

Augmentation of bursting pacemaker activity by egg-laying hormone in *Aplysia* neuron R15 is mediated by a cyclic AMP-dependent increase in Ca^{2+} and K^+ currents

(neuropeptide/intracellular messenger/voltage clamp)

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ABSTRACT Release of the neuropeptide egg-laying hormone (ELH) from *Aplysia* bag cell neurons augments the endogenous bursting pacemaker activity of neuron R15. We have studied the ionic mechanisms underlying the effect of ELH in voltage-clamped R15 neurons. Both electrical discharge of the bag cells, which releases endogenous ELH, and application of synthetic ELH on cell R15 result in an increase in two discrete ionic currents. One of these currents activates with hyperpolarization, reverses near the K^+ equilibrium potential, is sensitive to the external K^+ concentration, and is blocked by addition of 5 mM Rb^+ or 1 mM Ba^{2+} to the bathing medium. This current appears to be identical to the inwardly rectifying K^+ current I_{R} . The other current activates with depolarization and is blocked by replacement of external Ca^{2+} with Co^{2+} or Mn^{2+} . This current appears to be a voltage-gated Ca^{2+} current I_{Ca} . Both I_{Ca} and I_{R} in R15 have previously been shown to be enhanced by the neurotransmitter serotonin, acting via intracellular cyclic AMP. We now report that increasing cyclic AMP in R15, by applying either serotonin or the adenylate cyclase activator forskolin together with a phosphodiesterase inhibitor, mimics and occludes the action of ELH on neuron R15. Furthermore, application of ELH increases the cyclic AMP content of single R15 neurons, as measured by radioimmunoassay. Finally, the effects of ELH are potentiated by a phosphodiesterase inhibitor. These results suggest that ELH augments bursting activity in R15 by causing cyclic AMP-mediated increases in I_{R} and I_{Ca} .

The electrical activity of bursting pacemaker neurons can be modulated by a variety of neurotransmitters and hormones (1–3). Often the effect of neuromodulatory substances on bursting neurons is neither simply excitatory nor simply inhibitory. Instead, neuromodulators often cause changes in the pattern of rhythmic activity. The ionic and intracellular mechanisms of these complex changes in electrical activity of bursting cells are not well understood.

The endogenously generated bursting activity of neuron R15 from the abdominal ganglion of *Aplysia* can be modulated by biogenic amines (4–6), neuropeptides (7, 8), and synaptic stimuli (9, 10). The bursting activity of R15 is also modulated by repetitive firing (“afterdischarge”) of an electrically coupled cluster of neurosecretory “bag cell” neurons, also found in the abdominal ganglion (11). The bag cell neurons synthesize several neuropeptides, which are released during the afterdischarge. These peptides initiate egg-laying and associated behaviors by affecting both the reproductive organs and central neurons of *Aplysia* (12–15). Release of the neuropeptide egg-laying hormone (ELH) from bag cell neurons, or bath application of purified native ELH, results in a long-lasting (up to 3 hr) increase in the number and

frequency of spikes during bursts in R15 (7, 14). In addition, ELH induces an increase in the amplitude of the interburst hyperpolarization (7, 14). Hence, ELH augments the bursting activity of R15, apparently by affecting both the depolarizing and hyperpolarizing phases of the burst cycle.

In this study, the effects of ELH on ionic currents were examined in voltage-clamped R15 neurons. We show here that two ionic currents are enhanced by ELH: an inward (or anomalously) rectifying K^+ current (I_{R}) and a voltage-gated Ca^{2+} current (I_{Ca}). It has been demonstrated previously that both I_{R} and I_{Ca} are enhanced by serotonin (5-HT; 5-hydroxytryptamine) and that the increase in these currents is mediated by a 5-HT-stimulated increase in the intracellular level of cyclic AMP in cell R5 (5, 6, 16). Here we present evidence that the enhancement of I_{Ca} and I_{R} by ELH is likewise mediated by an increase in cyclic AMP.

MATERIALS AND METHODS

Aplysia californica (200–300 g) were obtained from Alacritty Marine Biologicals (Redondo Beach, CA). Individuals that had laid eggs during the previous 1 or 2 days were not used. The animals were injected with 100 ml of 400 mM MgCl_2 , the abdominal ganglion was removed and pinned to the Sylgard base of a 1-ml chamber, and the connective tissue sheath overlying the neuronal cell bodies was removed. In bag cell discharge experiments, only the portion of the sheath covering the soma of R15 was removed. The preparation was continuously superfused at 1 ml/min (unless indicated otherwise) with saline containing 460 mM NaCl, 55 mM MgCl_2 , 11 mM CaCl_2 , 10 mM KCl, and 10 mM Na HEPES (pH 7.4). In some experiments, the saline contained 100 mM MgCl_2 to reduce spontaneous synaptic activity, and NaCl was lowered to 392 mM to maintain osmolarity. Neuron R15 (17) was impaled with one or two electrodes (2–10 M Ω) containing either 0.5 M K_2SO_4 or 1.5 M KCl. Steady-state current vs. voltage (I – V) curves were constructed from ionic currents measured with the use of a conventional one- or two-electrode voltage clamp system. All experiments were performed at least three times unless otherwise indicated.

In most experiments, ELH was applied by “puffing” 1 μl of saline containing 40 μM synthetic ELH (kindly provided by F. Strumwasser, Boston University) over the soma of R15. Synthetic ELH produces egg-laying behavior indistinguishable from that seen after injection of native purified ELH into *Aplysia* (18). The ELH saline was applied from the drawn-out tip of polyethylene tubing positioned 400 μm away from the soma of R15. Tracer dye indicated that the ELH

Abbreviations: ELH, egg-laying hormone; 5-HT, serotonin; I_{Ca} , voltage-gated Ca^{2+} current; I_{R} , inward rectifying K^+ current.

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saline was washed away from R15 in 1–2 min. Puffing normal saline at R15 produced no effects.

Bag cell afterdischarges were elicited by electrically stimulating the left bag cell cluster with a suction electrode. Occurrence of the discharge was confirmed by extracellularly monitoring with a second suction electrode the activity of the right bag cell cluster, which is electrically coupled to the left cluster.

Application of ELH in experiments in which cyclic AMP content was measured was done by one of two protocols. In two experiments, R15 was voltage-clamped to -75 mV in a 200- μ l chamber. Then 100 μ l of a 24 μ M ELH solution was injected into the chamber. In a third experiment, 3 μ l of 40 μ M ELH was puffed over spontaneously bursting R15 neurons. In all three experiments, the preparation was allowed to incubate without superfusion for 10 min and then quickly frozen by addition of 70% propylene glycol/30% normal saline (vol/vol) cooled with dry ice to below -20°C (19). R15 neurons were removed from the ganglion and placed in a 98% ethanol/2% 0.2 M HCl solution (vol/vol) at 4°C . The cells were stored in this solution at -70°C and pools of five cells were assayed for cyclic AMP content by radioimmunoassay (DuPont RIANEN cyclic AMP RIA kit).

Forskolin was obtained from Calbiochem. 3-Isobutyl-1-methylxanthine was obtained from Sigma. Ro-20-1724 was kindly provided by W. Burkhard (Hoffman-LaRoche).

RESULTS

ELH Augments Bursting Activity in R15 and Affects Two Ionic Currents. Local application of a puff of synthetic ELH on the soma of cell R15 changes the bursting activity of R15 (Fig. 1A), as described with purified native ELH (7, 14). The ELH puff causes both an increase in the number and frequency of action potentials per burst and an enhancement of the amplitude of the interburst hyperpolarization. The effects of a brief application of ELH are long-lasting (>10 min), suggesting that modulation of bursting activity may involve an intracellular second messenger.

The steady-state I - V curve of voltage-clamped R15 neurons reveals two distinct effects of ELH (Fig. 1B). First, there is an increase in an inward current at membrane potentials more negative than the K^+ equilibrium potential (i.e., below -75 mV), which appears within 2 min after ELH application. Second, there is an increase in an inward current at membrane potentials more positive than about -60 mV, which first appears 2–5 min after applying ELH. The increase in both currents is maximal 7–12 min after the puff, and thereafter both currents slowly decrease, returning to their initial amplitudes after ≈ 45 min. Afterdischarge of the bag cells, which causes release of native ELH, produces similar effects on the I - V curve (Fig. 1C). Thus, two ionic currents appear to be modulated in response to the physiological release of ELH.

It has been shown that ELH produces burst augmentation by acting directly on R15 and not indirectly by affecting synaptic inputs onto R15 (14). Likewise, ELH continues to affect the two regions of the I - V curve when Ca^{2+} is reduced by a factor of 30 and 3 mM Mn^{2+} is added to the saline, which almost completely blocks synaptic activity. Hence, ELH appears to act directly on R15.

ELH Increases I_R . The I - V curve of R15 exhibits a region of steep slope (large conductance) at membrane potentials more hyperpolarized than about -75 mV. The large conductance below -75 mV has been attributed to a K^+ current, which has the unusual property of activating with hyperpolarization rather than with depolarization (the inward or "anomalously" rectifying K^+ current; I_R) (16). The current activated by ELH at potentials below -75 mV has several characteristics in common with those already described for I_R (16, 20). The ELH-induced current below -75 mV activates rapidly, within 10 msec of a hyperpolarizing step. The ELH-induced conductance at hyperpolarized potentials increases with elevation of K^+ in the saline and decreases with reduction of K^+ in the saline, as expected for I_R (16). Also, the current induced by ELH reverses at the K^+ equilibrium potential (E_K ; about -75 mV). I_R does not rectify perfectly but also reverses at E_K , such that there is some outward

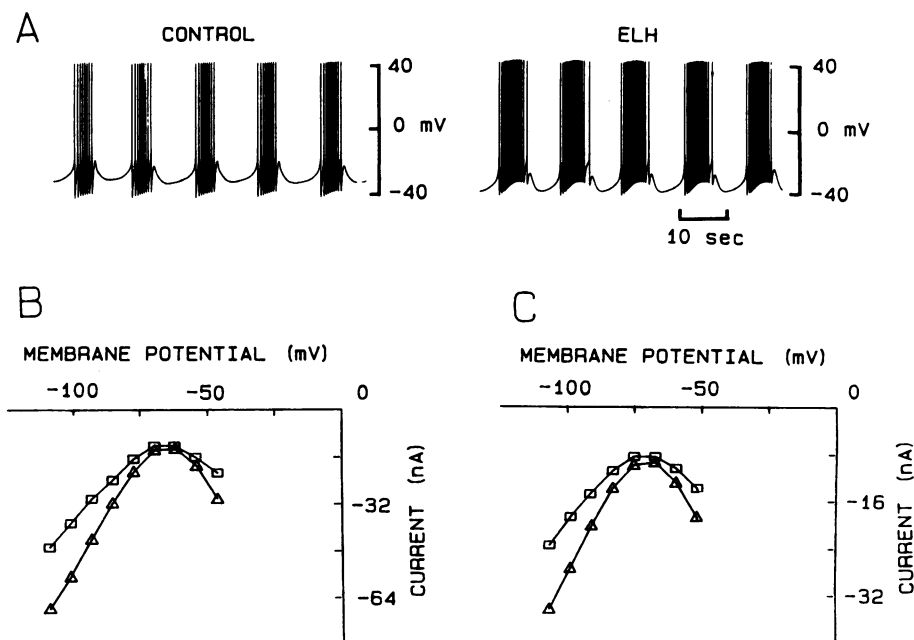


FIG. 1. Effects of ELH on bursting activity and I - V curve of neuron R15. (A) Bursting pacemaker activity of neuron R15 before and 5 min after local application of 20 μ l of 8 μ M synthetic ELH on the soma of R15. Note that ELH augments both the depolarizing and hyperpolarizing phases of bursting activity. (B) Steady-state I - V curve of neuron R15 before (\square) and 16 min after (Δ) local application of 1 μ l of 40 μ M synthetic ELH. Currents were evoked with 1-sec pulses from -80 mV. (C) Steady-state I - V curve of another R15 neuron before (\square) and 8 min after (Δ) bag cell discharge. Currents were evoked with 0.5-sec pulses from -60 mV.

current attributable to I_R between -75 and -50 mV (16, 20). We did observe a reversal of the ELH-induced current for 2–5 min after the ELH puff, but at later times the reversal of this current above E_K was obscured by the increase in the second ionic current.

We further tested the possibility that ELH increases I_R by examining the effect of ELH in the presence of agents that selectively block I_R . In normal saline, ELH increases the current evoked by a hyperpolarizing pulse from -75 to -125 mV (Fig. 2A). Addition of 5 mM Rb^+ to the bathing medium, which blocks I_R in R15 (16), causes a decrease in the current activated by hyperpolarization and blocks the effect of ELH at hyperpolarized membrane potentials (Fig. 2A). Likewise, 1 mM Ba^{2+} blocks I_R (16, 20) and also eliminates the ELH-induced current at membrane potentials below -75 mV, while leaving the second ELH-induced current activated above -60 mV intact (Fig. 2B). Thus, ELH induces an increase in the amplitude of I_R and also affects a second ionic current at depolarized membrane potentials.

ELH Increases I_{Ca} . The I - V curve of cell R15 exhibits a region of negative slope above -60 mV due to a persistent inward Ca^{2+} current (I_{Ca}), which is activated by depolarization (21). We tested the possibility that ELH increases I_{Ca} by examining the effect of ELH in the presence of divalent cation blockers of I_{Ca} , such as Mn^{2+} and Co^{2+} (21, 22). In normal saline, ELH causes an increase in the inward current activated by a depolarizing voltage clamp pulse from -52 to -44 mV (Fig. 3A). In saline in which Ca^{2+} is replaced with Mn^{2+} , the depolarizing pulse no longer elicits an inward current, but rather, an outward current is revealed, and ELH application fails to produce an effect (Fig. 3B). Similar results are obtained when I_{Ca} is blocked with Co^{2+} (data not shown).

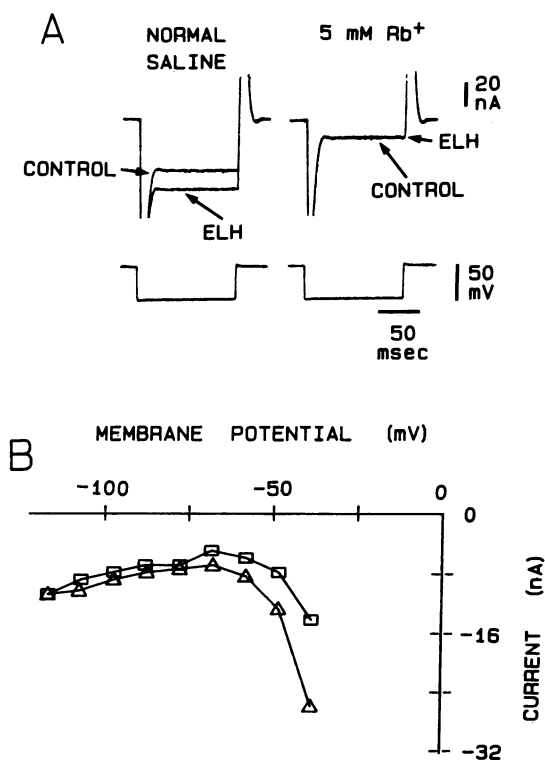


FIG. 2. ELH increases the inward rectifier K^+ current (I_R). (A) Currents elicited by a 50-mV hyperpolarizing voltage pulse from -75 mV before and after application of $40 \mu M$ ELH to an R15 neuron. (Left) Currents measured in normal saline. (Right) Currents measured in the presence of 5 mM Rb^+ , a blocker of I_R . (B) I - V curves obtained from a different R15 neuron before (\square) and 7 min after (Δ) $40 \mu M$ ELH application in the presence of 1 mM Ba^{2+} , another blocker of I_R . Currents were evoked with 1-sec pulses from -70 mV.

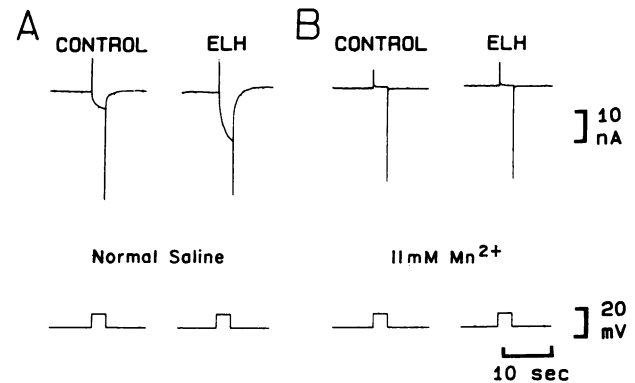


FIG. 3. ELH increases the voltage-gated calcium current (I_{Ca}). (A) Control current and current 7 min after ELH application. (B) Currents measured in saline in which Ca^{2+} was replaced with an equimolar concentration of Mn^{2+} , a blocker of I_{Ca} , before and 7 min after ELH application. In both A and B, the voltage clamp pulse was a 3-sec pulse from -52 to -44 mV and $40 \mu M$ ELH was applied locally to the soma of the same R15 neuron.

Hence, ELH has no effect on the current elicited by depolarizing pulses above -60 mV when I_{Ca} is blocked.

The effect of ELH on the inward current elicited by depolarization is probably due to an increase in I_{Ca} rather than a decrease in an outward K^+ current. First, the Ca^{2+} -dependent outward tail current following depolarization, which is due to the inactivation of resting I_{Ca} by accumulated intracellular Ca^{2+} (23, 24), is increased by ELH. In addition, the ELH effect is not reduced by addition of 10 mM tetraethylammonium ($n = 2$), which is sufficient to block the Ca^{2+} -activated K^+ current in R15 (25), or by addition of the K^+ current blocker 4-aminopyridine (5 mM) ($n = 2$). Decreasing the driving force for K^+ by increasing external K^+ does not decrease the ELH effect at membrane potentials more positive than -50 mV. Finally, the effect of ELH on the voltage-gated inward current above -60 mV is occluded by agents that increase I_{Ca} via internal cyclic AMP (see below). These data suggest that ELH increases I_{Ca} in R15.

The Effects of ELH on R15 Are Occluded by Agents That Increase Cyclic AMP. The neurotransmitter 5-HT enhances I_{Ca} and I_R in R15 via cyclic AMP (5, 6, 16). If ELH activates the same population of ion channels as does 5-HT, then application of 5-HT or other agents that increase cyclic AMP might occlude the action of ELH on R15. Fig. 4 shows that the effect of ELH on both I_{Ca} and I_R can be occluded by application of either 5-HT or the adenylate cyclase activator forskolin (26) together with a phosphodiesterase inhibitor. First, ELH was puffed on cell R15 to increase both I_R below -75 mV, and I_{Ca} above -60 mV (Fig. 4A). After 45 min of washing with normal saline, the I - V curve returns to normal. Then $100 \mu M$ 5-HT was added, also eliciting an increase in the two ionic currents (Fig. 4B). In the continued presence of $100 \mu M$ 5-HT, the ELH puff no longer has any effect on the I - V curve of R15 (Fig. 4C). The same experimental protocol was followed using $60 \mu M$ forskolin, together with $15 \mu M$ Ro-20-1724 (a phosphodiesterase inhibitor), to increase cyclic AMP and enhance both I_R and I_{Ca} (data not shown). ELH applied in the continued presence of these substances has no additional effect on the I - V curve of R15. Hence, the modulation of I_{Ca} and I_R can be saturated by increasing cyclic AMP with either 5-HT or forskolin, such that ELH no longer has an effect.

ELH Acts via Cyclic AMP in R15. To test further whether cyclic AMP mediates the actions of ELH, the cyclic AMP content of R15 neurons was measured with or without prior exposure to ELH. In two experiments, R15 cells were voltage clamped to -75 mV to prevent bursting activity, and

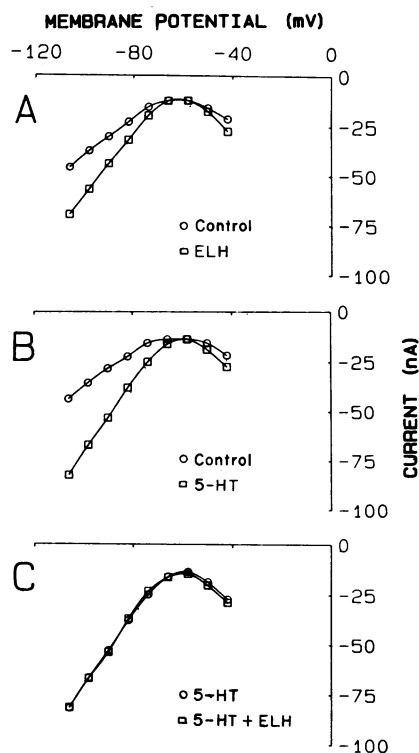


FIG. 4. ELH effects are mimicked and occluded by agents that increase cyclic AMP. (A) I - V curve in control saline (\circ) and after application of ELH (\square). (B) I - V curve in control saline (\circ) and after addition of $100 \mu\text{M}$ 5-HT (\square). (C) I - V curve in $100 \mu\text{M}$ 5-HT (\circ) and after application of ELH while in the continued presence of $100 \mu\text{M}$ 5-HT (\square). (A-C) Currents were evoked by 1-sec pulses from -80 mV . For each application, $40 \mu\text{M}$ ELH was applied locally to the soma of R15.

ELH was bath-applied (Fig. 5A, Exps. 1 and 2). In these experiments, ELH increased the cyclic AMP content by 72% over untreated cells. In a third experiment (Fig. 5A, Exp. 3), ELH was puffed onto spontaneously bursting cells, and their cyclic AMP content was compared to untreated bursting cells. ELH causes an increase in the cyclic AMP content of 70% over the control cells, although the basal cyclic AMP content of bursting R15 neurons is lower than that of hyperpolarized R15 neurons (see ref. 27). Hence, ELH does indeed increase the content of cyclic AMP in R15 cells.

Responses to neurotransmitters whose actions are mediated by cyclic AMP can be enhanced by addition of phosphodiesterase inhibitors. The effect of a transient application of ELH on I_{Ca} in the absence and presence of the phosphodiesterase inhibitor isobutylmethylxanthine is shown in Fig. 5B. Isobutylmethylxanthine increases the basal level of I_{Ca} , presumably by increasing the basal level of cyclic AMP in R15 (28), and potentiates the increase in the current induced by ELH. A similar potentiation of the ELH-induced increase in I_{R} also occurs (data not shown).

DISCUSSION

It has previously been determined that augmentation of the bursting pacemaker activity of cell R15 by discharge of the bag cell neurons is due at least in part to release of ELH (7, 14). This study provides evidence that application of synthetic ELH and bag cell afterdischarge increase both I_{Ca} and I_{R} in R15 and that these effects are mediated by cyclic AMP. We propose that augmentation of the depolarizing and hyperpolarizing phases of bursting pacemaker activity in R15 are due to the enhancement of I_{Ca} and I_{R} , respectively.

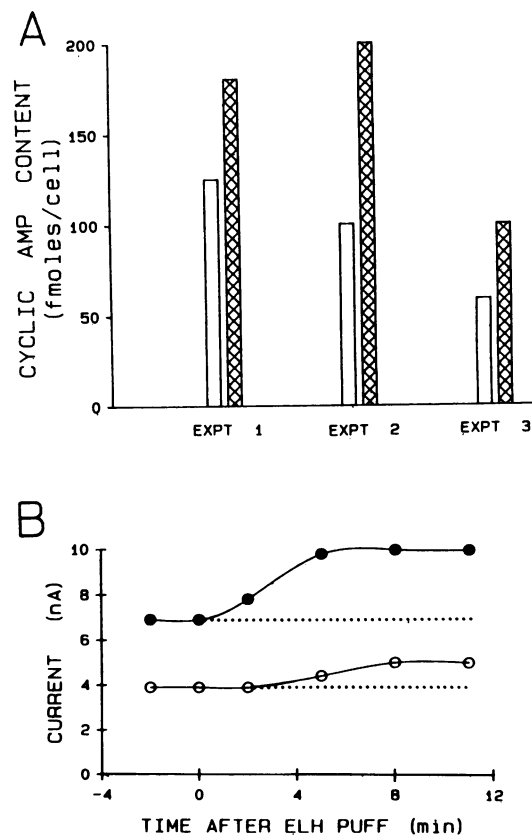


FIG. 5. ELH acts via cyclic AMP in neuron R15. (A) ELH increases the content of cyclic AMP in R15. Cyclic AMP content of control (open bars) and ELH-treated (cross-hatched bars) R15 neurons. Each bar represents a measurement from five pooled somata. In experiments 1 and 2, $8 \mu\text{M}$ ELH was bath-applied to R15 cells voltage clamped to -75 mV . In experiment 3, $3 \mu\text{l}$ of $40 \mu\text{M}$ ELH was applied locally on endogenously bursting R15 neurons (see *Materials and Methods*). (B) A phosphodiesterase inhibitor potentiates the effect of ELH on neuron R15. The effect of ELH on I_{Ca} measured before (\circ) and after (\bullet) 0.2 mM isobutylmethylxanthine was added to the saline. I_{Ca} was evoked with 0.5-sec pulses from -75 mV to -45 mV . ELH was applied locally by puffing $2 \mu\text{l}$ of $3 \mu\text{M}$ ELH at the soma of R15. Note that isobutylmethylxanthine increased the basal I_{Ca} current before ELH application and also potentiated the effect of ELH on I_{Ca} .

Enhancement of I_{Ca} , which activates with depolarization, is thought to be responsible for the increase in the number of action potentials per burst after 5-HT application to R15 (6), while inhibition of I_{Ca} by application of dopamine causes a decrease and eventual cessation of the depolarizing phase of bursting (29). There is also evidence that enhancement of I_{R} is responsible for the increase in the amplitude of the interburst hyperpolarization after 5-HT application (16). I_{R} activates with hyperpolarization at membrane potentials $10\text{--}20 \text{ mV}$ more depolarized than E_{K} (i.e., -65 to -55 mV ; refs. 16 and 20). Hence, when I_{R} is enhanced by ELH, the interburst hyperpolarization may be sufficiently large to activate enough I_{R} to initiate a regenerative hyperpolarization. Therefore, during burst augmentation, there may be an alternation between periods when I_{R} is regeneratively activated (the hyperpolarizing phase of bursting) and periods when I_{Ca} is regeneratively activated (the depolarizing phase).

The actions of ELH on R15 appear to be mediated by an increase in the intracellular level of cyclic AMP. Likewise, the actions of 5-HT on both I_{R} (5) and I_{Ca} (6) appear to be mediated by an increase in cyclic AMP. 5-HT increases the cyclic AMP content of R15 neurons by activating a 5-HT-sensitive adenylate cyclase (30). It is not known whether the

ELH-induced increase in cyclic AMP is due to a specific receptor for ELH, or if it is due to activation of the 5-HT receptor.

ELH is responsible for a large subset of the responses induced by bag cell afterdischarge, including egg-laying behavior (18), prolonged excitation of neurons in the abdominal (14) and head (12, 15) ganglia, and burst augmentation in R15 (7). ELH excites the buccal motoneuron B16 by activating a voltage-dependent slow Na⁺ current, possibly via intracellular cyclic AMP (15). It is possible that all of the effects of ELH in *Aplysia* are mediated by cyclic AMP. It will be of interest to test whether cyclic AMP mediates the response of other *Aplysia* neurons to ELH, such as the left lower quadrant neurons of the abdominal ganglion, which exhibit ELH-induced changes of the *I-V* curve that are similar to those seen in R15 (31).

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