


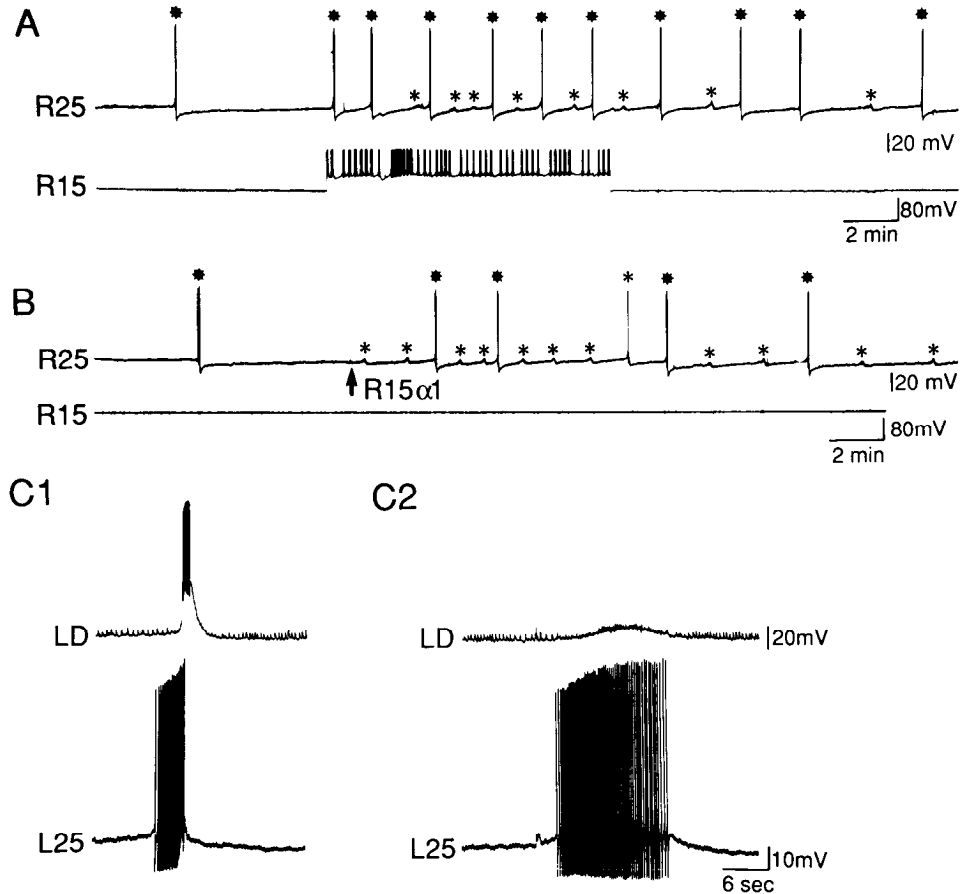
Figure 1. The chronically implanted extracellular electrode. The nerve is positioned in the groove and is isolated from the surrounding medium by means of a thin latex membrane. One side of the membrane is permanently glued to the electrode, and the other is attached, after the nerve is in place, by small pins inserted into the electrode block. One end of an insulated wire is embedded in the polymer, and its bare tip protrudes into a saline-filled chamber through which the nerve runs. The other end of the wire passes through the body wall of the animal and is connected to a preamplifier.

in the frequency of bursting of the R25/L25 cells that outlasted the firing of R15. In some experiments, the effect of R15 on the R25/L25 network was observed by monitoring the contractions of the gill, confirming that the bursting activity observed centrally does drive respiratory pumping (data not shown). R15 bursting increased the frequency of respiratory pumping in 94% of the preparations tested (120 preparations).

When R15 was allowed to burst, it increased the frequency of both weak and strong episodes of respiratory pumping. This is illustrated in Figure 2A, in which intense bursts recorded in an R25 cell are interspersed with weak conjoint excitatory/inhibitory synaptic input. An earlier study showed that subthreshold, conjoint synaptic input such as this is generated when weak episodes of respiratory pumping are driven by the L25 cells (Koester, 1989). During weak respiratory pumping, only the L25 cells burst, and they fire at a relatively low rate but with a longer burst duration than normal. In contrast, during the strong episodes of pumping, both the R25 and the L25 clusters fire intense, brief bursts of action potentials. The weak bursts in the L25 cluster are transmitted to RD and LD motoneurons that help drive the behavior as low-frequency EPSP bursts, which decay somewhat more slowly than the high-frequency bursts of EPSPs that are generated when both the R25 and the L25 clusters fire synchronously (A. Alevizos, unpublished observations). The difference between the strong and weak bursts recorded in an L25 cell is illustrated in Figure 2, C1 and C2, which also shows how the 2 burst types can be monitored in a postsynaptic follower of the R25/L25 cells (an LD cell in this case; see Koester, 1989).

The effect of R15 firing on the frequency of respiratory pumping when R15 is released after a 2-hr hyperpolarization period for 5, 10, 30, and 60 min is shown in Figure 3. The 10-, 30-, and 60-min firing periods of R15 produce the same maximum effects, which decay with similar time courses. None of the data points of these three curves are statistically different from each

Figure 2. R15 and the R15 α 1 peptide increase the frequency of both weak and strong episodes of respiratory pumping. **A**, Allowing R15 to fire for 10 min after a 2-hr hyperpolarization period elicits an increase in the rate of respiratory pumping, as monitored in an R25 cell. The weak bursts of the R25/L25 network are recorded as a conjoint excitatory/inhibitory synaptic input in this R25 cell. The strong bursts are marked by stars, and the weak bursts by asterisks. **B**, The R15 α 1 peptide mimicked the effect of R15 in the same preparation by increasing the rate of both strong and weak bursts. R15 was kept hyperpolarized throughout this part of the experiment. The peptide was added as a 50- μ l bolus of 2×10^{-5} M, 60 min after the end of R15 firing. **C**, The intensity of activity in an L25 cell can be monitored in an LD cell. **C1**, The brief, high-frequency bursting of the R25/L25 network as monitored in an L25 cell elicits strong bursting in the LD cell. **C2**, The lower-frequency but longer-duration bursting in an L25 cell contributes to a weaker, longer-lasting synaptic input in the LD cell. The LD cell was kept hyperpolarized to reduce background activity, so that the synaptic inputs from the R25/L25 network could be distinguished easily.



other at any time point after R15 has begun to fire, while they all differ from the responses produced by the 5-min release of R15. These results show that R15's effect reaches its maximum within 5–10 min after the start of spontaneous bursting and then decays over several minutes.

This effect of R15 on the rate of respiratory pumping has been overlooked previously, presumably because the effect decays with repeated R15 firing and it requires a long period of hyperpolarizing R15 to renew the responsiveness of the system. The response to firing R15 partially recovered when the interval between bursts was increased from 40 to 80 min (Fig. 4). These experiments began with a 2-hr hyperpolarization of R15, so they ran for several hours. As a result, the basal bursting frequency of respiratory pumping and the responsiveness of the R25/L25 cells decreased with time, which may account for the fact that recovery was not complete. In two additional experiments, R15 was not prevented from firing during the washing period, and it was hyperpolarized for periods of only 10 min, every 30 min (three R15 bursting periods of 30 min each separated by two hyperpolarization periods of 10 min each). No effect of R15 firing on the frequency of respiratory pumping was observed in these experiments (data not shown), consistent with the conclusion that the effect of R15 on the R25/L25 cells wanes upon continuous and prolonged spontaneous firing of R15.

R15 α 1 peptide may mediate the excitation of the R25/L25 network by R15

To test the possibility that R15 α 1 peptide may mediate the effect of R15 on the R25/L25 network, the peptide was applied to the

solution bathing the ganglion. R15 α 1 peptide increased the rate of respiratory pumping in a dose-dependent manner (Fig. 5), and it had the same qualitative effect as R15: it elicited both strong and weak bursts (see Figs. 2A,B; 10; 11). Thus, R15 α 1 peptide is a good candidate for mediating the excitatory effects of R15 on the R25/L25 network.

In order to determine whether the effects of R15 and of the R15 α 1 peptide on the R25/L25 network are direct, two experiments were performed in which polysynaptic transmission in the ganglion was reduced. In the first set of experiments, TTX was used to block Na⁺-dependent action potentials in the ganglion, thereby blocking most synaptic transmission. TTX completely blocks the action potentials in the R25/L25 cells, revealing the endogenously generated plateau potentials that underlie the bursting of these cells (Koester, 1989). Although the Na⁺ component of the action potentials in R15 is also blocked by TTX, R15 still can generate Ca²⁺-dependent action potentials. Firing R15 at 5 Hz while the preparation is bathed in TTX increases the frequency, duration, and amplitude of the plateau potentials monitored in an R25 cell (Fig. 6A). Similarly, bath application of the R15 α 1 peptide in the presence of TTX increases the frequency, duration, and amplitude of the plateau potentials recorded in R25 cells (Fig. 6B). In the second set of experiments, a solution of high divalent cations was used to block polysynaptic pathways. Allowing R15 to burst while the ganglion is bathed in this solution produces a long-lasting depolarization of an R25 cell (Fig. 6C). In a similar fashion, application of the R15 α 1 peptide while the ganglion is bathed in the same high-divalent-cation solution produces a long-lasting

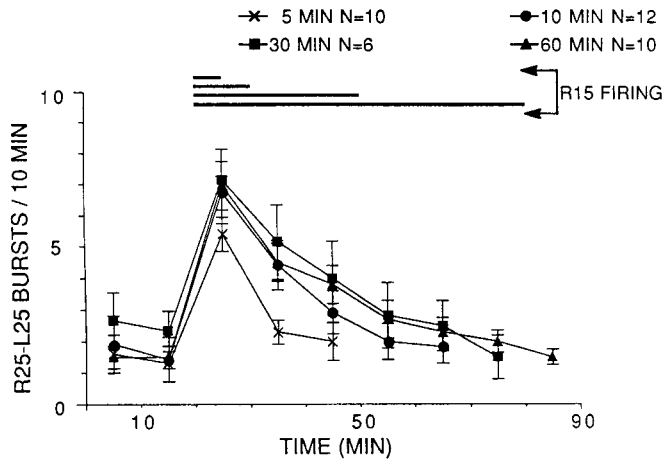


Figure 3. Modulation of the frequency of respiratory pumping by R15 decays during prolonged R15 activity. When R15 was allowed to burst spontaneously for various amounts of time after a 2-hr hyperpolarization period, it produced a long-lasting increase in the frequency of respiratory pumping. The amplitudes of the maximum effect and the time courses of decay of these increases were not significantly different for the 10-, 30-, and 60-min firing periods, while the effect produced by the 5-min firing period was significantly smaller in both amplitude and duration. These data indicate that the maximum effect of R15 bursting is exerted within the first 5–10 min of bursting, beyond which the response is independent of R15 activity. Respiratory pumping frequency was monitored via R25/L25 network followers. There was no significant difference in mean firing rate of R15 in the four groups, nor was there a trend for the firing rate of R15 to slow down over the course of the long burst periods (data not shown). The mean of the group firing rates of R15 under the four burst conditions was 0.7 Hz. Error bars represent SEM.

depolarization of the R25 cell (Fig. 6D). The effects of both R15 and the R15 α 1 peptide applied in high-divalent-cation solution are shorter lasting than when they are applied in ASW. This reduced time course of the effect may be due either to some direct effect of the divalent cations on the receptor, channel, or second messenger that mediates the effect, or to a polysynaptic component of the pathway that is blocked under high-divalent-cation conditions.

In order to test whether the excitatory effect of R15 on the R25/L25 network desensitizes, R15 was released from hyperpolarization for 10-min periods at brief time intervals. The responses of the R25/L25 network to this protocol then were compared to those for a similar protocol of application of exogenous R15 α 1 peptide. The response to the second period of R15 bursting is smaller than that to the first period (Fig. 7A). This decrement in response, which is statistically significant, could be due to presynaptic depression. However, a similar effect is produced by successive applications of a constant dose of R15 α 1 peptide. The second application of the peptide produces a significantly smaller effect than the first (Fig. 7B). Assuming that R15 α 1 peptide is the transmitter used by R15, these results suggest that the decay in responsiveness to R15 activity is more likely to result from desensitization, refractoriness, or slowly developing synaptic inhibition of the R25/L25 network than from presynaptic depression.

To examine whether these decrements in responsiveness to R15 activity can be attributed to refractoriness or inhibition of the R25/L25 cells, the R25/L25 network was excited by application of SCP_B following the second firing period of R15 (Fig. 7A). SCP_B is not present in R15 (Lloyd et al., 1985), but it is

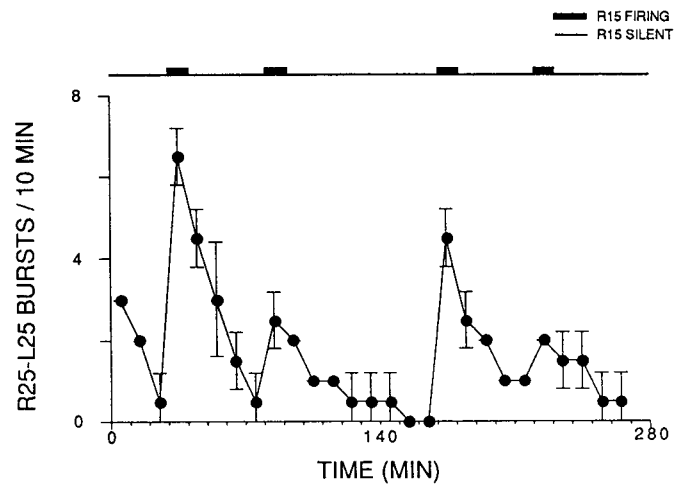


Figure 4. Recovery of the effect of R15 on respiratory pumping rate requires a prolonged hyperpolarization of R15. Allowing R15 to burst spontaneously for 10 min after a 2-hr hyperpolarization period produces a long-lasting increase in the rate of respiratory pumping ($N = 2$). After 40 min, R15 was released again from hyperpolarization for 10 min. The effect of R15 bursting was significantly smaller during the second firing period. Allowing an 80-min recovery period in R15 partially restored the initial effect on the respiratory pumping frequency. R15 was released from hyperpolarization for a fourth 10-min period, 40 min from the end of the third firing period. The effect of R15 on the respiratory pumping rate was again attenuated relative to the third firing period. The number of spikes in R15 did not change appreciably from the first bursting period to the last (328, 335.5, 355, and 337.5 spikes for each successive 10-min firing period), so the changes in the rate of respiratory pumping cannot be attributed to the small differences observed in the number of spikes in R15. Error bars represent SEM.

synthesized by the two R20 cells and is likely to be used as a transmitter by the R20 cells for exciting the R25/L25 network (Alevizos et al., 1989b). The frequency of respiratory pumping increased when SCP_B was applied in this protocol. This increase

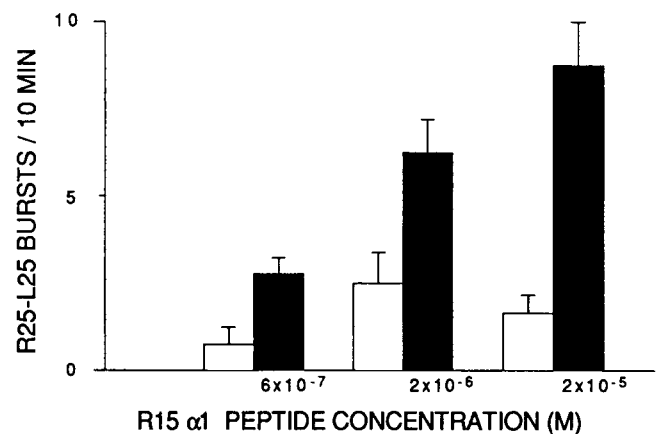
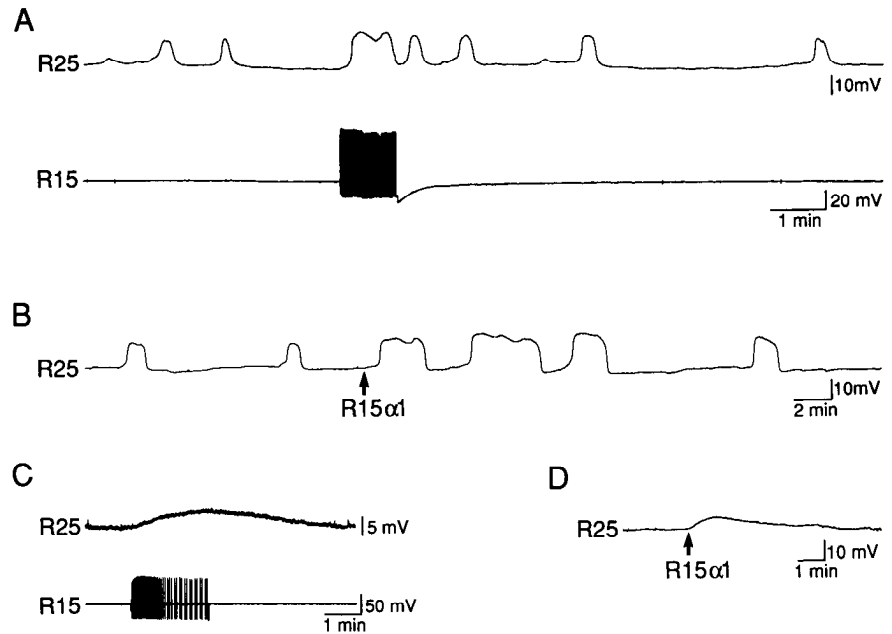


Figure 5. The R15 α 1 peptide mimicks the effect of R15 on the frequency of respiratory pumping. Open bars show the rates of respiratory pumping before adding the peptide. The R15 α 1 peptide was added near the ganglion from a pipette, as a 50- μ l bolus, at the concentrations indicated under the solid bars. The preparation was continuously superfused with ASW at a rate of 1–1.5 ml/min. The effect of the peptide is expressed in a dose-dependent manner, with higher concentrations producing stronger effects. The strong episodes of respiratory pumping were monitored in R25/L25 network followers ($N = 4$ for 6×10^{-7} M and 2×10^{-6} M; $N = 8$ for 2×10^{-5} M). R15 was kept hyperpolarized throughout these experiments. Error bars represent SEM.

Figure 6. R15 and the R15 α 1 peptide act directly on the R25/L25 cells. **A**, Bathing the ganglion in TTX (60 μ M) reveals endogenously generated plateau potentials that underlie the bursts in the R25/L25 network (Koester, 1989) and reduces polysynaptic activity by eliminating Na⁺-dependent spikes. Firing R15 at 5 Hz prolongs the duration and increases the amplitudes and the frequency of the plateau potentials in the R25/L25 network ($N = 5$). **B**, Applying R15 α 1 peptide mimicks the effects of R15 on the plateau potentials of the R25/L25 cells ($N = 5$). **C**, Releasing R15 from hyperpolarization elicits a long-lasting depolarization in an R25 cell, even with the ganglion bathed in a solution of high divalent cations to reduce polysynaptic transmission ($N = 3$). **D**, Adding R15 α 1 peptide while the ganglion is superfused with high-divalent-cation solution produces a long-lasting depolarization of an R25 cell ($N = 3$). A 50- μ l bolus of 2×10^{-5} M R15 α 1 peptide (arrows) was added near the ganglion in **B** and **D**.



was within the usual range of the SCP_B effect and not significantly different from the response when SCP application was not preceded by R15 firing ($N = 5$; data not shown).

Further evidence that the decreased responsiveness to successive R15 firings is not due to refractoriness of the R25/L25 cells comes from an experiment in which the synaptic actions of R15 and an R20 cell were interacted. Firing an R20 cell at 3 Hz for 10 min elicited a large increase in R25/L25 activity. Nevertheless, when R15 was released from hyperpolarization for 10 min, it produced an increase in respiratory pumping rate that was not statistically different from that which occurs when the R15 bursts are not preceded by R20 activity (Fig. 8).

The lack of specific antagonists for the R15 α 1 peptide receptor makes it difficult to test conclusively whether this peptide mediates the postsynaptic actions of R15. However, the fact that the effects of R15 and its peptide decrement with repetition suggested that application of a high concentration of the peptide and prolonged firing of R15 might cross-desensitize. Successive applications of the peptide do indeed desensitize the response of the R25/L25 cells. Allowing R15 to burst after the response to the peptide has been desensitized does not produce a significant increase in the respiratory pumping rate (Fig. 9A). Application of SCP_B at the end of the experiment shows that the R25/L25 cells are neither refractory nor does R15 α 1 peptide cause them to be inhibited via another pathway, because they still can be modulated normally by SCP_B. Similarly, allowing R15 to burst for 60 min desensitizes the R25/L25 cells, blocking the response to a second firing of R15 and to the application of the R15 α 1 peptide (Fig. 9B). This concentration of R15 α 1 peptide normally would be expected to produce a 2.5-fold increase in the rate of respiratory pumping (Fig. 5). The two cross-desensitization experiments illustrated in Figure 9 suggest that R15 and the R15 α 1 peptide use the same receptor or the same post-receptor signal-transduction mechanism to generate their effects, so it is likely that the R15 α 1 peptide mediates R15's synaptic actions on the R25/L25 cells. Moreover, these results support the hypothesis that the decay in the responsiveness of

the R25/L25 network to repeated firing of R15 results largely from desensitization, though an additional effect due to decreased transmitter release cannot be ruled out.

We also tested one of the other two neuropeptides known to be present in R15, the R15 β peptide, for its actions on the R25/L25 network. Bath application of this peptide produced no effect on the frequency of respiratory pumping, nor did R15 β peptide modulate the response of the R25/L25 network to the R15 α 1 peptide or to the firing of R15 (Alevizos, 1989). These experiments suggest that the R15 β peptide acts neither presynaptically, to inhibit or promote release from R15, nor postsynaptically, to modulate the response of the R25/L25 cells to R15. Of course, other peptides synthesized or processed (posttranslationally modified) in R15 may play a presynaptic or postsynaptic role in synaptic transmission by R15. The R15 γ peptide was not tested because it is not available in synthetic form.

Comparison between the effects of R15 and the R20 cells on respiratory pumping

As an additional test of the hypothesis that R15 α 1 peptide mediates the synaptic actions of R15 on the R25/L25 network, the synaptic actions of R15 were compared to the actions produced by direct application of the peptide and to the effects produced by the only other known excitatory modulator of the network, the R20 cells, and one of their putative transmitters, SCP_B. As described above, the R25/L25 network exhibits two distinct modes of activity: weak bursts (L25 cells bursting alone) and strong bursts (R25 and L25 cells bursting together). R15 bursting was found to increase the frequency of both weak and strong bursts in the R25/L25 network, with about the same time courses (Figs. 2A, 10A). A similar effect was produced by application of R15 α 1 peptide onto the abdominal ganglion (Figs. 2B, 10B). Weak bursting was observed in 78% of the preparations in which R15's synaptic actions were recorded ($N = 120$) and 80% of the preparations in which the effects of R15 α 1 peptide were recorded ($N = 75$). In those preparations in which only strong bursting was observed, we cannot exclude the possibility that

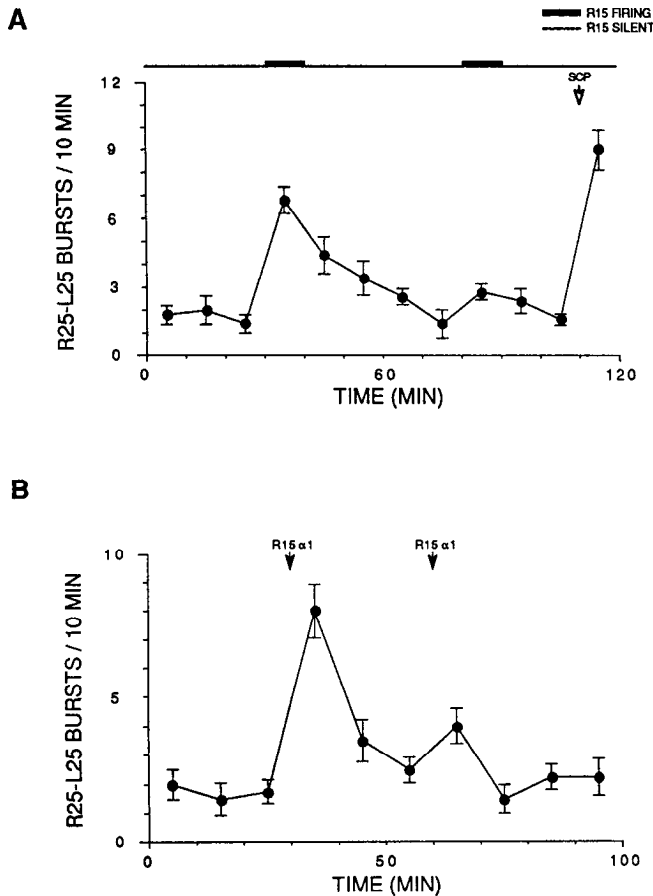


Figure 7. The effects of R15 and the R15 α 1 peptide on the frequency of respiratory pumping decay with repetition. *A*, Allowing R15 to burst spontaneously for 10 min after a 2-hr hyperpolarization elicits a long-lasting increase in the frequency of respiratory pumping. Releasing R15 from hyperpolarization 40 min after the end of the first firing period produces an effect on the respiratory pumping rate that is significantly smaller than that produced during the first firing period ($N = 4$). The number of spikes in R15 increased during the second firing period by 2.8%. Application of SCP_B produced a large increase in the rate of respiratory pumping (not significantly different from the effect of applying SCP_B without prior R15 activity; $N = 4$), suggesting that the R25/L25 cells are not refractory or inhibited by another pathway. *B*, Application of R15 α 1 peptide elicits a fourfold increase in the respiratory pumping rate. A bolus of R15 peptide added 40 min after the first bolus produces a significantly smaller effect ($N = 4$). All SCP_B and R15 α 1 applications in *A* and *B* consisted of a 50- μ l bolus of 2×10^{-5} M delivered near the ganglion. Error bars represent SEM.

weak bursting occurred but could not be recorded in the follower cells selected to monitor R25/L25 network activity.

Firing R15 or applying R15 α 1 peptide produces effects on the R25/L25 network different from those produced by the R20 cells or by SCP_B. The R20 cells and SCP_B exclusively generate strong bursting with a decelerating pattern, while R15 and the R15 α 1 peptide produce both weak and strong R25/L25 bursts that are more evenly spaced in time. The weak bursts, as monitored by the synaptic potentials in RD or LD followers, are not stereotyped in amplitude and form, while the strong bursts are more homogeneous (Fig. 11).

Another indication that R15 and the R20 cells have qualitatively different effects on the R25/L25 network comes from comparing the time courses of their synaptic actions. If R15 is

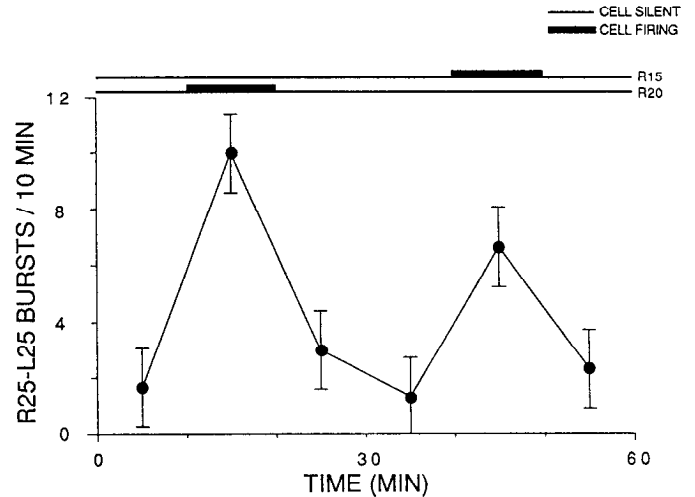


Figure 8. Previous activity elicited in the R25/L25 network by another input does not reduce the responsiveness of the network to R15. Firing an R20 cell at 3 Hz for 10 min elicits a fivefold increase in the frequency of respiratory pumping. R15 was kept hyperpolarized from the start of the experiment and was released from hyperpolarization 20 min after the end of firing in the R20 cell. R15 bursting produced a 3.5-fold increase in the frequency of respiratory pumping, which was not significantly different from the effect produced by R15 bursting that was not preceded by R20 firing (data not shown; $N = 3$). Error bars represent SEM.

fired at 3 Hz for 10 min, it produces an increase in the rate of bursting of the R25/L25 cells that decays over 60 min, while firing an R20 cell at the same frequency for the same period of time produces an effect that decays over 20 min (Fig. 12). This result is consistent with the conclusion that the two cell types use different transmitters.

Physiological function of activation of the R25/L25 network by R15

The fact that R15 is excited by the bag cells (Branton et al., 1978) suggests that it might mediate excitatory actions of the bag cells on the R25/L25 network. To test this hypothesis, we initiated a population burst in the bag cells by bathing the head ganglia with AGE, as described by Painter et al. (1988). In these experiments, R15 was kept hyperpolarized for a period beginning 2 hr before the application of AGE. The bag cell burst triggered indirectly by AGE had no effect on the frequency of R25/L25 burst activity until R15 was released from hyperpolarization, 15 min after the beginning of the bag-cell burst. As soon as R15 was allowed to burst, the frequency of R25/L25 bursting began to increase (Fig. 13; see also Alevizos et al., 1991b). In a separate set of experiments, when R15 was kept hyperpolarized throughout the experiment, the bag-cell burst had no significant effect on the frequency of respiratory pumping ($N = 3$).

R15 does not burst spontaneously in the intact animal

Because R15 is excited by the neuroendocrine bag cells when they trigger egg laying, we suggest that R15's motor effects described here and in the two companion papers (Alevizos et al., 1991a,b) may be involved in egg-laying behavior. Two observations, considered together, argue against this hypothesis. First, the synaptic actions of R15 decrement rapidly with prolonged firing of R15, apparently due to desensitization. Second, R15

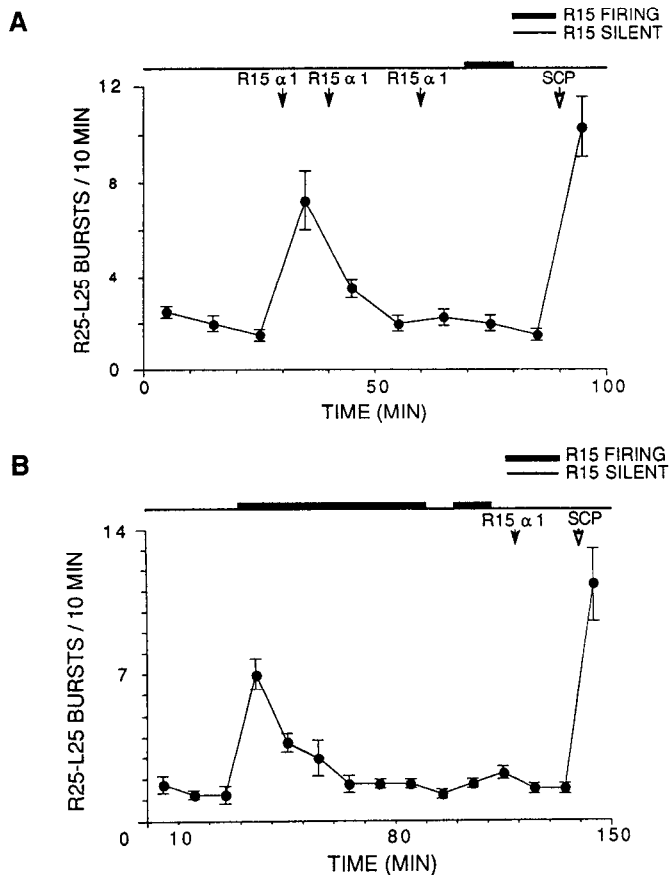


Figure 9. R15 and R15 α 1 peptide act on the R25/L25 network through the same receptor or postreceptor mechanism. **A**, Two successive applications of the R15 α 1 peptide render the R25/L25 network insensitive to a third application of peptide. Releasing R15 from hyperpolarization after the third application results in no effect on the frequency of respiratory pumping ($N = 4$). SCP_B applied 10 min after the R15 firing increases significantly the rate of respiratory pumping. R15 was kept hyperpolarized throughout the experiment. The preparations were not perfused during these experiments, and the peptides were presented as 50- μ l boluses of 2×10^{-5} M applied near the ganglion. **B**, Allowing R15 to fire spontaneously for 60 min after a 2-hr hyperpolarization period results in an increase in the frequency of respiratory pumping, which decays over a 60-min period. The R25/L25 network becomes insensitive to a second 10-min period of firing R15, and application of the R15 α 1 peptide as a 50- μ l bolus of 2×10^{-6} M, a concentration that normally elicits a 2.5-fold increase in the rate of respiratory pumping, has no effect. SCP_B (50 μ l of 2×10^{-5} M) applied 10 min later produces a significant increase in the rate of respiratory pumping ($N = 4$). Error bars represent SEM.

bursts continuously in the isolated ganglion and in the isolated nervous system (Beiswanger and Jacklet, 1975; Lickey et al., 1976). Thus, R15's synaptic actions are chronically in a refractory state *in vitro*, and they can be demonstrated only if R15 is hyperpolarized for 1–2 hr to prevent it from firing. Given this combination of continuous bursting activity and robust desensitization, can the synaptic actions of R15 ever be expressed physiologically? Perhaps R15 bursting is less active in the intact, freely behaving animal than in the various reduced preparations from which it has been recorded. We recorded the activity of R15 in the intact animal in order to test this hypothesis.

In earlier studies, Pinsker and colleagues attempted to monitor R15 activity in intact animals by recording extracellularly from the axons of R15 in peripheral nerves or from its cell body

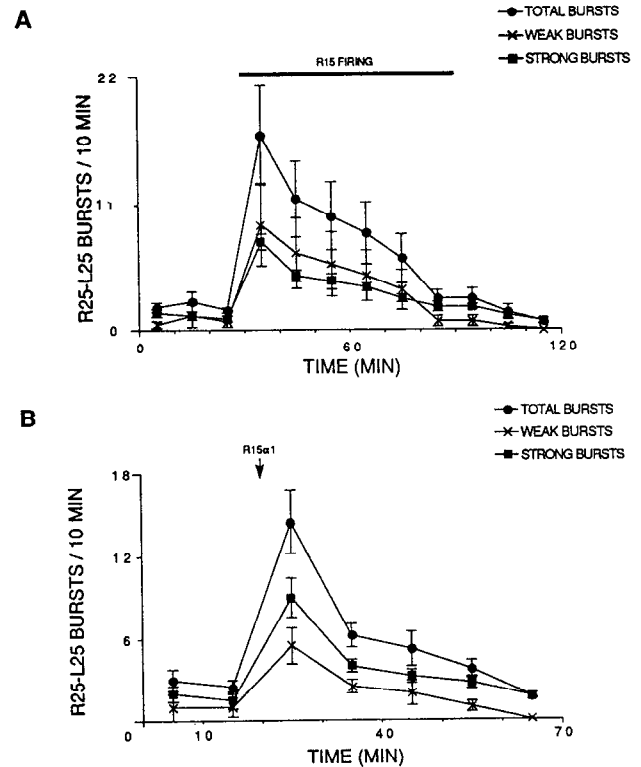


Figure 10. R15 and the R15 α 1 peptide produce both strong and weak bursting of the R25/L25 network. **A**, Firing R15 by brief current pulses at 1.8 Hz for 60 min after a 2-hr hyperpolarization elicits an increase in the frequency of respiratory pumping, which consists of both strong and weak bursting. The weak and strong bursting decay with similar time courses. The graph shows the amplitudes and durations of the increases in frequency of weak, strong, and total number of bursts of the R25/L25 network in response to R15 firing ($N = 4$). **B**, Application of R15 α 1 peptide mimicks the effect of R15 on the frequency of respiratory pumping, in that it generates both weak and strong bursts in the R25/L25 network. The graph shows the amplitudes and durations of the increases in frequency of weak, strong, and total number of bursts of the R25/L25 network in response to R15 α 1 peptide, which was given as a 50- μ l bolus of 2×10^{-5} M, presented close to the ganglion ($N = 4$). Error bars represent SEM.

(ter Maat et al., 1983; Parsons et al., 1983). They could not definitively identify R15 spike activity in the genital and pericardial nerves (Ter Maat et al., 1983), and attempts to record chronically from the soma of R15 also yielded ambiguous results (D. W. Parsons, personal communication). In acute experiments performed by Bablanian and Treisman (1983, 1985), intracellular recordings were made from R15 while the abdominal ganglion was exposed through a slit on the body wall in animals that were restrained by pinning the foot and the parapodia. R15 burst occasionally in these experiments, but fired primarily in a synaptically driven mode. However, potentially noxious afferent stimuli resulting from opening and pinning out the animal are likely to have influenced the firing of the cell.

We chose to record axonal activity of R15 in the intact animal. The main branches of the pericardial, genital, and siphon nerves have high background activity, making it difficult to identify the R15 spike in these branches. Therefore, we attempted to improve the signal-to-noise ratio by recording from nerves that had relatively few axons other than that of R15. The best nerves in this regard are the fine branches of the pericardial nerve that innervate the sheath of the hepatopancreas. As demonstrated

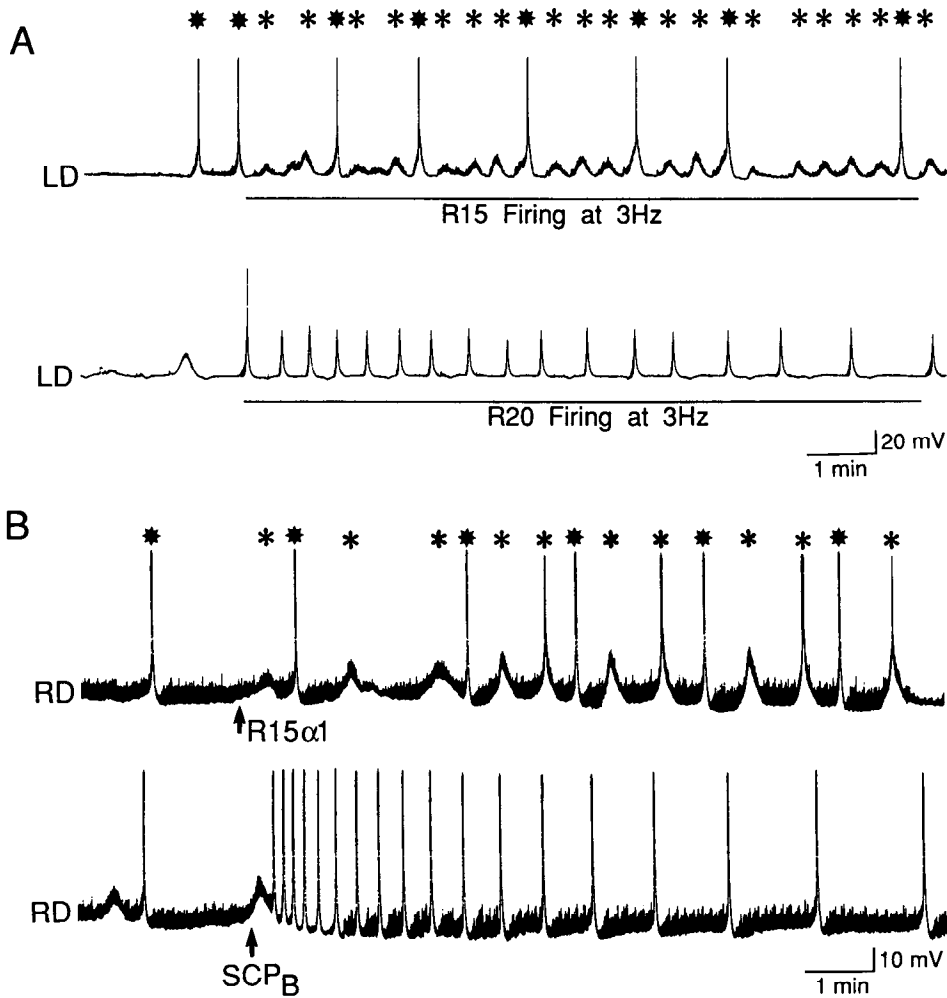


Figure 11. The R25/L25 network responds differently to R15 and the R20 cells and to their putative transmitters. *A*, Firing R15 for 10 min at 3 Hz after a 2-hr hyperpolarization generates weak and strong bursting of the R25/L25 cells, monitored in an LD cell. Strong bursts are marked by stars and weak bursts are marked by asterisks. Note that the weak bursts are not stereotyped in amplitude and shape. In the same preparation, firing an R20 cell for 10 min at 3 Hz produces only strong bursting, which produces synaptic input that is stereotyped in shape and amplitude. The strong bursts elicited by firing R20 are less effective in triggering spikes in the LD cell because R20 weakly inhibits this LD cell. *B*, The R15 α 1 peptide mimicks the effect of R15 on the frequency of respiratory pumping by eliciting both strong (stars) and weak bursts (asterisks), as monitored in an RD cell. SCP_B generates only strong bursts, as monitored in the same RD cell. Both peptides were applied in 50- μ l boluses of 2×10^{-5} M. The records in *A* and *B* are from different preparations. In both *A* and *B*, the LD and the RD follower cells were hyperpolarized to reduce spike activity, thereby making the synaptic input from the R25/L25 network more apparent.

by Rittenhouse and Price (1985), there is very little electrical activity *in vitro* in these branches, other than that attributed to R15. Although there is some variability in which branches contain R15 axons, the low background activity in these nerves makes them ideal for recording the spontaneous firing pattern of R15 in the intact animal.

In six successful recordings from the hepatopancreas nerve branch, activity of the R15 axon could be identified easily in the reduced preparation, but in none of them could R15-like bursting activity be identified in the intact animals. For up to 4 d of recording (approximately 3 hr of intermittent recordings per day) for one animal, R15 was either silent or beating at extremely low firing rates. Figure 14*A* shows three random 1.5-min periods selected from a 2.5-hr recording. Figure 14*B* shows the activity of R15's soma spike in the reduced preparation and its bursting pattern recorded extracellularly from the fine nerve branch. No such bursting activity was recorded in the intact animal.

One possible explanation of why we recorded no R15 bursting in the intact animals could be branch-specific block of the R15 axon in the hepatopancreas nerve. To address this issue, we recorded from other R15 nerve branches in three additional experiments. In one animal the electrode was placed on the spermathecal nerve, which often contains an axon branch from R15 (Rittenhouse and Price, 1985). In two additional experi-

ments, we recorded from the ventricular branch of the pericardial nerve (Mayeri et al., 1974). Although R15 bursting was clearly visible in all three animals in the reduced preparation examined at the end of the experiment, no R15 bursts were recorded in the intact animal. In all, 52 hr of recording from nine animals failed to reveal any sign of R15 bursting like that recorded in the isolated ganglion.

Several manipulations were used in an effort to trigger R15 bursting in the chronic preparations: exposure of the animal to low salinity (10% hypotonic) ASW for 30 min and return to normal ASW, prolonged contact with another animal, feeding to satiation, warming of the ASW from 14°C to 18°C, and recording from the animals at different times of the day/light cycle. None of these experimental conditions triggered R15 bursting activity. Although we cannot rule out the possibility that R15 bursting is suppressed by some unforeseen side effect of the surgery, our results are consistent with the hypothesis that R15 does not burst continuously in the intact animal. If it bursts at all, it apparently does so infrequently.

Discussion

R15 α 1 peptide may mediate R15's actions on the R25/L25 network

Four types of evidence support the conclusion that R15 exerts its excitatory effect on the R25/L25 network by the release of

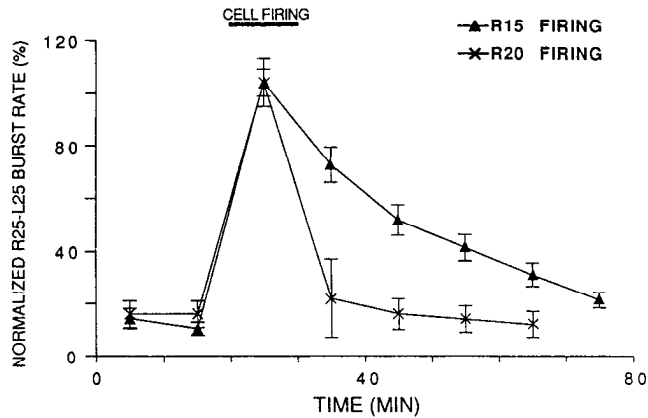


Figure 12. R15 produces a longer-lasting modulation of respiratory pumping than do the R20 cells. Firing R15 for 10 min at 3 Hz after 2 hr of hyperpolarization elicits an increase in the rate of respiratory pumping that decays over a 50-min period. In contrast, firing an R20 cell for the same amount of time at the same frequency elicits a change in respiratory pumping that decays shortly after R20 stops firing ($N = 4$). The amplitudes of the two responses have been normalized so that their time courses of decay can be compared. Mean baseline and maximum values of respiratory pumping frequency for the R15 experiment were 0.7 and 5.8 per 10 min; for the R20 experiment, the values were 1.6 and 10.4 per 10 min. Error bars represent SEM.

R15 α 1 peptide: (1) R15 has been shown to synthesize the R15 α 1 peptide (Weiss et al., 1989), and application of the peptide to the ganglion mimicks the effect on the network produced by firing R15, in sign (excitatory), in form (both weak and strong bursts are elicited), and in duration (both are long lasting). (2) Both R15 and exogenously applied R15 α 1 peptide appear to act directly on the R25/L25 network, because both effects persist under conditions in which polysynaptic activity in the ganglion is greatly reduced. (3) The actions of R15 and the R15 α 1 peptide cross-desensitize. (4) Another of R15's three identified neuropeptides, R15 β peptide, has no effect on the frequency of respiratory pumping. Taken together, these data are consistent with the hypothesis that the R15 α 1 peptide mediates the excitatory modulatory action of R15 on the R25/L25 network.

Modulation of the form of respiratory pumping by R15 and the R20 cells

Different modulatory inputs or substances applied to the R25/L25 circuit can produce different modes of behavioral output. The R20 cells and their putative transmitter, SCP, generate exclusively strong bursts in the R25/L25 network (Alevizos et al., 1989b). In contrast, R15 and the R15 α 1 peptide elicit a mixture of strong and weak bursting. The production of different modes of behavior by the same neuronal circuit also has been reported for other central pattern generators. For example, the circuit that generates escape swimming in *Tritonia* can be modulated to generate withdrawal reflexes (Gettings and Dikin, 1985; Gettings, 1989), and the gastric mill oscillator of lobster can be switched between two different patterns of behavioral output by modulatory inputs (Selverston, 1989).

The different effects exerted by R15 and the R20 cells on respiratory pumping suggest that the two cell types may serve different physiological functions. R15 generates a long-lasting increase of both the strong and the weak R25/L25 bursting frequency, while the R20 cells produce a briefer increase, predominantly in the rate of strong bursts. Visual observations of

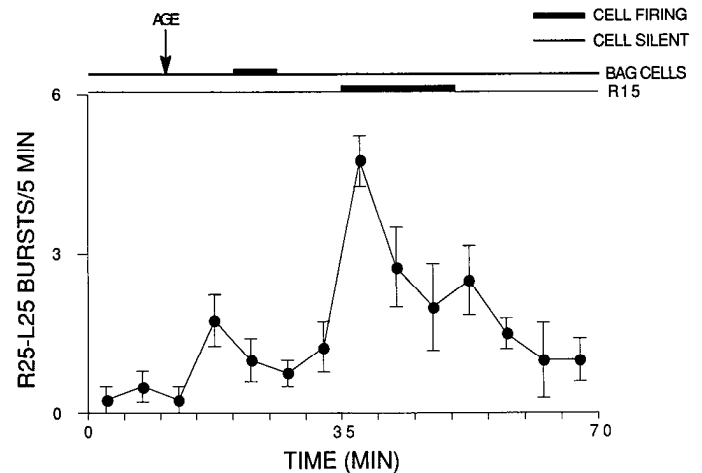


Figure 13. R15 activity is necessary and sufficient for modulation of respiratory pumping rate during bag cell discharge. The abdominal ganglion remained connected to the head ganglia via the pleuroabdominal connectives in this preparation. The head ganglia were kept isolated from the rest of the nervous system in a 1-ml subchamber that was physically isolated from the main chamber. R15 was kept hyperpolarized from the start of the experiment while respiratory pumping rate was monitored in postsynaptic followers of the R25/L25 network. Bag cells were induced to discharge by application of AGE (1/8 of a homogenized atrial gland) on the head ganglia. Bag cell discharge began 9 ± 2 min after AGE application. When R15 was released from hyperpolarization and allowed to burst spontaneously for 15 min, beginning 15 min after the start of the bag cell discharge (which lasted 6 ± 1 min), there was an increase in the respiratory pumping rate. The change in the frequency of respiratory pumping elicited by R15 bursting was not significantly different from the change recorded without previous firing of the bag cells ($N = 4$). The small increase in the rate of respiratory pumping immediately after AGE application was not studied further. Error bars represent SEM.

the contractions of the mantle organs and the heart during the weak bursting elicited by R15 revealed no obvious qualitative difference from the contractions during the strong bursts. Rather, the difference seems to be mostly quantitative; that is, heart inhibition and siphon and gill contractions were weaker during weak bursting than during strong bursting. Further work will be required to determine the functional significance of the two different types of respiratory pumping.

The R15 and the R20 cells also differ in their ability to elicit repeated responses in the R25/L25 network. The synaptic action of R15 exhibits pronounced desensitization that may limit the time course of R15's effect on the frequency of respiratory pumping. In contrast, the effects produced by the R20 cells do not desensitize appreciably (Alevizos, 1989).

Physiological roles of R15 and the R20 cells in modulating the frequency of respiratory pumping

The long-lasting nature of the modulatory effects of R15 and the R20s cells, as well as the different patterns of modulation they produce, suggests that variable patterns of activity in these modulatory inputs to the R25/L25 cells may contribute to the variability observed across and within animals in the rate of respiratory pumping (Eberly et al., 1981). In addition, the identification of these two sources of modulatory input should prove useful in determining the mechanisms by which environmental and physiological stimuli modulate respiratory pumping behavior. The physiological conditions under which the R20 cells

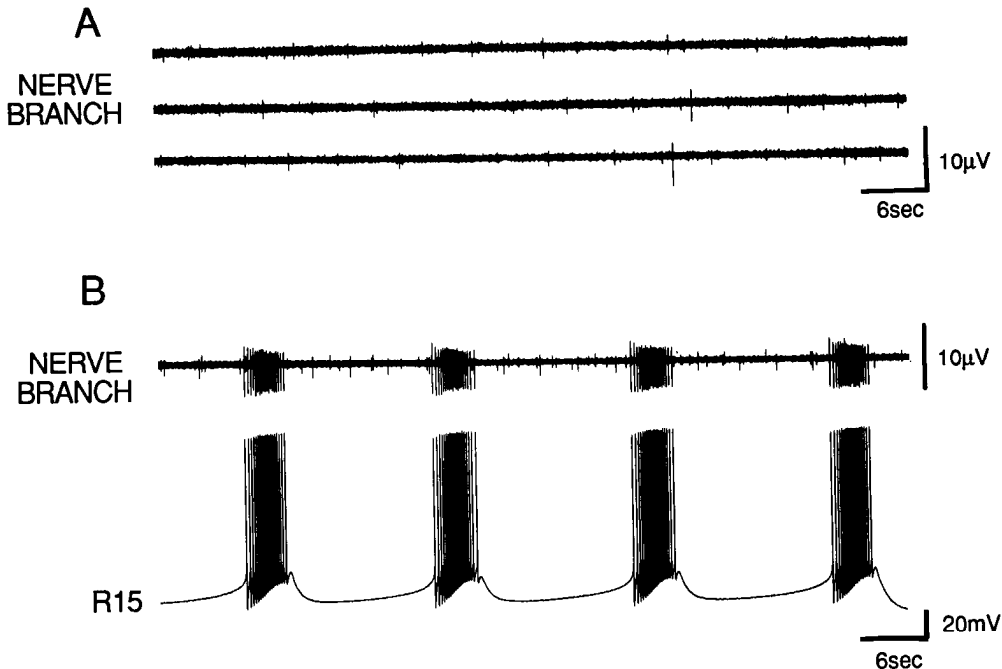


Figure 14. R15 does not burst spontaneously in the intact animal. An extracellular electrode chronically implanted into the subject was used to record activity from a small branch of the pericardial nerve that runs from the major nerve trunk to the mesentery of the hepatopancreas, which is innervated by R15's processes. *A*, In the intact animal there was no activity in R15 under a variety of experimental conditions. Three random 1.5-min samples are shown from a 2-hr recording in the intact animal. *B*, At the end of the experiment, the animal was killed, and the abdominal ganglion was dissected from the animal, along with the electrode still attached to the nerve branch. Bursting activity was recorded in the nerve of the isolated ganglion. Intracellular recording from the R15 soma showed that the bursting activity in the nerve was due to R15. These data are representative of 52 hr of recording from nine animals.

are activated are not yet known, though indirect evidence (Alevizos, 1989) suggests that they may be responsible for the spontaneous seizures of respiratory pumping observed in the intact animal (Kanz and Quast, 1990). R15, on the other hand, is likely to be activated during egg laying (Branton et al., 1978). Although there is no direct evidence that respiratory pumping increases during spontaneous egg-laying behavior, it has been reported to increase following bag-cell bursts triggered *in vitro* (Schaefer and Brownell, 1986). Our results, together with the data showing that the bag cells excite R15 (Branton et al., 1978), suggest that, if the bag cells increase the rate of respiratory pumping *in vivo*, they may do so in part by exciting R15.

R15 bursting is suppressed in the intact animal

We could find no evidence that R15 bursts continuously in the intact animal. Although we cannot rule out nonburst firing of R15, its frequency must be quite low, as illustrated by Figure 14*A*. Our working hypothesis is that, *in vivo*, R15 is a conditional burster, and that its burst activity is normally suppressed. It seems likely that R15 burst generation is triggered *in vivo* when a bag-cell burst initiates egg laying. However, because no spontaneous bouts of egg laying occurred during our chronic recording experiments, this question remains open.

The lack of R15 bursting in the intact animal has two important consequences for its physiological actions. First, were R15 to burst continuously, its synaptic outputs would be permanently blocked by desensitization. The lack of continuous bursting in the intact animal means that, when R15 does burst, the synaptic actions described here and in the two companion papers can be expressed. Second, Kramer and Levitan (1990) have shown that there is an activity-dependent suppression of R15's responses to two excitatory modulators, egg-laying hormone (ELH) and 5-HT. If R15 is silent when ELH or 5-HT is applied, the modulation of the currents that generate the bursting is maximal, while when R15 is bursting the effects of these modulators are minimal. To exhibit a maximum response to

ELH during the bag cell discharge, R15 must be silent when ELH is released.

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