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The Neuropeptide Egg-Laying Hormone Modulates Multiple Ionic Currents in Single Target Neurons of the Abdominal Ganglion of *Aplysia*

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The bag cell neurons of the abdominal ganglion of *Aplysia* are a useful system for the study of peptidergic neurotransmission. A 20 min burst of impulse activity in the bag cells induces or augments repetitive firing in LB and LC neurons in the abdominal ganglion for up to several hours. Previous experiments have indicated that this effect is mediated by the putative bag cell transmitter egg-laying hormone (ELH). Using voltage-clamp analysis we found that bag cell bursts (BCBs) evoke long-lasting changes in membrane current in these neurons that are mimicked by the application of ELH. The combined ELH-evoked current is inward at all membrane potentials between −110 and −10 mV and consists of 3 separable currents persisting for 30–120 min. They include (1) a depolarizing current that is activated at membrane potentials above −40 mV. This current, termed I_{SI}, is blocked by prolonged exposure to 10 mM Ni^{2+}/0 mM Ca^{2+} and is not abolished by 0 mM Na^{+} or 100 mM TEA^{+}/0 mM Na^{+} in the bathing medium. It is therefore a Ca^{2+}-sensitive current and does not involve Na^{+} as a charge carrier. (2) There is a hyperpolarizing current that is activated at membrane potentials below approximately −70 mV. This current, termed I_{R}, is blocked by external Rb^{+} (5 mM) and Cs^{+} (10 mM) and has a chord-conductance that shifts with the external [K^{+}] according to the Nernst potential for potassium. It is therefore an inwardly rectifying K^{+} current. (3) There is a small, steady depolarizing current, termed I_{X}. This current is the only one that remains after prolonged exposure to 10 mM Ni^{2+}/0 mM Ca^{2+}-containing bathing medium. It is Na^{+} dependent and is associated with a small increase in membrane conductance that is largely independent of membrane voltage. All 3 currents are slow to inactivate; they appear to sum algebraically to produce the net BCB- or ELH-evoked current.

Various neuropeptides have been found in the CNS of vertebrates and invertebrates, and peptides are widely accepted as potential neurotransmitters (Krieger, 1983; Iversen, 1984; Schmitt, 1984; O'Shea and Schaffer, 1985). However, relatively little is known about their exact physiological roles or their cellular mechanisms of action. From studies in identified peptidergic systems, where the effects of the application of peptide can be compared with the neurally evoked response, it is clear that the actions of some of these peptides are distinct from those of "classical" neurotransmitters in that peptides can modulate electrical signaling for seconds, minutes, or longer (Branton et al., 1978; Jan and Jan, 1982; Konishi and Otshuka, 1985; Sigvardt et al., 1986). We studied the long-lasting changes in ionic currents that are caused by the release of the neuropeptide egg-laying hormone (ELH) from the bag cell neurons of the marine mollusc *Aplysia.*

The bag cells are neuroendocrine cells that release 4 neuroactive peptides derived from a common precursor protein (Stuart and Strumwasser, 1980; Scheller et al., 1983; Rothman et al., 1985; Brown and Mayeri, 1986; Sigvardt et al., 1986). These bag cell peptides are released during a 20–30 min period of repetitive, high-frequency impulse activity. *In vivo* such bag cell bursts (BCBs) invariably precede a stereotyped behavior called egg-laying (Pinsker and Dudek, 1977). One of the released peptides, ELH, is a 36 amino acid peptide that fulfills almost all the strict criteria for transmitter identification (Branton and Mayeri, 1978; Branton et al., 1978; Mayeri et al., 1979a, b; Stuart and Strumwasser, 1980; Mayeri and Rothman, 1982; Mayeri et al., 1985). ELH is a wide-acting neuropeptide that acts directly on the ovotestis (Rothman et al., 1983b) and on different parts of the CNS (Branton and Mayeri, 1978; Branton et al., 1978; Stuart and Strumwasser, 1980) at the initiation of the egg-laying behavior (Cobbs and Pinsker, 1982a, b; Ferguson et al., 1986).

The bag cells are organized in 2 clusters of about 400 neurons each, located at the rostral margin of the abdominal ganglion. Previous investigations indicate that ELH is released from the bag cells' axons into interstitial and vascular spaces of the abdominal ganglion, where the peptide diffuses a relatively long way (up to tens of microns) to LB, LC, and R_{15} target neurons and induces or augments repetitive spike activity for up to several hours (Branton and Mayeri, 1978; Branton et al., 1978; Mayeri et al., 1979a, b; Mayeri and Rothman, 1982; Mayeri et al., 1985). Application of ELH to buccal neuron B_{16} of the buccal ganglion also induces repetitive firing (Stuart and Strumwasser, 1980; Ram, 1983; Kirk and Scheller, 1987).

To further identify ELH as neurotransmitter in the abdominal ganglion, we studied the ionic mechanisms that underlie responses to neurally evoked release and to application of ELH to LB or LC target neurons in the abdominal ganglion. These neurons include siphon, kidney pericardial and vasoconstrictor motorneurons (Koester and Kandel, 1977), and they have been established as target neurons for ELH (Mayeri et al., 1979b). We found that the ionic mechanism of the neurally evoked

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response is apparently identical to that of ELH action and that the response involves at least 3 different currents. These currents differ from the current modulated by ELH in the buccal neuron B_{16} (Kirk and Scheller, 1986). Parts of this study have been published in abstract form (Jansen and Mayeri, 1986, 1987).

Materials and Methods

Animals and dissection. Abdominal ganglion were dissected from sexually mature *Aplysia californica* weighing 200–400 gm, obtained from Sea Life Supply (Sand City, CA). The connective tissue sheath overlying the left hemiganglion was removed, and cells on the dorsal surface of the left lower (caudal) quadrant, which includes the LB and LC neurons, were axotomized by cutting the commissure and the genital–pericardial nerve with a razorblade scalpel. In some preparations an additional cut between the left upper and left lower quadrant of the abdominal ganglion was made. The isolated cluster of cells corresponded to the caudal- and medial-most group, which were all usually responsive to ELH and bag cell discharges. The preparation was kept at room temperature (20–22°C); the animals were kept at 19°C.

Bag cell stimulation. BCB discharges were triggered by brief extracellular electrical stimulation via an electrode placed on the cell bodies of the left bag cell cluster (Mayeri et al., 1979a). A 1–2 sec train of 5 msec pulses was applied at a rate of 5/sec through a constant-current device. Bag cell spike activity was monitored by switching the stimulating electrodes on the bag cell cluster to an A/C amplifier. In some experiments, BCBs were triggered by extracellular stimulation of the pleural–abdominal connective. In those experiments, the pulse duration was 0.5 msec, and bag cell activity was monitored with an extracellular electrode placed on the bag cells. The LB or LC cell was released from voltage clamp for a short period during bag cell stimulation. The long-lasting nature of the responses to BCBs and ELH applications usually do not permit the triggering of more than one BCB or more than one ELH application in a given preparation.

Data collection and analysis. Cells were voltage-clamped with a 2-electrode system (Dagan 8100). Cells were penetrated with one electrode to record the membrane potential and a second electrode to pass current. The electrodes were filled with 2M KCl, and the current electrodes had resistances of 1–2 MΩ. The electrodes were shielded with aluminum foil to within 1–2 mm of the tip. This shielding method results in capacitive transients that typically last less than 1–2 msec. The membrane potential was measured differentially with respect to a second microelectrode positioned close to the ganglion. The drift in the voltage recording system was typically less than 1 mV/d. The preamplifier had a high-frequency cutoff of 30 kHz. The membrane current was measured with a virtual ground circuit that was connected to the recording chamber through an Ag-AgCl half-cell. No compensation for series resistance was made. In the ramp mode, the monitored current was filtered with a low-pass filter with a high-frequency cutoff of 13 Hz, except for the data shown in Figure 3*C*, which was filtered at 30 Hz. In the square-wave mode, the current monitor filter had a high-frequency cutoff of either 3 kHz or 300 Hz. The data were digitized with a 12-bit analog-to-digital converter (Labmaster, Scientific Solutions) using a sampling interval of 55 msec (ramp mode) or 1.0 msec (square-wave mode). Capacitive transients were measured using sampling intervals of 0.04 msec. Data acquisition and analysis were done using Pclamp software (Axon Instruments, Burlingame, CA) and an IBM PC-AT computer.

Depolarizing ramp command voltages between −110 and −10 mV were applied every 2.5 min (Fig. 1). The rate of change of the membrane potential was 6.6 mV/sec. Stability during the ramp experiments was monitored by plotting superimposed current sweeps on a Hewlett-Packard 7470A *X-Y* plotter. For analysis, multiple successive *I-V* relations obtained immediately before application of peptide or triggering of a bag cell burst (indicated as "baseline") were averaged. Evoked currents were then obtained by subtraction of this averaged baseline from *I-V* relations generated after a BCB was triggered or peptide was applied. Families of square-wave voltage commands were similarly applied at regular intervals, usually 2.5 min. Averaging and subtraction methods were the same as for ramp experiments.

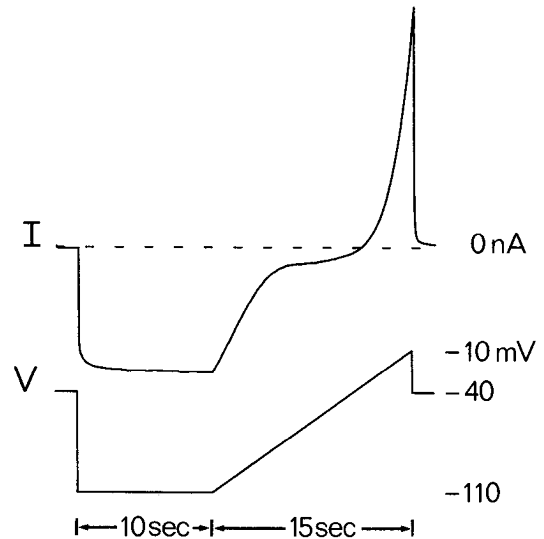

Figure 1. The ramp command potential used in most experiments and the current produced in a LB or LC neuron. The membrane potential was stepped from a holding potential of −40 or −50 mV to −110 mV, where the cell was held for 10 sec. The ramp command had a duration of 15 sec and depolarized the membrane with a rate of 6.6 mV/sec to −10 mV. At the end of the ramp, the membrane potential was stepped back to the holding potential. Note the marked inward rectification of the current below about −70 mV.

Solutions. ELH was purified from homogenized bag cell clusters by HPLC (Rothman et al., 1985). A polyethylene pipette (diameter ~ 100 μm) was used to apply 10 μl of ELH (concentration in pipette, 100 μM) to the abdominal commissure area exposed by the axotomy. Prior to the actual experiment, the recording chamber (~1 ml) was perfused steadily with artificial seawater (ASW, Instant Ocean), and perfusion was stopped when the experiment was started. In control experiments, stopping the perfusion or applications of ASW never produced the long-lasting effects of BCBs and ELH. The compositions of the bathing media are shown in Table 1.

Results

BCBs and ELH cause apparently identical changes in membrane conductance

The bag cell neurons are normally silent. When a brief electrical stimulus is applied to 1 of the 2 bag cell clusters, the bag cells

Table 1. Composition of bathing media

Solution	KCl	$MgCl_2$	$CaCl_2$	Tris	TMACl	HEPES	TEACl	$CoCl_2$	NaCl	RbCl	Na_2SO_4
ASW	10	53	9	—	—	—	—	—	420	—	27
10 Co/0 Ca	10	50	—	—	—	—	—	10	495	—	—
Na-free (Tris)	10	50	10	375	—	—	100	—	—	5	—
Na-free (TMA)	10	50	10	—	495	10	—	—	—	—	—
0 Na	10	50	10	495	—	—	—	—	—	—	—

All solutions have pH 7.6 (adjusted with HCl); all concentrations are in mM. Single additions of rubidium (5), cesium (10), cobalt (10), nickel (10), or potassium (20) were added hyperosmotically to normal ASW. The 50% Na^+-containing medium was obtained by making a mixture (50:50) of 0 Na medium and normal ASW.

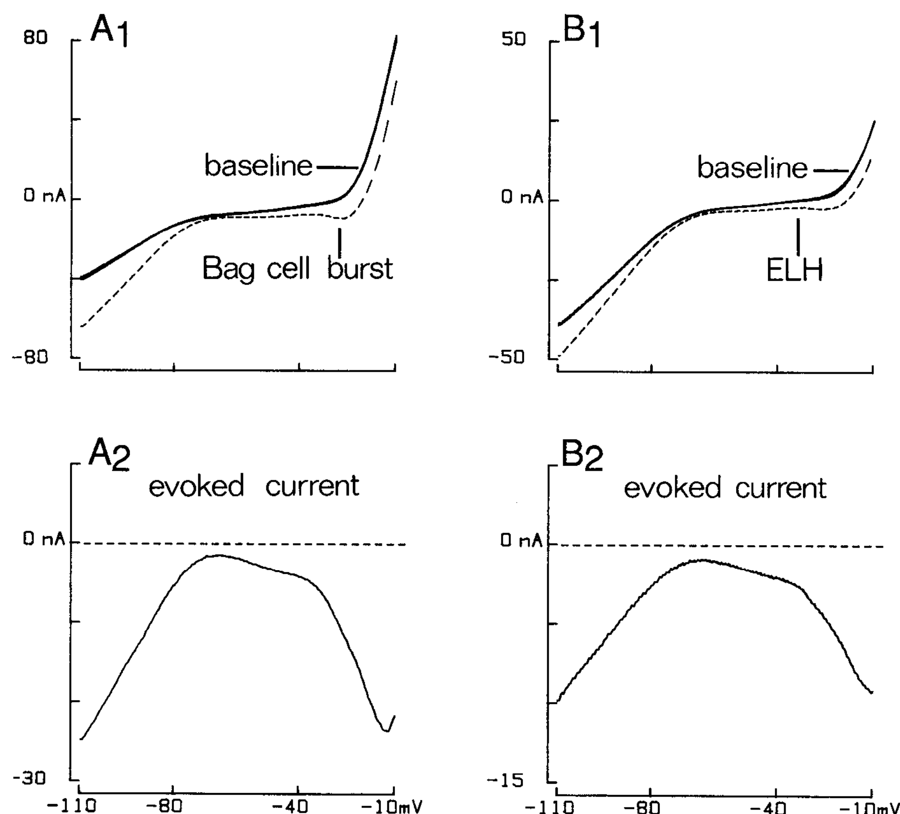

Figure 2. Voltage-clamp records show that currents evoked by a BCB discharge in an LB/LC target neuron are apparently identical to those evoked by ELH. *I-V* relations were recorded by applying slow depolarizing ramp command potentials every 2.5 min before and after electrical triggering of a BCB or application of ELH. In both A_1 and B_1, the baseline (*solid line*) is composed of 5 consecutive, superimposed *I-V* relations to show baseline stability. A_1, After triggering of a BCB (see Materials and Methods), the membrane current changed slowly over the course of 20 min. The *dotted line* represents the current 25 min after the BCB was triggered. A_2, The BCB-evoked current was calculated by subtraction of the *I-V* curve generated after a BCB was triggered from an average of the baseline *I-V* relations. Note the different scale in the evoked current plots. B_1, In a different preparation, 10 μL ELH was applied to the abdominal commissure area with a polyethylene pipette (concentration in the pipette 100 μM). B_2, The ELH-evoked current was calculated as in A_2.

discharge in near unison for 20–30 min, releasing ELH and other peptides derived from the ELH/BCP precursor protein (see Mayeri et al., 1985). Such a bag cell burst, or applied ELH, produces long-lasting repetitive spiking in LB and LC neurons, which are normally silent or fire irregularly at low frequencies (Branton et al., 1978, Mayeri et al., 1979b). We found that BCBs and applied ELH induce persistent (apparently noninactivating) currents in these neurons, which develop over the course of 5–20 min and last for 30 min up to 2 hr.

It was important to verify that the baseline *I-V* relations recorded before a BCB or application of ELH were stable, so that even small changes in long-lasting current induced by a BCB or application of ELH could be detected. Each preparation was therefore given ample time (typically 1–2 hr) to stabilize before the baseline data were taken. To measure the long-lasting currents, *I-V* relations were generated using a depolarizing ramp command voltage. This ramp command voltage was applied every 2.5 min before and after application of ELH or stimulation of a BCB (Fig. 1). The baseline stability typically obtained is shown in Figure 2, A_1, B_1, in which 5 successive baseline *I-V* relations are superimposed. These baseline *I-V* relations, obtained immediately before application of peptide or triggering of a BCB, were averaged. The net current evoked by the peptide or bag cell activity was then calculated by subtraction of the averaged baseline *I-V* relation from *I-V* relations generated after application of peptide or after bag cell activity.

The net currents evoked by BCBs ($n = 6$) or ELH ($n = 6$) were inward in 10 of 12 experiments over the entire voltage range tested (Fig. 2, *A*, *B*). In the other 2 experiments (one BCB, one ELH-application), a small (<0.5 nA) outward current could be observed between about −75 and −55 mV (data not shown).

The currents evoked by BCBs and ELH were apparently identical in their voltage dependence. This provides additional evidence that ELH is a neurotransmitter in the abdominal ganglion.

The ELH-evoked current was found to be composed of three separable slow currents. The inflections in the evoked current curve at −70 and −40 mV (Fig. 2, A_2 and B_2) indicated that the evoked current has at least 2 components with a steep voltage dependence. One component activates at membrane potentials below −70 mV and has an apparently linear voltage dependence; it was subsequently identified as an inwardly rectifying potassium current, and termed I_R. A second component activates at membrane potentials higher than about −40 mV, and was termed I_{SI} (for slow inward). Additionally, we isolated a third, voltage-independent inward current, termed I_X. As will be shown below, all 3 currents are slowly or noninactivating; they appear to sum algebraically to produce the ELH-evoked current shown in Figure 2.

Currents evoked by ELH are slowly or noninactivating

The ramp command voltage used provides no information about the activation and inactivation kinetics of the ELH-modulated currents. In particular, transient currents are likely to be inactivating continuously during the ramp and will therefore not contribute significantly to the ramp-generated membrane current. The time dependence of the ELH-evoked currents was therefore investigated with conventional square-wave command voltage steps of 250 msec duration between −110 and −10 mV (Fig. 3, $n = 3$). Figure 3*A* shows the currents before and after application of ELH. The net ELH-evoked currents shown in Figure 3*B* were calculated by subtraction. As with the ramp command potential, the ELH-evoked current was inward

over the entire voltage range tested and caused a small inward shift of the holding current, which is apparent in the evoked current of Figure 3*B* at the far left of each trace.

The currents evoked by ELH at membrane potentials below −50 mV activated rapidly and showed no sign of inactivation during the 250 msec pulse. The currents evoked at −30 and −10 mV, however, activated slowly, in about 75 msec, and showed no inactivation for the duration of the pulse. With pulses >0 mV and >250 msec (data not shown), slow inactivation of the ELH-evoked current could be seen. This inactivation process had a time constant of about 250 msec. Currents above −10 mV were not, however, analyzed further in this study. These data indicate that the 2 voltage-dependent currents (activated below −70 mV and above −40 mV, respectively) have different activation kinetics.

To compare currents generated with square-wave pulses with those generated with the ramp command potential used in most of the experiments, a series of ramps and a series of square-wave command pulses were applied alternately before and after an ELH application. The currents measured at the end of each 250 msec pulse were plotted together with the evoked current measured with the ramp command potential in Figure 3*C*. The currents obtained by the two methods compare well over the entire voltage range, indicating that using the ramp command voltage is an accurate way of measuring the slowly inactivating ELH-evoked currents.

Nickel or cobalt ions block the 2 voltage-dependent currents evoked by ELH and reveal a third, steady inward current

The data in Figures 2 and 3 are consistent with the idea that the ELH-evoked current is composed of at least 2 voltage-gated currents. The following experiments illustrated that there is a third ELH-evoked current, separate from I_R and I_{SI}. To investigate the ionic mechanism of the response to applied ELH, we tried blocking ELH-evoked currents with Ca^{2+}-channel blocking agents. Adding 10 mM Ni^{2+} or 10 mM Co^{2+} to the ASW bathing medium for 15–30 min dramatically changed the normal *I-V* relation observed before the peptide is applied (Fig. 4A_1, dashed line). The outward current was greatly reduced, most likely as a result of indirectly blocking the Ca^{2+}-dependent K^+-current. In addition, a region with negative slope conductance appeared in the *I-V* relations of some, but not all, cells.

With Co^{2+} (or Ni^{2+}) present in the bathing medium (Fig. 4*A*), the voltage-dependent components of the ELH-evoked current seen in normal ASW were substantially reduced. However, they were not blocked completely. There was also a residual evoked current that resulted in a downward shift of the entire *I-V* relation (Fig. 4A_1). This downward shift was also observed in experiments in which ganglia were washed for long periods of time (>1 hr) with a medium in which all the Ca^{2+} was replaced with Ni^{2+} or Co^{2+} (Fig. 4*B*). The voltage-dependent currents were blocked completely by this procedure; the only response that remained was a small inward current whose conductance was independent of membrane voltage. The amplitude of this current, termed I_X, was 2.2 ± 0.7 nA (SEM) measured at −80 mV ($n = 5$). As in Figure 4B_2, the *I-V* relation of I_X usually had a small, positive slope. Although the variability was such that the extrapolated reversal potential of this current could not be estimated accurately from these data, it is likely to be higher than 0 mV. The data thus indicate that the 2 voltage-dependent components of the ELH-evoked current are sensitive to Ni^{2+} and Co^{2+}, albeit when applied for a prolonged period of time, and that I_X is an additional current, separate from the other components.

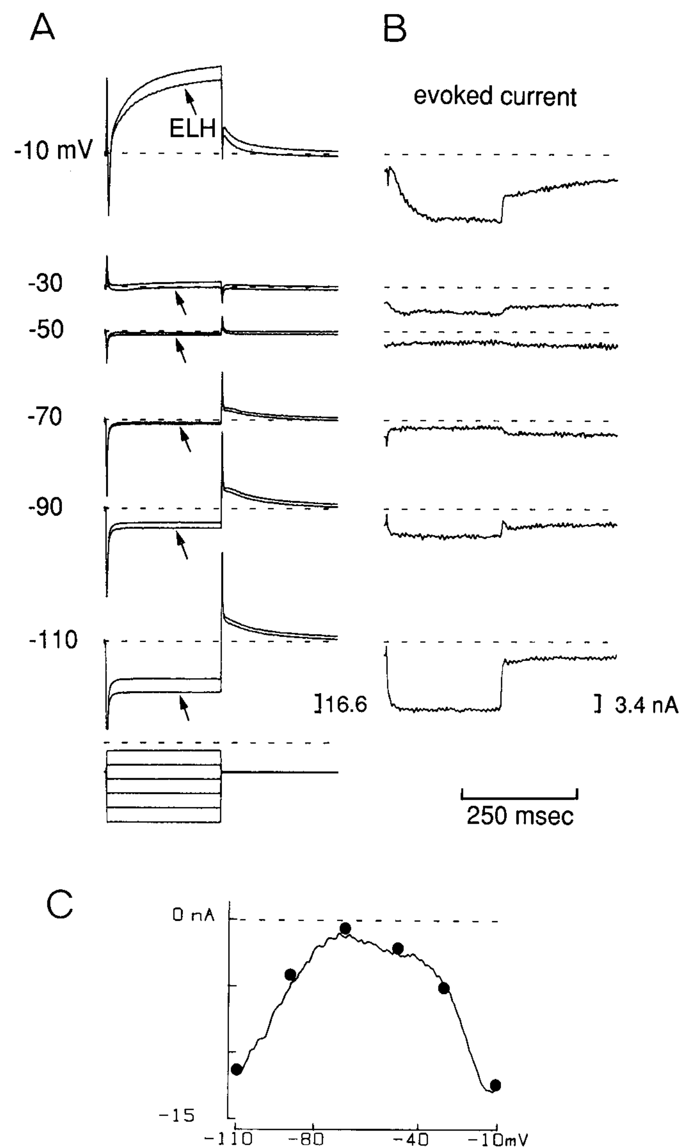

Figure 3. Currents investigated with square-wave voltage steps show the activation and inactivation characteristics of the ELH-evoked currents. *A*, The membrane potential was stepped from a holding potential of −40 mV to the indicated membrane potentials for 250 msec. The *arrows* indicate the current record after application of ELH. Zero current is indicated by the *dashed line* in each set of traces. The superimposed traces at the bottom show the command voltages. *B*, The ELH-evoked currents were calculated by subtraction of the records in *A* (note the different vertical scale). The currents evoked at −10 and −30 mV were activated in about 75 msec. At membrane potentials below −50 mV, the evoked currents activated rapidly. *C*, Comparison of square-wave and ramp-generated currents. The ELH-evoked currents, measured at the end of each square-wave step, were plotted together with ELH-evoked current measured with the ramp command potential in the same cell, during the same ELH application data from the same preparation as *A* and *B*. This shows that using the ramp command potential is an accurate way of measuring slow ELH-evoked currents.

I_{SI} is a slow inward current separate from I_R and I_X and does not depend on the external Na^+ concentration

We next attempted to isolate the current evoked at membrane potentials above −40 mV from the other 2 postulated currents.

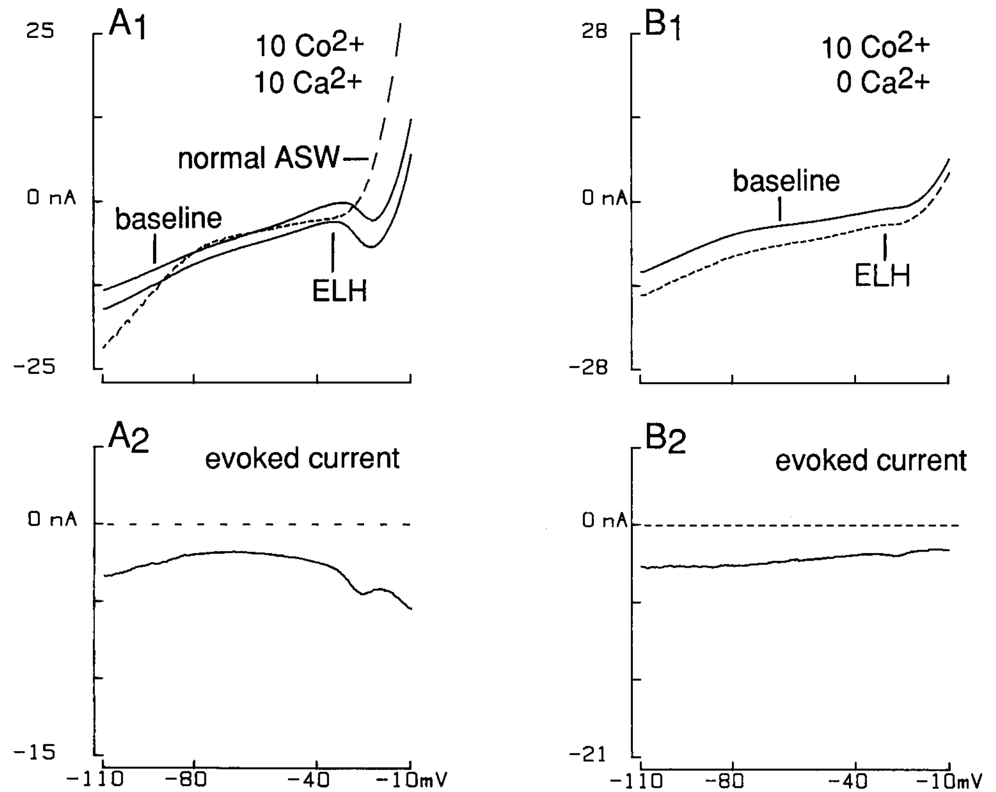

Figure 4. Isolation of a steady, inward ELH-evoked current, I_X, by application of calcium channel blocking agent Co^{2+} to the bathing medium. A_1, The normal baseline *I-V* relation (*dotted line*) was changed dramatically when 10 mM Co^{2+} was added to normal seawater (*upper solid line*). The outward current at more depolarized membrane potentials is reduced, most likely by blocking the Ca^{2+}-dependent K^+ current. Additionally, the inwardly rectifying current, seen below −70 mV, was blocked. After ELH was applied (*lower solid line*), the *I-V* relation shifted downward. A_2, The ELH-evoked current was reduced in size compared with normal ELH responses but was not blocked completely. B_1, I_X could be isolated completely by washing the ganglion for extended periods of time with a medium in which all the Ca^{2+} was replaced with Co^{2+}. The *dashed line* indicates the *I-V* relation after application of ELH. B_2, The voltage-dependent currents that ELH normally evokes were blocked completely, and the only current that remained was a small inward current, I_X, with an apparently voltage-independent conductance.

It is possible that this current is caused by a true inward current carried by Ca^{2+} and/or Na^+, by a decreased outward current, or a combination of these. Since I_{SI} appears to be at least Ca sensitive (Fig. 4), we raised the external Ca^{2+} concentration from 10 to 30 mM. In addition, Na^+ currents were eliminated by replacing all external Na^+ with Tris, 5 mM Rb^+ was added to block the inwardly rectifying K^+ current, and 100 mM TEA^+ was added to block the delayed rectifier and the Ca^{2+}-dependent K^+ currents (Hermann and Gorman, 1981; $n = 4$).

Figure 5A_2 shows that these ion substitutions blocked the

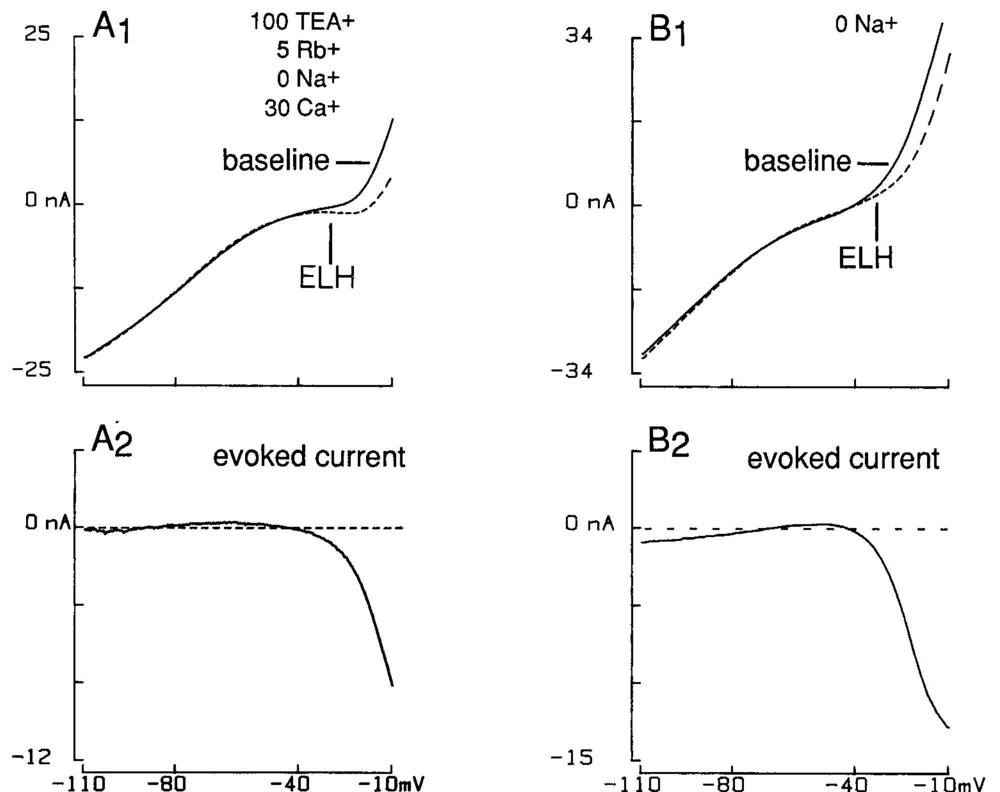

Figure 5. Isolation of a slow inward current, I_{SI}, by agents that block Na^+ and K^+ currents. A_1, The ganglion was bathed in a medium containing 5 mM Rb^+, 100 mM TEA^+, and 375 mM Tris to block the inwardly rectifying K^+ current, the delayed rectifying, and the Ca^{2+}-dependent K^+ currents and all Na^+ currents, respectively. Additionally, the Ca^{2+} concentration was raised to 30 mM. The baseline *I-V* relation (*solid line*) shows the absence of any inward rectification. The *dashed line* represents the *I-V* relation after application of ELH. A_2, The current that ELH evokes in this medium was termed I_{SI}, a current separate from I_X and I_R. B_1, In a different preparation, the ganglion was bathed in a medium in which all external Na^+ was replaced with TMA^+. B_2, Application of ELH showed that both I_X and I_R were blocked but that I_{SI} was apparently unaffected.

ELH-evoked current below about −70 mV and the inward current I_X. The voltage-dependent current that is evoked above −40 mV was still present, however, and appeared to be of about the same magnitude in this voltage range as the current evoked in normal seawater (Figs. 2, 3). This indicates that I_{SI} is a separate current and that it is not likely to be a Na^+ current. Although I_{SI} is Ca^{+2}-sensitive, its dependence on Ca^{2+} or other ions as a charge carrier remains to be determined. Replacing Na^+ with TMA^+ in otherwise normal ASW did not appear to affect I_{SI} either, but it did block I_R and I_X (Fig. 5*B*, $n = 2$). Possibly, the blocking of the latter 2 currents was due to nonspecific effects of the high (495 mM) TMA^+ concentration.

I_R *is an inwardly rectifying* K^+ *current*

The current remaining after subtracting I_{SI} (Fig. 5A_2) and I_X (Fig. 4B_2) from the total evoked current shown in Figure 2 has an inwardly rectifying *I-V* relation with a reversal potential that is close to the reversal potential of K^+. Three criteria have been used to identify inwardly rectifying K^+ currents: (1) It is activated when the membrane is hyperpolarized to potentials that are near or below E_K, (2) its chord conductance depends on the external K^+ concentration, and (3) the current is blocked by externally added rubidium and cesium (Hagiwara and Takahashi, 1974; Hagiwara and Yoshii, 1979; Stanfield et al., 1985).

We used these criteria to determine if the ELH-evoked current that is activated below approximately −70 mV is an inwardly rectifying K^+ current. To determine if it is a K^+ current, we increased the potassium concentration outside the cell ($[K^+]_o$) from 10 (normal ASW) to 30 mM and applied ELH (Fig. 6*a*; $n = 3$). The membrane potential at which I_R activates in the baseline *I-V* relation (Fig. 6*A*, solid lines) was shifted from about −70 mV in 10 mM K^+ to −45 mV in 30 mM K^+. In addition, the extrapolated reversal potential of the ELH-evoked current shifted from about −70 mV in 10 mM K^+ (Fig. 2), to about −50 mV in 30 mM K^+ (Fig. 6A_2). The change in extrapolated reversal potential compares well with the shift predicted from the Nernst potential for potassium, from −76 to −48 mV. In addition, in low external K^+ (1 mM), the activation of inward rectification was shifted to lower membrane potentials, consistent with the idea that I_R is a K^+ current (data not shown).

Associated with the change of $[K^+]_o$ from 10 to 30 mM, there was a dramatic change in the slope of the *I-V* relation below E_K, the reversal potential for K^+ (E_K is indicated by the arrows in Fig. 6). At both the whole-cell and the single-channel level (Hagiwara and Takahashi, 1974; Fukushima, 1982) it has been shown that the conductance of the inwardly rectifying K^+ current is roughly proportional to $([K^+]_o)^{-1/2}$. This is in contrast with the linear dependence on $[K^+]_o$, predicted by the constant-field assumption (see Hagiwara and Takahashi, 1974). Accordingly, we found that the slope of the *I-V* relation below E_K in Figure 6 A_2 increased 1.7-fold upon changing $[K^+]_o$ from 10 to 30 mM, exactly as predicted from the square-root relation for a 3-fold increase in $[K^+]_o$.

Chord conductance of I_R *depends on the external* K^+ *concentration*

Inwardly rectifying K^+ channels are unique in that the gating of the channel depends not only on the membrane potential, but on the external potassium concentration as well (Hagiwara and Takahashi, 1974). It was not possible for us to isolate I_R pharmacologically from the other ELH-evoked currents. We therefore used substraction methods to isolate I_R to investigate this

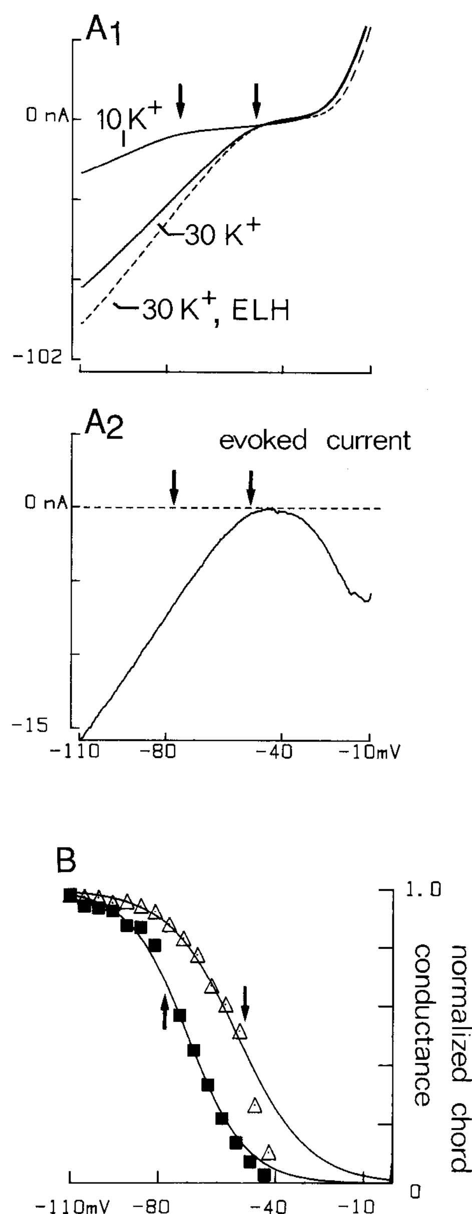

Figure 6. ELH modulates an inwardly rectifying K^+ current. A_1, In normal (10 mM K^+) seawater, the inwardly rectifying current activates below about −70 mV. When the external K^+ concentration is raised to 30 mM, the theoretical reversal potential for K^+ (E_K), shifts from −76 to −48 mV (indicated by the *arrows*). The slope of the *I-V* relation below E_K increases about 1.7-fold, with a 3-fold change in $[K^+]_o$, whereas at membrane potentials above −48 mV, the *I-V* relation shows very little change (*solid lines*). The *dashed line* indicates the *I-V* relation in 30 mM K^+ after application of ELH. A_2, The membrane potential at which the ELH-evoked I_R activates shifted with E_K, from about −70 mV (compare Fig. 2) to about −50 mV. *B*, To isolate I_R, a voltage-independent current with the characteristics of I_X was subtracted to make the current at the theoretical E_K zero. The chord conductance of the ELH-evoked current was then calculated by dividing the current by the driving force on K^+, V-E_K. Plotting the normalized chord conductance measured in 10 mM K^+ (*solid squares*) and 30 mM K^+ (*open triangles*) against the membrane potential indicates that the chord conductance shifts with the external potassium concentration, a unique property of the inwardly rectifying K^+ current. The normalized chord conductances in 10 and 30 mM K^+ were fitted with Boltzmann equations (*solid lines*). The parameters used are explained in the text. The *arrows* indicate E_K in 10 and 30 mM K^+, respectively.

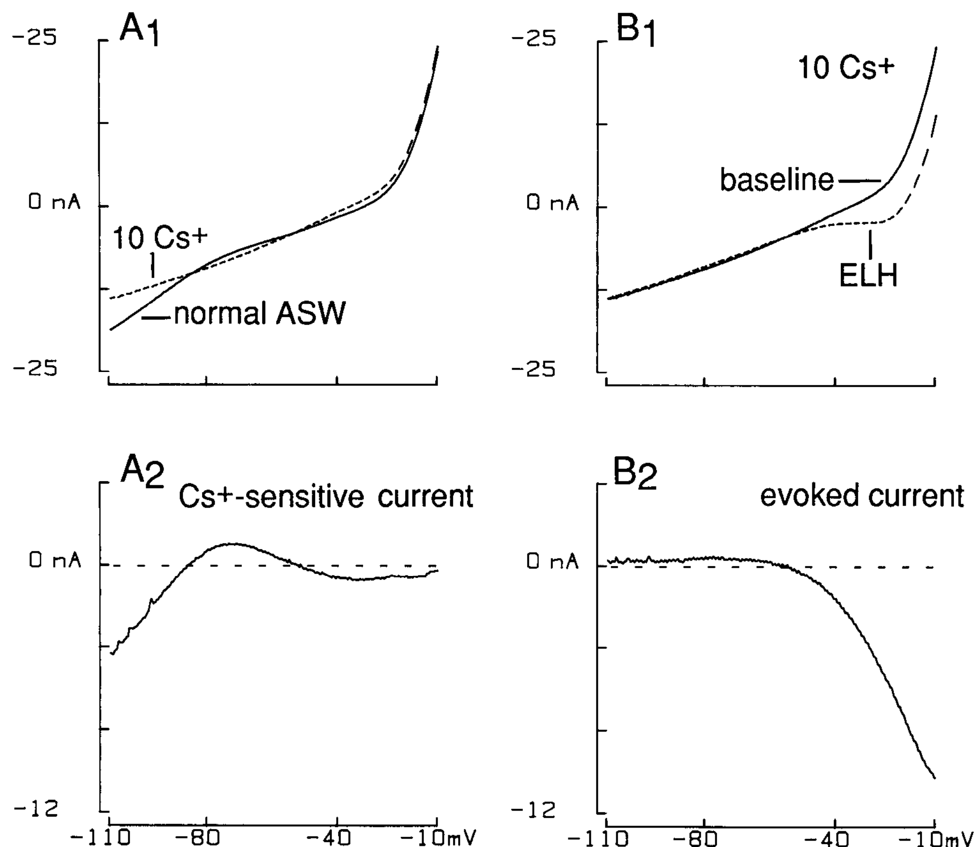

Figure 7. The ELH-evoked I_R is blocked by external Cs^+. A_1, When 10 mM Cs^+ is added to normal seawater, the inward rectification, normally present in these cells (*solid line*), is blocked (*dashed line*). A_2, The cesium-sensitive current shows that the blocked current indeed is inwardly rectifying and has a reversal potential of about −86 mV. B_1, When ELH is later applied to the same ganglion bathed in 10 mM cesium-containing seawater, ELH no longer evokes the voltage-dependent current below about −70 mV that is seen in normal ASW (*solid line*, baseline; *dotted line*, ELH). B_2, With cesium present, the ELH-evoked current only shows an I_{SI}, whereas I_X and I_R are apparently blocked.

feature. At membrane potentials below −50 mV, we postulated that 2 ELH-evoked inward currents are active: I_R and I_X (see Figs. 4 and 6). To isolate the K^+-sensitive current, a theoretical voltage-insensitive current similar to I_X was substracted from the ELH-evoked current. The value of I_X was set so as to make the net evoked current zero at the theoretical E_K. The values used for E_K in normal and high K^+-containing seawater were −77.3 and −51.9 mV, respectively. These values are close to the values of E_K in normal ASW (−76 mV, measured directly in *Aplysia* neurons; Kunze and Brown, 1971) and 30 mM K^+ (−48 mV) and were used in the Boltzmann fit, described below. The chord conductance at each membrane potential, *V*, was then obtained by dividing the current by driving force on K^+ ($V - E_K$). Plotting the chord conductance of I_R, isolated in this way, in normal (Fig. 6*B*, solid squares) and 30 mM K^+ ASW (Fig. 6*B*, open triangles) against the membrane potential, indicates that there is a sigmoidal decrease in the chord conductance at membrane potentials near and above E_K. This sigmoidal chord conductance shifted with the external K^+ concentration. The shift is a unique property of inwardly rectifying potassium currents (data from Figs. $2B_2$ and $6A_2$).

Hagiwara and Takahashi (1974) found that the chord conductance of the inwardly rectifying K^+ current in starfish egg could be described by Boltzmann expressions of the form

$$g_k = g'_k\{1 + \exp[V - V'/k]\}^{-1}$$

where g_k = potassium conductance; g'_k = limiting potassium conductance, V = membrane potential, and V' = membrane potential at half-maximal g'_k. The parameter k determines the shape (steepness) of the curve. Correspondingly, we found that the ELH-evoked currents in 10 and 30 mM, isolated with the subtraction method, could be fitted by this expression (Fig. 6*C*, solid lines) using the following parameters: normal K^+: V' = −70 mV, k = 10 mV, E_K = −77.3 mV; high K^+: V' = −53 mV, k = 12 mV, E_K = −51.9 mV. The data were fit by eye. The subtraction procedure used separates I_R from I_X but not from I_{SI}, which activates at more depolarized membrane potentials. Activation of I_{SI} therefore potentially interferes with the curve fitting, and presumably caused the data points at membrane potentials less negative than −50 mV to deviate from the Boltzmann fit.

I_R *is blocked by external rubidium and cesium ions*

Cesium and rubidium have been used to block inwardly rectifying K^+ currents (Hagiwara and Takahashi, 1974; Gay and Stanfield, 1977; Benson and Levitan, 1983). When added to normal seawater, Rb^+ (5 mM) or Cs^+ (10 mM) blocked an inwardly rectifying current that is normally present in LB and LC neurons in the absence of the peptide (Fig. 7*A*; total n = 7). This current (Fig. $7A_2$) had an average reversal potential of −86 mV (±2.9 mV SEM). With the Cs^+-sensitive current blocked, the ELH-evoked current showed no inward current below −70 mV (Fig. 7*B*), whereas the inward current evoked above −40 mV did not seem to be reduced compared with responses to ELH in normal seawater (Fig. 2*B*). This indicates that Cs^+ and Rb^+ block the inwardly rectifying K^+ current that is normally enhanced by ELH.

Additionally, Cs^+ and Rb^+ also appear to block the postulated current I_X: The ELH-evoked current does not show any inward current below −50 mV. In support of this interpretation, the Rb^+- or Cs^+-sensitive current has an average reversal potential that is about 10 mV more negative than expected from the

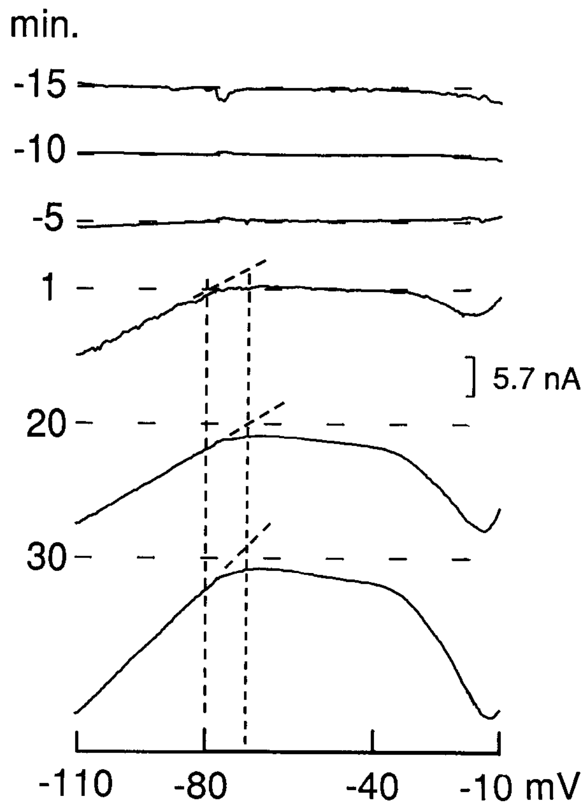

Figure 8. Differential development of BCB-evoked currents also suggests the existence of I_X. Using data from the same preparation as in Figure 2*A*, the baseline *I-V* relations were averaged, and the difference between the individual baseline records and the average was calculated to demonstrate baseline stability. Three of those records, marked 15, −10, and −5 min, are shown. At t = 0, a BCB was triggered, and the development of the different components of the ELH-evoked current was followed by plotting successive evoked current relations. After 1 min, only I_R and I_{SI} were visible. I_R had a reversal potential (calculated by extrapolation of the linear part of the evoked current below −80 mV) of −80 mV, and there was no current evoked between about −80 and −40 mV. After 20 min, the ELH-evoked current was inward at all membrane potentials, and I_R now had an extrapolated reversal potential of about −70 mV. After 30 min, the ELH-evoked current was fully developed in this example.

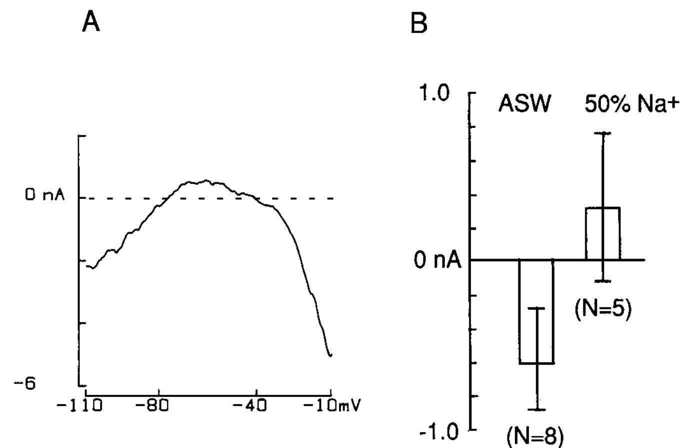

Figure 9. Reducing external Na^+ to 50% abolished the ELH-evoked downward shift of the *I-V* relation. *A*, The ganglion was bathed in a medium in which 50% of the external Na^+ was replaced with Tris, and ELH was applied. In contrast with the ELH-evoked current in normal seawater (see Fig. 2*B*), the ELH-evoked current in 50% Na^+ showed a reversal potential close to −76 mV, the reversal potential for K^+. The evoked current was outward between about −75 and −55 mV. *B*, Comparison of the ELH-evoked currents seen in normal and in 50% Na^+-containing seawater, measured close to E_K. In normal seawater, the average current at −72.5 mV was inward: −0.6 nA (±0.3 nA SEM; N = 8); in 50% Na^+-containing seawater, the average current at −72.5 mV was outward: +0.3 nA (±0.4 nA SEM; n = 5).

Nernst potential for K^+, −86 rather than −76 mV. This is as expected if Cs^+ and Rb^+ were to block I_X, since it would shift the *I-V* relation of the Cs^+-sensitive current upward and the reversal potential to more negative values.

Differential development of BCB- or ELH-evoked currents also suggests the existence of I_X.

In most preparations, the total BCB- or ELH-evoked current was fully developed in 5–30 min after triggering a BCB or applying ELH. Figure 8 shows a typical example of the development of BCB-evoked current in normal seawater (data from Fig. 2*A*). Baseline *I-V* relations were averaged, and baseline stability was demonstrated by calculating the differences between the baseline average and each of the individual baseline *I-V* relations. Three such evoked current relations are plotted in Figure 8 for baseline relations recorded at 15, 10, and 5 min before the BCB, showing that the baseline was stable. At 0 min, a BCB was triggered and the development of the evoked current was followed for 30 min.

After 1 min, two distinct voltage-dependent currents were visible: I_R, which was discernible at membrane potentials below −80 mV, and I_{SI} at membrane potentials above −40 mV. However, no evoked current was visible between −70 and −40 mV. After 20 min both I_R and I_{SI} currents were increased in size (as judged by their respective slope conductances), and the evoked current was now inward over the entire voltage range. Interestingly, the reversal potential for I_R, extrapolated from the straight-line *I-V* relation below −80 mV, had shifted from its original value of −80 mV at 1 min to −70 mV at 20 min. At 30 min the current was fully developed, and when released from voltage clamp, the cell was found to be firing spontaneously. (In 5 experiments the extrapolated reversal potential measured at maximal response was −68 ± 3.8 mV SEM). The shift in extrapolated reversal potential can be accounted for by a downward shift in the *I-V* relation (see Fig. 4) due to the onset of I_X. As I_X sums with I_R, the extrapolated reversal potential is shifted from near the equilibrium potential for K^+ to more positive values.

An alternative explanation for the shift in the reversal potential is that it is caused by the accumulation of K^+ ions at the outside of the cell membrane. However, the ramp command starts at −110 mV, below E_K, and is preceded by a hyperpolarizing step that lasts for 10 sec. During this 10 sec phase a considerable K^+ influx, not efflux, is expected due to the inward rectification of the *I-V* relation. Therefore, extracellular K^+ accumulation does not seem to be a reasonable explanation for the relatively depolarized extrapolated reversal potential. The I_{SI} current activates only above −40 mV (Figs. 5, A_2, B_2; 7B_2) and it cannot account for the shift in the reversal potential of I_R. We therefore conclude that E_K is the real reversal potential for I_R and that the shift of the extrapolated reversal potential to −70 mV, visible in the 20 and 30 min traces, results from the development of I_X.

Low external Na^+ selectively blocks the downward shift of the I-V *relation*

The idea that a part of the ELH-evoked current serves to shift the entire *I-V* relation downward is borne out by another experimental approach that appears to selectively block I_X. We applied ELH to ganglia that were bathed in a medium in which 50% Na^+ was replaced with Tris. As opposed to the ELH-evoked current in normal seawater (Fig. 2), the ELH-evoked current in 50% Na^+ medium was outward rather than inward in the −80 to −40 mV range in 5 out of 6 experiments (Fig. 9*A*). Moreover, the average reversal potential was −74.5 mV (±5.6 mV SEM; $n = 5$), close to the theoretical value of −76 mV for E_K. This result indicates that the downward shift in the *I-V* relation is produced by I_X and that it was abolished in 50% Na^+. Figure 9*B* shows the size of the ELH-evoked current in normal and 50% Na^+ seawater, measured close to E_K. In normal seawater, the average current at −72.5 mV was inward: −0.6 nA (±0.3 nA SEM; $n = 8$). In 50% Na^+, the average current at −72.5 nV was outward: +0.3 nA (±0.4 nA SEM; $n = 5$). These experiments thus suggest that I_X is a Na^+-sensitive current that shifts the whole *I-V* relation downward and causes a shift of the extrapolated potential for the *I-V* relation extrapolated from below −80 mV.

Discussion

The present study shows that the current evoked by the neuropeptide ELH comprises at least 3 different components I_{SI} (Figs. 5, 7), I_R (Fig. 6), and I_X (Fig. 4). Additionally, this study showed that BCBs and application of ELH evoke apparently identical currents in target neurons of the abdominal ganglion. Since each component of the ELH-evoked current was slowly inactivating or noninactivating, it was possible to analyze the currents using a ramp command potential. However, since the ramp selectively measures slow currents, it is possible that other, rapidly inactivating currents are also modulated by ELH. In particular, ELH modulates a transient potassium current in addition to the currents described here. Analysis of this current and of I_{SI} will be described in a subsequent paper (R. F. Jansen and E. Mayeri, unpublished observations).

Mayeri et al. (1985) showed that ELH still affects LB and LC neurons when fast synaptic transmission was blocked or when the cells were surgically isolated in a small cluster. In the present study, the cells were usually surgically isolated in a small cluster, but left within the ganglion. It therefore seems likely that all the effects of BCBs and ELH are direct. With few exceptions all of the currents modulated by ELH appeared to be modulated in each cell.

Modulation of the slow inward current

The ELH-evoked I_{SI} current was apparently unaffected by complete removal of external Na^+ in the presence of 100 mM TEA^+, which is reported to block close to 90% of the delayed rectifier K^+ current and almost 100% of the Ca^{2+}-dependent K^+ current in *Aplysia* neuron R_{15} (Hermann and Gorman, 1981). It thus appears that I_{SI} is not produced by an increase of a Na^+ current or by reduction of either of these 2 K^+ currents. However, there have been several reports of relatively TEA-resistant K^+ currents in *Aplysia* (Klein and Kandel, 1980; Deitmer and Eckert, 1985), and the present data do not exclude the possibility that a TEA^+-resistant K^+ current underlies I_{SI}. Since I_{SI} was blocked by replacing external Ca^{2+} with Co^{2+}, we conclude that I_{SI} is a Ca^{2+}-sensitive current and that the charge carrier is not Na^+.

In the buccal motorneuron B_{16} of *Aplysia*, applied ELH causes repetitive firing that is caused by a slow inward current. Contrary to the present results, however, this ELH-evoked current was blocked by removing external Na^+ and was not reduced by Ca^{2+} channel blocking agents (Kirk and Scheller, 1986). Furthermore, currents similar to I_R or I_X were not evoked by ELH in this neuron. It thus appears that ELH modulates different currents in different parts of the brain of *Aplysia* to induce repetitive firing.

Modulation of an inwardly rectifying K^+ current

The most complete analysis in this study was done for the ELH-evoked current I_R. This current appears to be a pure K^+ current and has all the properties that have been described for inwardly rectifying K^+ currents. Both the sensitivity of the chord-conductance of I_R to $[K^+]_o$ and the fact that the slope conductance of the *I-V* curve changes proportionally with the square root of $[K^+]_o$ are unique properties of inwardly rectifying K^+ currents. Additionally, the experiments with the blocking agents Cs^+ and Rb^+ indicate that I_R is similar to the inwardly rectifying K^+ current found in frog muscle and starfish egg (Adrian, 1969; Hagiwara and Takahashi, 1974).

Modulation by peptides of K^+ currents similar to I_R has been described in a number of preparations: I_R is increased by 5-HT in the cell R_{15}, by alpha-bag cell peptide in the bag cells (Benson and Levitan, 1983; Kauer and Kaczmarek, 1986), and suppressed by substance P in cultured magnocellular neurons (Stanfield et al., 1985) and by L-glutamate in *Carassius auratus* (goldfish) retinal horizontal cells (Kaneko and Tachibana, 1985). Unlike some of these examples, however (Benson and Levitan, 1983; Stanfield et al., 1985), in the present study both the inwardly rectifying current present in the baseline *I-V* relation and the I_R evoked by ELH were shown to be substantially reduced by Ca^{2+} channel blocking agents like Co^{2+} and Ni^{2+}. At present, we do not know the mechanism of this action. There are, however, reports to suggest that inwardly rectifying K^+ currents are sensitive to intracellular calcium (Kramer and Levitan, 1986) and magnesium levels (Matsuka et al., 1987; Vandenberg, 1987).

Modulation of the steady inward current

Several lines of evidence indicate that I_X is a third ELH-evoked current, separate from I_R and I_{SI}. First, I_X is apparently the only ELH-evoked current that remains in 10 Co/0 Ca medium (Fig. 4). Second, the other ELH-evoked currents (I_{SI} and I_R) cannot account for the net inward evoked current that occurs in the −70 to −40 mV range: The inwardly rectifying K^+ current is outward, not inward, above the reversal potential for K^+ ions, −76 mV. The isolated I_{SI} current (Figs. 5, 7) becomes activated only at membrane potentials above −40 mV. Third, the extrapolated reversal potential of the current evoked below −80 mV shifts to more positive values as the response to ELH develops (Fig. 9). The onset of a steady inward current (I_X), but not an inwardly rectifying K^+ current, would explain such a shift. Other mechanisms that would explain this shift, such as extracellular K^+ accumulation, were ruled out by holding the cells at −110 mV for 10 sec before the onset of the ramp.

In addition, an I_X-like inward current was reduced or absent in all experiments where the external Na^+ concentration was reduced (Fig. 8) or eliminated (Fig. 5), and the current remaining

in 10 Co/10 Ca medium and the current that is apparently blocked by 50% Na^+ were of comparable size. We therefore conclude that I_X is a third ELH-evoked current that needs external Na^+ to operate. Although we did not measure a reversal potential for I_X, the reversal potential extrapolates to a positive membrane potential. This is consistent with the possibility that Na^+ is a charge carrier for I_X.

An isoprenaline-modulated, Na^+-dependent current, with some properties similar to those of I_X, has recently been described in ventricular myocytes (Egan et al., 1987). However, as an inward current with a voltage-independent conductance, I_X appears to represent a novel type of current to be modulated by neuropeptides. A current modulated by the neuropeptide LHRH with characteristics similar to those of I_X has been briefly described in the bullfrog sympathetic ganglion (Jones, 1985).

ELH modulates opposing currents to control neuronal excitability

Application of ELH or the triggering of a BCB induces or augments long-lasting repetitive spiking in LB and LC neurons (Mayeri et al., 1979b). The currents modulated by ELH are likely to be essential for generating the pacemaker currents that result in repetitive activity, but the relative contributions of the currents are not entirely clear. The currents I_X and I_{SI} provide the depolarizing drive that tends to increase spike rate. The voltage-independent current I_X provides a steady "background" current that is active at all membrane potentials between -110 and -10 mV and depolarizes the membrane, even at very negative membrane potentials. Although I_X and I_{SI} are relatively small at resting membrane potentials, the slope of the I-V relation of these neurons in the -60 to -30 mV region is small; consequently, relatively small currents can result in large depolarizations (see Figs. 1 and 2).

As a result of its inwardly rectifying I-V relation, I_R is expected to be activated during the undershoot at the end of each spike. Analysis in Figures 6*C* and 7A_2 indicates that I_R may already be activated for 25% at -60 mV, and I_R may therefore contribute to the mechanism of repetitive spiking by prolonging the interval to the next spike, as in Purkinje fibers of the vertebrate heart (Noble and Tsien, 1968). In contrast to I_{SI} and I_X, the I_R would tend to decrease spike rate. Additionally, as mentioned above, we cannot exclude the possibility that ELH may modulate other, transient currents that are involved in spike generation.

ELH as a neurotransmitter

The present study shows that BCBs and ELH induce apparently identical changes in membrane conductance. This provides additional evidence that ELH is a neurotransmitter. Earlier investigations (see Mayeri et al., 1985) showed that ELH meets almost all of the strict criteria that have been used to identify substances as neurotransmitters (Werman, 1966). This includes synthesis, release, mimicry, and a mechanism for inactivation (which in this case is slow or ineffective). Although a receptor antagonist for ELH is lacking, there is the unusual added evidence that, as a consequence of the neuroendocrine nature of the bag cells, ELH is released in more than sufficient quantities to act as a neurotransmitter within the ganglion (Mayeri et al., 1985). It therefore seems reasonable to conclude that ELH is an identified neurotransmitter. Despite the fact that peptides are now widely accepted as potential neurotransmitters, the number of peptides that have been shown to fulfill the strict criteria for transmitter identification is small. The peptides for which a role as neurotransmitter is most clear include LHRH (Jan and Jan, 1982), substance P (Konishi and Otsuka, 1985), and alpha-bag cell peptide (Rothman et al., 1983a; Sigvardt et al., 1986).

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