Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3017–3021, May 1986
Neurobiology

Egg-laying hormone of *Aplysia* induces a voltage-dependent slow inward current carried by Na^+ in an identified motoneuron

(neuropeptides/ion-channel modulation/voltage clamp)

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Communicated by Donald Kennedy, December 20, 1985

ABSTRACT **This report presents studies on ionic currents in *Aplysia* motoneuron B16 that are modulated by the neuropeptide egg-laying hormone (ELH) of *Aplysia*. ELH induces an inward current that persists in the presence of the peptide and that decays slowly after ELH is removed from the bath. The effect is not due to a decrease in the delayed potassium current, the calcium-activated potassium current, or the transient potassium current. Current-voltage measurements indicate that ELH produces increased inward currents from −80 mV to ≈0 mV. The effect is particularly enhanced in the region from −40 mV to −25 mV where a negative slope conductance due to a voltage-dependent slow inward current is observed. The slow inward current and the response to ELH persist in saline solutions in which Ca^{2+} is replaced with Co^{2+} but are eliminated when Na^+ is replaced with equimolar concentrations of either Tris or *N*-methyl-D-glucamine. The response to ELH is unaffected by replacing chloride with equimolar acetate; by increasing the potassium concentration; or by adding tetraethylammonium chloride, CsCl, 4-aminopyridine, or tetrodotoxin to the saline bath. In addition, the reversal potentials for the ELH response (range, −28 to +46 mV), obtained from difference current-voltage relationships, are consistent with an increase in the Na^+-dependent slow inward current. We conclude that at least one of the effects of ELH on B16 is to increase a slow inward current carried by Na^+.**

Peptides are used as neurotransmitters in the central and peripheral nervous systems and are involved in the production or modulation of various behaviors. While neuropeptides are ubiquitous features of both vertebrate and invertebrate nervous systems, only in relatively few cases have the cellular actions of a peptide been related to its behavioral effects (1–6).

The neuropeptide egg-laying hormone (ELH) of *Aplysia* is synthesized in a cluster of neurosecretory neurons, the "bag cells," which are located on the rostral margin of the abdominal ganglion (7). The bag cells fire in a burst of activity termed afterdischarge (8), and they release ELH (and associated peptides) into the interstitial spaces of the ganglion. ELH diffuses into the hemolymph where, acting as a hormone, it induces egg-release from the ovotestis (9). Purified ELH induces egg-laying behavior when injected into a sexually mature *Aplysia* (10, 11). In addition, an inhibition of feeding also occurs, which begins before the appearance of eggs in the genital groove and persists for the duration of egg-laying behavior (11).

Mayeri and colleagues (5) have documented several direct, neurotransmitter-like effects of ELH on identified neurons in the abdominal ganglion. ELH also acts on neurons in the buccal ganglion (11), including the motoneuron B16 (12, 13). B16 mediates contraction of the accessory radula closer muscle (ARC), which closes and retracts the radula (12), the organ used to grasp food.

ELH, at nanomolar concentrations, induces a prolonged excitation of B16, an effect that has a slow onset (1–4 min) and a duration of several minutes (11, 13). The consequential retraction or closing of the radula by B16 would prevent the ingestion of the egg string during egg laying and is consistent with the inhibition of feeding behavior observed. Therefore, the ELH action on B16 may be an important component of the egg-laying fixed-action pattern.

In this study, the effects of ELH on voltage-dependent ionic currents in B16 were investigated. We show here that the excitatory effect of ELH is due to an increase in a voltage-dependent slow inward current carried largely by sodium ions.

METHODS

Aplysia californica (75–200 g; Sea Life Supply, Seaside, CA) were used for the physiological experiments. The results presented here are taken from a total of 64 preparations. All experiments were performed at room temperature (22°C–25°C).

Dissection Procedures and Motoneuron Identification. To unambiguously identify B16, we found it necessary to correlate its firing with the occurrence of excitatory junction potentials (EJPs) in the ARC (12, 13). Therefore, we removed the buccal ganglia and the ARCs bilaterally with the peripheral nerves [B4; nomenclature according to Kandel (14)] to the ARCs attached (Fig. 1*A*). The preparation was placed in a two-chambered dish lined with sylgard. The muscles were put in a separate bath from the buccal ganglia, and the baths were isolated by an intervening partition formed with vaseline. The volume of the bath containing the buccal ganglia was 1 ml. Extracellular electrodes were used to record EJPs from the ARC and to stimulate the axons in nerve B4. EJPs followed B16 action potentials (Fig. 1*A*, *Inset*) at a fixed latency with a mean ± SD of 21 ± 3 msec (n = 55).

Solutions. The normal saline solution had the following composition: 490 mM NaCl/11 mM KCl/19 mM $MgCl_2$/30 mM $MgSO_4$/11 mM $CaCl_2$/10 mM Tris·HCl, pH 7.6. The saline used to block Ca^{2+} currents contained 10 mM $CoCl_2$ with no added $CaCl_2$. In reduced sodium saline solutions, sodium was replaced with equimolar amounts of either Trizma-7.8 (pH 7.8) (Sigma) or *N*-methyl-D-glucamine (pH adjusted to 7.6 with concentrated HCl) (Sigma). For saline solutions low in Cl^-, the NaCl was replaced with 490 mM sodium acetate (final Cl^- concentration, 51 mM). When used,

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Abbreviations: ELH, egg-laying hormone; ARC, accessory radula closer muscle; EJP, excitatory junctional potential; I_{K^+}, delayed potassium current; I_A, transient potassium current; $I_{K^+(Ca^{2+})}$, calcium-activated potassium current; I_{IN}, voltage-dependent slow inward current.

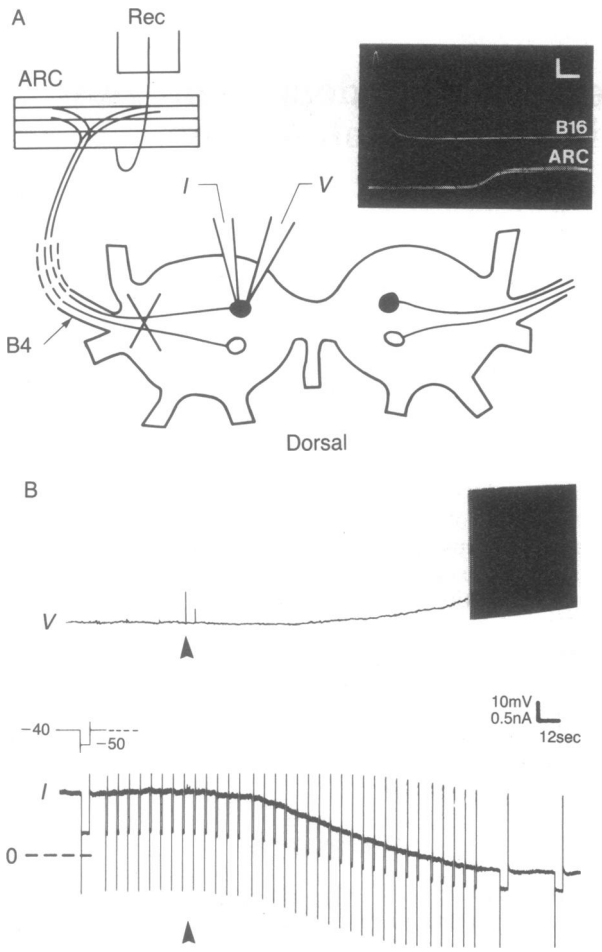

FIG. 1. (*A*) Scheme of the experimental preparation. The ARC motoneurons B16 (●) and B15 (○) and the ARC muscle are illustrated. A hook-in-oil electrode (Rec) was used to record EJPs from the ARC muscle. (*Inset*) B16 action potentials and corresponding ARC EJPs (three traces superimposed). B16 was distinguished from B15 by the relative positions of their somata and properties of their EJPs (12, 13). ×, site of axotomy; *V*, voltage recording electrode; *I*, current passing electrode. (*B*) ELH induces a slow depolarization that leads to repetitive firing recorded in current clamp (*V*) and induces a slow inward shift in holding current recorded in voltage clamp (*I*). These recordings were taken from different preparations, both bathed in saline with $CaCl_2$ replaced with 10 mM $CoCl_2$. ELH was bath-applied (arrowheads) and remained in the bath for the duration of the recordings. Repeated 10-mV hyperpolarizing steps (1-sec duration) were applied in voltage clamp from a holding potential of −40 mV, and the resulting step current was monitored. Note at the beginning and end of the current record (*I*) that the chart speed was increased 5 times to show the detail of the step current. In this and subsequent figures, 0--- next to the current trace indicates zero current level.

tetraethylammonium chloride (100 mM), 4-aminopyridine (4 mM), CsCl (10 mM), and tetrodotoxin (60 μM) (all from Sigma) were added hypertonically to the saline. When changing solutions, the equivalent of at least 10 bath vol was perfused across the preparation before measurements were made.

Voltage Clamp. We found that proper space clamp was not achieved (indicated by the occurrence of spike-like currents generated in unclamped regions of the axon) unless the axon of the motoneuron was removed. Therefore, most of the results presented here are taken from preparations in which B16 was surgically axotomized. Axotomy was achieved by making a cut with iridectomy scissors or by applying a suture with a single strand of dental floss near where nerve B4 left the ganglion (≈300 μm from the B16 soma). Before axotomy, the means (±SD) for resting membrane potential, peak-to-peak spike height, and spike duration measured at one-half amplitude were −45 ± 4 mV (n = 27), 75 ± 8 mV (n = 21), and 2.8 ± 0.6 msec (n = 18), respectively. The means (±SD) for cells that had been axotomized were −44 ± 5 mV (n = 21), 71 ± 11 mV (n = 18), and 3.2 ± 0.9 msec (n = 17), respectively. Cells were used only if they had a resting membrane potential of at least −35 mV after axotomy.

After B16 was axotomized, it was voltage-clamped with two microelectrodes by using a voltage clamp similar to that described by Barish and Thompson (15). Membrane potential was recorded differentially between the voltage-sensing microelectrode filled with 3 M KCl (3–8 MΩ) and a saline–agar bridge placed in the bath. The current-passing electrode was filled with 3 M potassium acetate (5–10 MΩ), and current was monitored with a virtual ground circuit. The signals from the virtual ground were first low-pass filtered by using an 8-pole Bessel filter (Freq. Dev. Inc.) (cutoff frequencies were 10–100 Hz for measurements of currents during 1-sec voltage pulses and 1 KHz for all other current measurements). The currents obtained during voltage pulses of long duration (e.g., 1 sec) were plotted with a Brush pen recorder (Gould). Transient potassium current (I_A) and delayed potassium current (I_{K+}) recordings were digitized at 1 KHz and stored on diskettes.

ELH Purification. Large (0.5–2 kg) *A. californica* were collected at Moss Landing, CA. Bag cell clusters from these animals were used to purify ELH according to the procedures of Chiu *et al.* (16). The ELH was tested for purity using HPLC and electrophoresis in acrylamide gel containing $NaDodSO_4$ and urea. ELH was dialyzed into the appropriate saline solutions and stored at −70°C until used. Aliquots (5–25 μl) of these ELH stock solutions (20 μM) were brought to room temperature and were applied with final concentrations in the bath between 100 and 500 nM.

RESULTS

ELH Induces an Inward Shift in Holding Current. When applied to the bath, ELH acted on the motoneuron B16 and produced a slow depolarization that led to repetitive firing (Fig. 1*B*, V) with a latency to the first spike of ≈3 min (11, 13). When B16 was voltage-clamped, bath application of ELH produced a slow inward shift in the holding current (Fig. 1*B*, I) with a mean peak amplitude (±SD) of 1.9 ± 1.0 nA (n = 21 observations) and with a mean latency (±SD) of 98 ± 90 sec (n = 28). The response to ELH was not due to indirect synaptic effects because the effects occurred in saline containing 10 mM Co^{2+} substituting for Ca^{2+}. All synaptic transmission was blocked in this solution.

The Inward Current Does Not Result From Effects on I_A, I_{K+}, or Calcium-Activated Potassium Current [$I_{K+(Ca^{2+})}$]. The ELH-induced inward current could result from a decrease in potassium conductance at rest (17). Both I_A and I_{K+} were monitored during the response to ELH, but the amplitudes and time courses of these currents were essentially unchanged (data not shown). We also found that blocking Ca^{2+} currents [and thus $I_{K+(Ca^{2+})}$] had little effect on the response to ELH. In fact, the effects of ELH on B16 appeared to be enhanced in saline with 10 mM Co^{2+} replacing Ca^{2+}, even though this saline composition eliminated $I_{K+(Ca^{2+})}$ (data not shown).

ELH Induces or Enhances a Voltage-Dependent Slow Inward Current (I_{IN}). Repeated hyperpolarizing steps (10 mV, 1 sec) from a holding potential of −40 mV were applied under voltage clamp to monitor the input resistance of B16 during bath application of ELH. As shown in Figs. 1*B* and 2 before ELH was applied, the response to a hyperpolarizing voltage step was an inward current step (step current, see arrowhead in Fig. 2). During the development of the ELH effect in Fig.

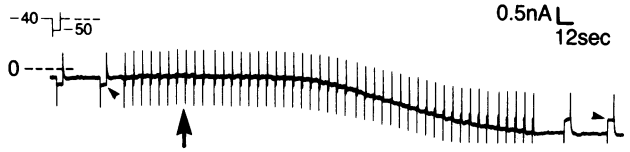

FIG. 2. The inward shift in holding current in response to ELH is associated with the induction of a negative slope resistance. Repeated 10-mV hyperpolarizing steps (1-sec duration) were given before and during the response to ELH. ELH was bath-applied (arrow). The step current changed from inward prior to the application of ELH to outward during the ELH effect (arrowheads), indicating the induction of a negative slope resistance at this holding potential (−40 mV). $CoCl_2$ (10 mM) replaced $CaCl_2$ in the bathing solution. The chart speed was increased 5 times at the beginning and end of the trace.

1*B*, the step current decreased in amplitude, suggesting a decrease in conductance. That is, less inward current was generated by the 10-mV hyperpolarizing steps. However, in 14 out of 19 cases tested, the step current not only became less inward during the response to ELH, it actually reversed direction and became outward with respect to the new holding current level (Fig. 2). The outwardly directed step current in response to a hyperpolarizing voltage step indicated that a negative slope resistance had appeared at this membrane potential. These results suggested that ELH induced a voltage-dependent slow inward current (I_{IN}) that deactivated during the hyperpolarizing voltage steps; this resulted in the decrease in step-current amplitude observed in Fig. 1*B* as well as the negative slope resistance seen as the outwardly directed step currents illustrated in Fig. 2. The differences in the observed changes in step current in the two preparations from which Figs. 1*B* and 2 were taken appeared to be due to the presence of some slow inward current at rest (before the application of ELH) in the preparation from which Fig. 2 was taken (see below).

The induction of a negative slope resistance region by ELH was confirmed by constructing current–voltage (*I–V*) curves (using 1-sec-long voltage pulses) before, during, and after the response to ELH (Fig. 3 *A* and *B*). ELH produced an inward shift in the *I–V* relationship throughout the range from −80 to −10 mV with a very pronounced increase in inward current at voltages between −40 and −20 mV where a negative slope resistance was seen (Fig. 3*B*). The negative slope resistance region of the *I–V* curve was due to a noninactivating I_{IN}, which was especially prominent within this voltage range (Fig. 3*A*). In other preparations (including the one from which Fig. 2 was taken), I_{IN} was present with a lesser amplitude before the application of ELH and was dramatically enhanced by the peptide throughout the activating voltage range. The enhancement or induction of the slow inward current and the negative slope resistance region in the *I–V* curves were apparent in all preparations, including those with step currents that failed to reverse direction during the response to ELH (e.g., see Fig. 1*B*).

Endogenous pacemakers and bursting neurons invariably possess a slow inward current and a negative slope resistance region in the *I–V* relationship measured with 1-sec pulses (18). Likewise, in current clamp mode, B16 was a conditional burster (19); the motoneuron occasionally produced a series of endogenously generated bursts (pacemaker potentials followed by clustered firing without synaptic input) upon impalement, and endogenous bursting was frequently observed as the ELH effects subsided (data not shown).

The reversal potential for the ELH effect was determined from difference *I–V* curves. These curves were constructed by subtracting the *I–V* curves in control conditions from the *I–V* curves obtained during the ELH response. The reversal potential is the point where the difference *I–V* curve crosses

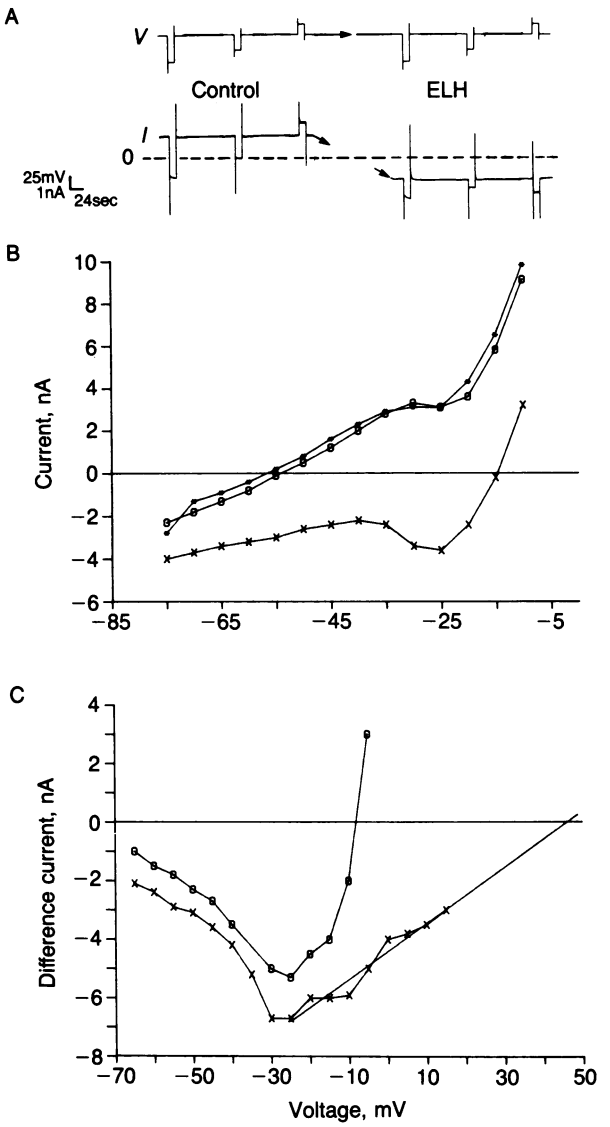

FIG. 3. ELH induces a slow inward current and alters the *I–V* relationship, and the effect has a depolarized reversal potential. (*A*) Selected records illustrating how the *I–V* curves were obtained (using voltage pulses 1 sec in duration) and the appearance of the slow inward current with depolarizing pulses to −30 mV. Control records were taken before and ELH records were taken ≈5 min after the application of ELH. $CoCl_2$ (10 mM) replaced $CaCl_2$ in the bath, and the holding potential was −40 mV. (*B*) *I–V* curves were obtained before (control; ○) and during (X) the ELH effects and after the ELH effects had subsided (*). Note that the currents during the ELH response throughout the range measured were more inward compared to those before or after the ELH response. The maximal ELH effects occur in the region of the *I–V* relationship where a slow inward current is activated (peak effect at −30 to −25 mV). (*C*) Difference *I–V* curves reveal reversal potentials for the ELH effects in two different preparations. In one case (○), the difference *I–V* curve shows a real reversal potential at −12 mV. In the other preparation (X; same as in *B*), an estimate of the reversal potential (+46 mV) was obtained via extrapolation with a linear regression using the data points from −25 to +15 mV (correlation coefficient, 0.98).

zero current, as shown for two cells in Fig. 3*C*. In nine experiments, the difference *I–V* curves actually crossed zero, and the mean (±SD) reversal potential obtained was −14 ± 8 mV. However, in eight other preparations, the reversal potentials had to be estimated by extrapolating the difference *I–V* curves to the point of intersection with zero current. The mean (±SD) reversal potential obtained from these cases

was +9 ± 18 mV (Fig. 3C). Our reversal potential measurements may vary because the response to ELH is due to a change in more than one current. However, the reversal potentials suggested that the effect of ELH was to increase the membrane conductance to an ion with a relatively depolarized equilibrium potential (e.g., Na^+ or Ca^{2+}).

The ELH Effects Are Sodium Dependent. The response to ELH was not reduced in saline solutions in which Ca^{2+} currents were blocked by substituting Co^{2+} for Ca^{2+} (see above). Therefore, we investigated saline solutions that were low in sodium. When the preparation was perfused with solutions in which all the sodium was replaced with equimolar Tris or *N*-methyl-D-glucamine, the mean (±SD) holding current shifted outward by 1.2 ± 1.0 nA (n = 17). A typical example of the effects of ELH in saline with sodium replaced with *N*-methyl-D-glucamine is presented in Fig. 4. In Fig. 4*A*, ELH was applied at the point indicated by the arrow. There was no apparent change in input resistance as monitored with the step currents, although there was a slight outward drift in holding current. *I–V* curves, constructed with voltage pulses 1 sec in duration, revealed that, in the absence of sodium, the slow inward current and the negative slope resistance region were eliminated as was the inward current response to ELH (Fig. 4*B*). The *I–V* curves constructed before and after the application of ELH differed only at depolarized voltages where a slight increase in outward currents was apparent (Fig. 4*B*). This may represent outward sodium movement through the I_{IN} channels; however, since no reversal potentials were obtained in these cases, this point remains open. In four cases, the response to ELH was restored to near normal levels when the preparation was subsequently perfused with saline having a normal sodium concentration (Fig. 4*C*). In one experiment in which some sodium (50 mM) remained in the saline, a reduced response to ELH was observed.

One possible reason for the relatively low reversal potentials obtained for the ELH responses is that the I_{IN} channels are permeable to both sodium and potassium ions, in which case the reversal potentials would be less depolarized than the +60 mV expected for a pure sodium conductance. However, we did not see a substantial depolarizing shift in the reversal potential when extracellular potassium concentration was increased (see below). The reversal potentials for the ELH responses were similar to those observed by Gerschenfeld and Paupardin-Tritsch (20) for 5-hydroxytryptamine (serotonin)-induced currents carried by Na^+ (0 mV).

Further evidence that the response to ELH was sodium dependent was obtained from a number of additional ion substitution experiments and from the use of channel blockers. Specifically, the response to ELH was not affected by the following manipulations: (*i*) reducing external Cl^- from 541 mM to 51 mM by replacing NaCl with sodium acetate (n = 3); (*ii*) removing external K^+ with no replacement (n = 1); (*iii*) increasing external K^+ hypertonically to 22, 50, or 100 mM (n = 4); (*iv*) application of 100 mM triethylammonium chloride, 10 mM CsCl, and 4 mM 4-aminopyridine (n = 2); (*v*) application of 60 μM tetrodotoxin (n = 2). Tetrodotoxin frequently fails to block Na^+-dependent slow inward currents (21).

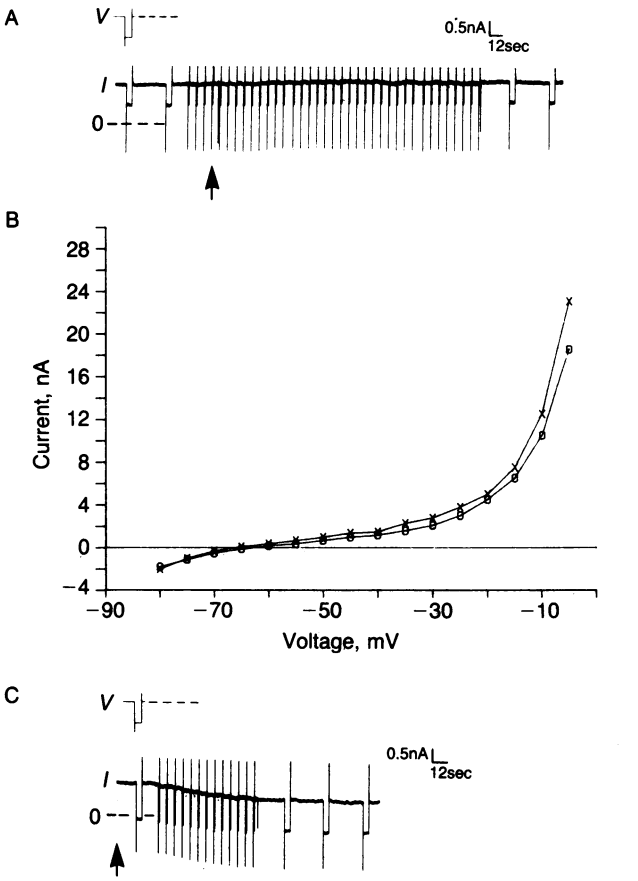

FIG. 4. The ELH effects are sodium dependent (*A–C* taken from the same preparation). (*A*) The slow inward shift in holding current is eliminated in saline with no sodium. Sodium was replaced by substituting 490 mM *N*-methyl-D-glucamine for NaCl, and 25 μl of ELH stock solution (no Na^+, no Ca^{2+}) was applied (arrow). The holding current remained constant and then drifted slightly outward. The step currents (from 10-mV hyperpolarizing voltage pulses, 1-sec duration) maintained a constant amplitude, indicating no significant change in input resistance and no induction of a negative slope resistance at this holding potential (−40 mV). (*B*) The inward shift in current and increased negative slope resistance region of the *I–V* curves that normally occur in response to ELH are eliminated when sodium is replaced with an impermeant ion. The *I–V* curve constructed before the ELH was applied (Control; ○) showed no negative slope resistance region. The *I–V* curve obtained in the presence of ELH (X) showed only a slight increase in outward currents at depolarized potentials. No negative slope resistance region was induced by ELH. (*C*) The response to ELH partially recovers when the preparation is perfused with saline having a normal Na^+ concentration. ELH (15 μl) was applied (arrow). An additional 10 μl of ELH had been applied to the preparation 2 min prior to this application. This resulted in the same final concentration as in *A* (500 nM). Note the inward shift in holding current and the decrease in step current during the response to ELH. The chart speed was increased 5 times at the beginning and end of the traces in *A* and *C*.

DISCUSSION

We have found that the response of B16 to the neuropeptide ELH is due at least in part to the induction or enhancement of a voltage-dependent slow inward current (I_{IN}) carried by sodium ions. This slow inward current is responsible for the negative slope resistance region found in the *I–V* relationship. I_{IN} exhibits a small amount of activation at the resting membrane potential; therefore, as it increases in response to ELH, the B16 membrane slowly depolarizes until it reaches threshold for repetitive firing. Although it appears that the ELH effects on B16 are due to increased sodium currents, we cannot exclude a possible contribution of other currents that have not been tested, including the serotonin-sensitive potassium current (17, 22).

During the response to ELH, the increased inward currents found in the *I–V* curves at hyperpolarized levels (−80 to −60 mV) could be due to a significant activation of I_{IN} at these voltages. Alternatively, there may be another sodium-dependent current (e.g., an inward leakage current) that increases its conductance in response to ELH.

Other Neuronal Effects That Resemble the Response to ELH. Arginine vasopressin appears to have effects on cell 11 of *Otala* and R15 in *Aplysia* that are very similar to the effects of ELH on B16 (23). These effects include increased inward current in *I–V* curves found throughout the range of voltages tested (23). Cyclic AMP-stimulated voltage-dependent inward currents carried by Na^+ have been observed in other molluscan neurons (24, 25).

The voltage-dependent response to serotonin observed in RB, LB, and LC neurons in the abdominal ganglion of *Aplysia* resembles the response of B16 to ELH (with the exception that the serotonin-induced current is carried by Ca^{2+}) (26). Interestingly, we found that B16 also responds to serotonin in a manner very similar to that of ELH ($n = 5$).

ELH as a Peptide Neurotransmitter. ELH released during a bag cell discharge (or perfused through the ganglionic artery) acts directly on several identified neurons in the abdominal ganglion (5). The effects include prolonged excitation of LB and LC cells (which resembles the B16 response to ELH) and burst augmentation in R15. The ELH effects are selective; the other neuronal effects produced following a bag cell discharge (e.g., inhibition of left upper quadrant neurons) are due to the release of other peptides encoded by the ELH gene (5, 27). Additional results suggest that the rate of ELH degradation following release is low (5). It appears, therefore, that inactivation of ELH may occur very slowly, and the concentration of ELH in the hemolymph may reach titers sufficient to produce an effect on B16 as well as induce egg release from the ovotestis. However, there is ELH immunoreactivity in other parts of the central nervous system (28), including the buccal ganglia (29), and ELH-encoding mRNA from buccal ganglia has recently been characterized (30). Therefore, ELH could be released by neurons that are directly apposed to B16. The long latency, long duration, and lack of desensitization exhibited by the ELH effects on B16 suggest that a second messenger mechanism is involved. Agents that increase intracellular cAMP (e.g., 8-bromo-cAMP and isobutylmethylxanthine) depolarize B16 and have effects similar to those of ELH (unpublished observations). It is possible that ELH acts through the cAMP second messenger system, and it will be interesting to determine whether the same second messenger pathway is utilized in the response of B16 to serotonin.

We thank Drs. Tom Abrams, Rick Aldrich, Cynthia Bishop, Corey Goodman, John Kuwada, and Stuart Thompson for critically reading an early draft of the manuscript. ELH was purified with the help of Dr. Rashad-Rudolf Kaldany. Wayne Sossin provided expert computer assistance and useful discussions. We thank Dr. Jeff Ram for sharing his unpublished results with us. Special thanks to Dr. Stuart Thompson for helping in the construction of the voltage clamp and useful discussions. This work was supported by a National Institutes of Health grant (R.H.S.). R.H.S. is a McKnight Foundation Scholar and a Klingenstein Fellow in the Neurosciences.

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