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Authors: Kinugawa, Aiko, and Nagahama, Tatsumi

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A CPG Component LE Generates Depolarization of Buccal Neurons by Producing Constant Plateau Potentials During Feeding Responses of *Aplysia kurodai*

Aiko Kinugawa[1] and Tatsumi Nagahama[2*]

[1]*Department of Life Science, Graduate School of Science & Technology, Kobe University, Kobe 657-8501, Japan*
[2]*Department of Biophysics, Faculty of Pharmaceutical Science, Toho University, Miyama, Funabashi 274-8510, Japan*

In the buccal ganglia of *Aplysia kurodai* we have identified neurons (here termed LE neurons, or LE) producing plateau potentials lasting several seconds by application of short depolarizing currents. Results obtained from experiments using various bath solutions suggest that generation of these plateau potentials may be an endogenous property of LE. Application of various intensities or lengths of depolarizing currents induced in LE almost constant plateau potentials with fixed duration and depolarizing size. LE spikes produced monosynaptic EPSPs in the ipsilateral multi-action neuron (MA) and the jaw-closing motor neuron (JC) in the buccal ganglia. Conversely, MA spikes produced monosynaptic IPSPs in LE. There was electrical coupling between LE and both MA and JC. During the feeding-like response elicited by electrical stimulation of the nerve, LE showed rhythmic depolarization almost simultaneously with MA and JC, and firing on the plateau potentials occurred during the period of JC firing, the later phase of radula retraction. Hyperpolarization of LE during the feeding-like response suppressed generation of plateau potentials, though rhythmic small depolarization was still induced. During LE hyperpolarization, the duration of the depolarization of MA and JC was shortened. These results suggest that LE may be an element of the feeding CPG circuit and may contribute to part of the depolarization of MA and JC by generating constant plateau potentials during the feeding response, though LE may not have rhythm-generating ability.

Key words: pattern, action potential, behavior, interneuron, identification, central nervous system

INTRODUCTION

It has been generally accepted that rhythmic motor activities seen in animal behaviors such as walking, flying, swimming, and biting (swallowing) can be generated in the absence of sensory inputs by specific neural circuits termed central pattern generators (CPGs) (Grillner, 1977, 1981; Kristan *et al*., 1977; Delcomyn, 1980). CPG neural circuits and their modulation have been well studied in many vertebrates and invertebrates (Getting, 1989; Harris-Warrick, 1993; Pearson, 1993; Grillner *et al*., 1995). The functional element of a CPG related to generation of stable, long-lasting depolarization will be important for generation of the basic long-lasting firing activity of motor neurons during a rhythmic response. In several cases, it has been reported that long-lasting depolarization is generated by neurons producing plateau potentials, known to be triggered by brief depolarizing inputs and terminated by hyperpolarizing inputs or spontaneously (Russell and Hartline, 1977, 1978; Susswein and Byrne, 1988; Hounsgaard and Kiehn, 1989; Plummer and Kirk, 1990; Kiehn, 1991; Hurwitz and Susswein, 1996).

The marine gastropod *Aplysia* shows clear food preferences (Audesirk, 1975; Carefoot, 1967, 1970; Kupfermann and Carew, 1974; Nagahama and Shin, 1998). For example, the East Asian species *Aplysia kurodai* feeds well on *Ulva* but rejects *Gelidium* or *Pachydictyon*, with different patterned, rhythmic movements of the jaws and radula following taste recognition of the seaweeds (Nagahama and Shin, 1998). Our interest was in elucidating the neural mechanism modulating the CPG circuit concerned with the patterned jaw movements in the food preference behaviors. We have previously demonstrated that a change of the patterned jaw movements from ingestion to rejection is caused by an advance of the onset time of firing of jaw-closing (JC) motor neurons at each depolarizing phase of the rhythmic response (Nagahama and Shin, 1998). During the ingestive response, the delay of onset of JC firing is produced by the firing of multi-action (MA) neurons that monosynaptically inhibit the JC neurons (Nagahama and Takata, 1988, 1989, 1990). The MA neurons are probably equivalent to the B4/5 neurons in *A. californica* (Gardner, 1971). We have found that the firing advance in JC during the rejective response is

* Corresponding author. Phone: +81-47-472-2894;
Fax : +81-47-472-2894;
E-mail: nagahama@phar.toho-u.ac.jp
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caused by suppression of synaptic transmission from MA in the case of *Gelidium* and by decrease in firing activity of MA in the case of *Pachydictyon* (Nagahama *et al*., 1999).

We recently demonstrated that synaptic transmission can be suppressed by a cerebral dopaminergic neuron during the *Gelidium* rejective response (Narusuye and Nagahama, 2002). However, we have not explored the mechanism of decrease in MA firing activity during the *Pachydictyon* response. Some CPG component regulating MA firing activity may contribute to a change from the ingestive to the rejective response. In addition, we have not explored the neural mechanism generating depolarization of JC during these responses.

We searched for neurons impinging on MA and JC and found a unique neuron whose firing produces excitatory postsynaptic potentials (EPSPs) in both neurons. This neuron generates constant plateau potentials lasting for several seconds, independently of the length or intensity of the depolarizing currents. The morphological and electrophysiological characteristics of this neuron are similar to those of the B51 neuron in *Aplysia californica* (Plummer and Kirk, 1990), except for some differences in neuronal connections.

In *Aplysia kurodai* and *Aplysia californica*, we previously found a discrepancy in the number of equivalent neurons (four MA and two B4/B5 in the buccal hemiganglion), suggesting that the total number of neurons in the buccal ganglion may be different in the two species (Gardner, 1971; Nagahama and Takata, 1989). Therefore, we could not use the same numbering of equivalent neurons in *Aplysia kurodai*, and we named the identified neuron LE. Here we show that LE may be a CPG element and may contribute to generation of the later phase of depolarization of MA and JC during the feeding response, though the decrease in LE spike activity cannot explain the change in JC firing patterns from the ingestive to the rejective response. Some of the findings in this article have been presented in a preliminary communication (Kinugawa and Nagahama, 2004).

MATERIALS AND METHODS

Animals and seaweed

Aplysia kurodai individuals weighing 50–660 g were collected from the Pacific coast of Japan. Animals were maintained at 14–16°C in aquaria filled with aerated and filtered artificial seawater (ASW). *Ulva pertusa (Ulva)* was used for animal food and was supplied once daily. Raw *Ulva* was maintained in aquaria with ASW at room temperature, and frozen *Ulva* was stored below –20°C in a freezer.

Preparations

In the present experiments, isolated preparations were used. The animals were anesthetized by injection of isotonic $MgCl_2$ solution (30% of body weight) into the body cavity and dissected at room temperature. The preparation consisted of cerebral ganglia, buccal ganglia, and buccal mass. The buccal mass was then cut into halves along the midline to separate the paired symmetrical buccal musculatures. The peripheral nerves were severed, except for buccal nerves 2 and 3 (n2 and n3). The ganglia and the paired buccal mass were separately pinned to the silicone elastomer (Sylgard, Dow) surface of each compartment of a Lucite recording chamber, and petroleum jelly (Vaseline) was placed on the partition between each set of ganglia and the buccal musculature. The sheath overlying the buccal ganglia was surgically removed. In control experiments, ganglia and buccal musculatures were bathed in ASW.

Electrophysiology

Recordings from individual neurons were performed using conventional electrophysiological techniques. For intracellular recording and stimulation, neurons were impaled with glass microelectrodes filled with 2 M potassium acetate. These electrodes were beveled to 3–6 MΩ. For intracellular recording and dye injection, double-barrel glass microelectrodes filled with 2 M potassium acetate (3–6 MΩ) and 3% Lucifer Yellow CH (SIGMA) in distilled water (20–30 MΩ) were used. For electrophysiological experiments, a compartment with 2 M potassium acetate was used for recording and current stimulation of the cell, and for intracellular staining, another compartment with Lucifer Yellow solution was used for iontophoretic injection of the dye. In experiments exploring the effects of inhibition of neuronal spike activity, long-lasting hyperpolarizing currents (–15 to –40 nA) were applied to the neuron during responses. Suction electrodes (tip diameter, 100–150 μm) were used for electrical stimulation of nerve trunks. In order to induce feeding-like responses, the esophageal nerve was repetitively stimulated with 5 ms current pulses at 2 Hz (2–5 V).

The composition of ASW used in the experiments was as follows (in mM): 470 NaCl, 11 KCl, 11 $CaCl_2$, 25 $MgCl_2$, 25 $MgSO_4$, and 10 Tris-HCl (pH 7.8–7.9). In some experiments, polysynaptic pathways were suppressed using 5×Ca^{2+}, 2×Mg^{2+} or 3×Ca^{2+}, 3×Mg^{2+} solutions. These solutions increase the threshold of neurons without enhancing synaptic transmission, because Mg^{2+} counteracts Ca^{2+} action at the synapse, but both act to raise the threshold (Kandel, 1976). Therefore, they block the firing of an interposed interneuron when the connections are polysynaptic. The osmotic balance in these solutions was maintained by replacement of Na^+. Ca^{2+}-free or Ca^{2+}-deficient, Mg^{2+}-rich (1 mM Ca^{2+}, 200 mM Mg^{2+}) solutions were also used to block chemical transmissions. Ca^{2+}-free solutions were produced by replacing Ca^{2+} with Mg^{2+}. In Ca^{2+}-deficient, Mg^{2+}-rich solutions, the osmotic balance was maintained by replacement of Na^+. All experiments were performed at room temperature.

Morphology

Dye was injected using a 5–20 nA hyperpolarizing DC current for 40–60 min. The preparations were fixed in phosphate-buffered 4% formaldehyde (pH 7.8) at 4°C for at least 12 h, dehydrated through a standard alcohol series, and finally cleared with methyl salicylate. The structures of the neurons were visualized with a laser-scanning microscope (LSM-310; Zeiss). Based on photographs, the fine structures of cells were drawn with the use of imaging software (Photoshop, Adobe).

RESULTS

Morphology of the LE neuron

Neurons were stained with 3% Lucifer Yellow CH by iontophoretic injection from microelectrodes. A photo image and drawings of typical LE neurons are shown in Fig. 1. The soma of LE was located in the rostro-ventral side of each right and left buccal ganglion (long axis 100±21 μm (SD), short axis 50±15 μm; n=10). LE projected a major axon into the radular nerve (Fig.1A) in five preparations, but not in the other five preparations (Fig.1B). LE usually had several processes close to the cell body and sometimes at various points along the major axon. In the present experiments, we could not locate two LE neurons simultaneously in an ipsilateral ganglion. Therefore, we do not know whether there are two types of LE in an ipsilateral ganglion, or a single LE showing diverse peripheral structures. However, the LE neurons studied always showed coincident electrophysio-

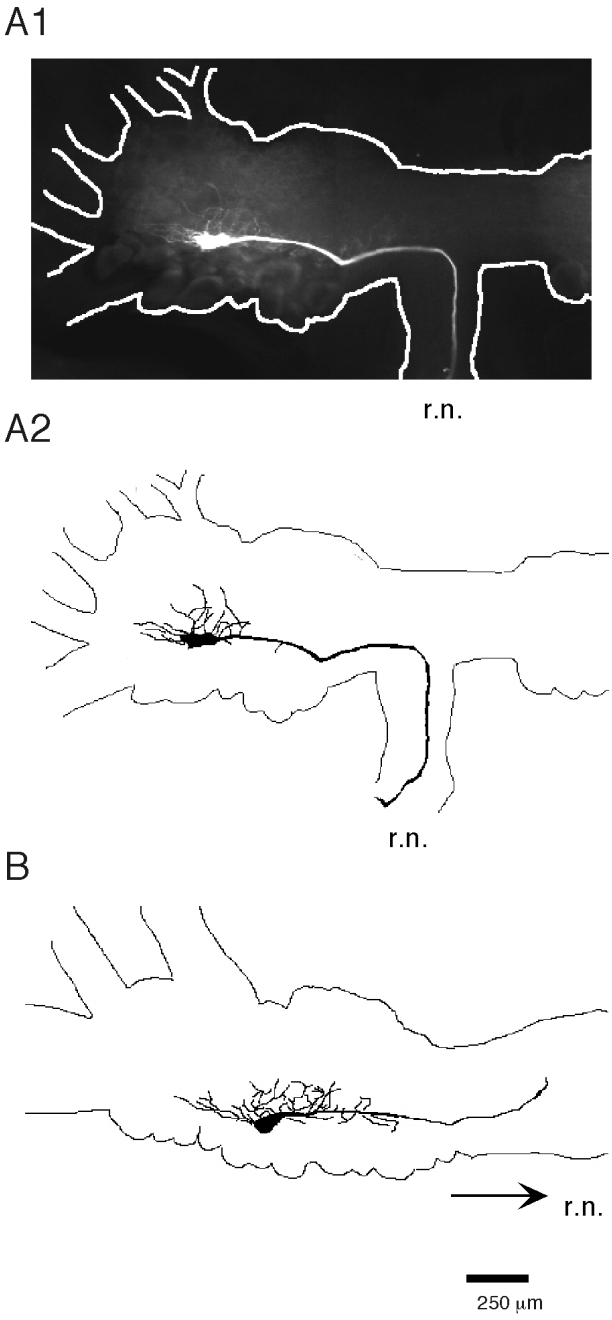

Fig. 1. Cellular morphology of LE neurons. **A)** fluorescence photo image (1) and drawing (2) of an LE neuron projecting a major axon into the radular nerve. **B)** drawing of an LE neuron with no projection of a major axon into the radular nerve. The neurons were filled with Lucifer yellow CH. Buccal ganglia were mounted with rostral surface upward.

logical properties within the ganglia. We therefore treated all LE neurons as a single group. The structures of the LE projecting a major axon into the radular nerve are similar to those of B51 reported in *Aplysia californica* (Plummer and Kirk, 1990).

Physiological characteristics

The average resting membrane potential of LE neurons

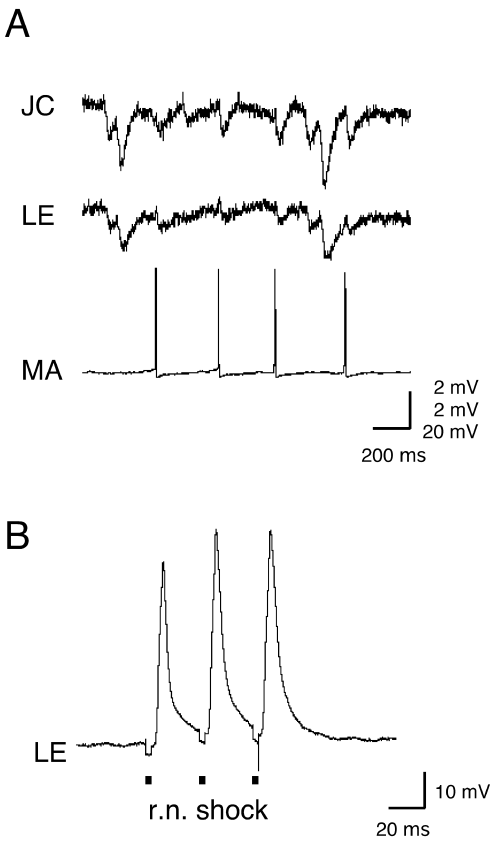

Fig. 2. Physiological properties of LE neurons. **A)** inhibitory postsynaptic potentials (IPSPs) spontaneously induced in LE. Synchronous IPSPs were often observed in JC, some of which followed the spikes of MA firing one-for-one. **B)** antidromic spikes elicited by repeated electrical stimulation of the radular nerve (3 ms, 2 V, 3 pulses) in the LE projecting a major axon to the radular nerve.

was –51±7 mV (SD) (n=21). Spontaneous inhibitory inputs were usually observed in LE (Fig. 2A). When simultaneous recordings were performed on MA, JC and LE, we often observed synchronous IPSPs in LE and JC, some of which followed spontaneous firing of MA in a one-for-one manner. Electrical stimulation of the radular nerve elicited antidromic spikes in the LE projecting the axon into the radular nerve (Fig. 2B). Similar stimulation did not elicit these spikes in the LE projecting no axon into the radular nerve.

When LE was depolarized with short DC currents, plateau-like depolarizing potentials lasting for several seconds were usually induced (Fig. 3A). The minimum time required for these currents to induce plateau-like potentials was 200~300 ms (16 nA). The LE spikes usually became smaller and then often disappeared with an increase in firing frequency. The small hyperpolarization of LE could restore LE spikes that faded away during the plateau-like potential (Figs. 3B1 and 3B2), suggesting that hyperpolarization may be able to restore some inactivated ion channels. The plateau-like potentials could be terminated by a large hyperpolarization of LE with short DC currents applied to it (Fig. 3B3). The minimum length of hyperpolarizing currents required to terminate plateau-like potentials was about 250 ms (–35 nA). The features that long-lasting depolarization is

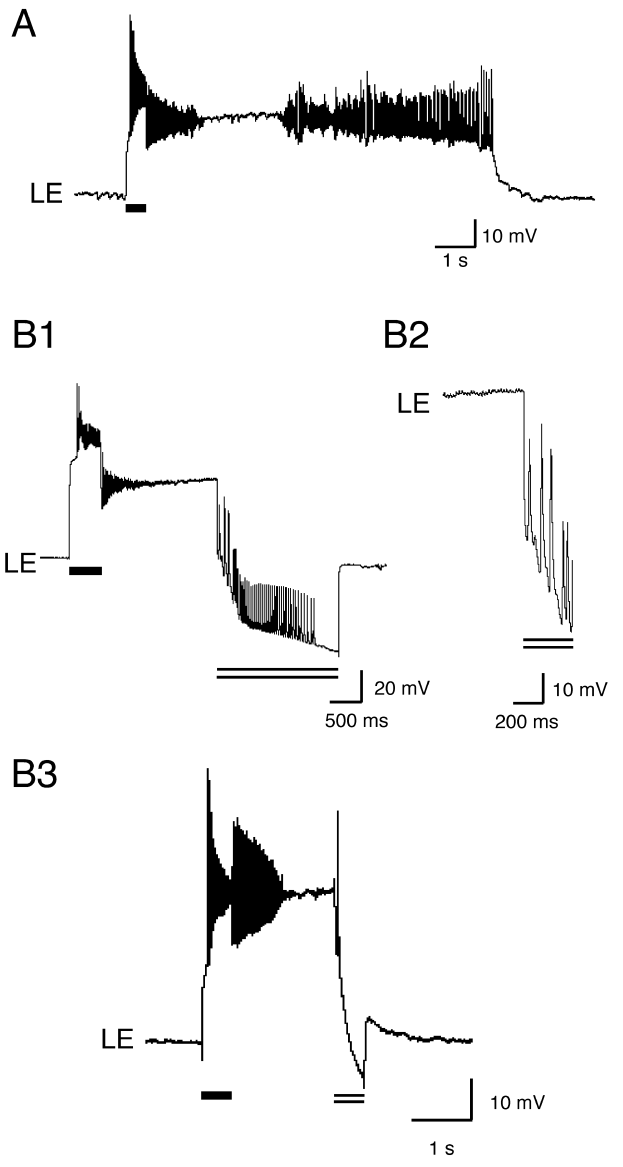

Fig. 3. Generation of plateau-like potentials in LE. **A)** a plateau-like potential induced in LE by brief depolarization (500 ms, bar) with short DC currents in normal ASW. **B1)** recovery of LE spikes that faded away with increasing firing frequency during the plateau-like potential by small hyperpolarization (double line, –15 nA) of LE. The large negative change in the membrane potential during hyperpolarization resulted from the wrong bridge balance of the amplifier. **B2)** a faster-speed recording of a part of B1, in which individual spikes can be seen. **B3)** termination of the plateau-like potential by large hyperpolarization of LE (double line, –35 nA). Depolarizing currents to induce the plateau-like potentials were applied at bars.

produced by a short depolarizing current and terminated by a short hyperpolarizing current are consistent with those generally reported for plateau potentials. We therefore refer to the depolarizing potentials induced in LE as plateau potentials in this text. It has been reported that B51 in *Aplysia californica* also generates plateau potentials (Plummer and Kirk, 1990).

Firing properties of the plateau potentials

We explored the properties of the plateau potentials in more detail, and initially the relationship between current intensity and firing frequency. In these experiments, LE was excited with 500 ms depolarizing pulses, and average firing frequencies during the initial 500 ms were obtained. As a control, similar experiments were also performed on JC. Fig. 4A shows a comparison of the firing properties between LE and JC for two preparations each. The threshold current intensities for firing were similar in both neurons. In JC neurons, firing frequencies increased smoothly with increasing current intensity, and the slope gradually declined at large currents (40–50 nA). In LE neurons, firing frequencies increased sharply with increasing current intensity, and the slope suddenly became gentle at much smaller currents (10–20 nA), though the frequencies showed dispersion.

Constant properties of the plateau potentials

We explored whether the length of injected currents affects the duration or depolarizing size of the plateau potentials. In these experiments, the duration was determined as the length from time of onset of the stimulation to the time when the membrane potential returned to the resting level, as shown in Fig. 4B. Depolarizing size was determined as the change from the resting membrane potential to the most depolarized potential in the levels of afterhyperpolarization of the spikes (also shown in Fig. 4B). Depolarizing currents of constant intensity were injected for various lengths of time (Fig. 4C). Figs. 4C2 and 4C3 show the relationships between current length and average duration of the plateau potential, and between current length and average depolarizing size, in three preparations. Current length (>200 ms) scarcely affected the duration and depolarizing size of the plateau potentials, in all preparations.

We also explored whether the intensity of the injected current affects the duration or depolarizing size of the plateau potential. In these experiments, depolarizing currents of constant length were injected at various intensities (Fig. 4D). The relationships in three preparations between current intensity and average duration, and between current intensity and average depolarizing size, are shown in Figs. 4D2 and 4D3. The duration of the plateau potential was almost constant at sufficient current intensity (>20 nA), though it tended to lengthen near the threshold (about 11 nA). In addition, current intensity scarcely affected the depolarizing size of the plateau potential. These results suggest that LE may produce constant plateau potentials of fixed duration and depolarizing size independently of the length or the intensity of the excitatory synaptic inputs, when the length and intensity of the inputs are appropriate to the current conditions, as in the present experiments.

Generation mechanisms of the plateau potentials

LE plateau potentials were sustained even when ganglia were bathed in Ca^{2+}-free solution (Fig. 5A2), suggesting that extracellular calcium ions may not contribute to generation of these plateau potentials. In addition, LE also showed plateau potentials when ganglia were bathed in Ca^{2+}, Mg^{2+}-rich solutions ($5\times Ca^{2+}$, $2\times Mg^{2+}$ or $3\times Ca^{2+}$, $3\times Mg^{2+}$) (Fig. 5B2). The Ca^{2+}-free and Ca^{2+}, Mg^{2+}-rich solutions blocked chemical and polysynaptic transmission, respectively (see Materials and Methods). Therefore, these results suggest that the plateau potentials may be generated by an endog-

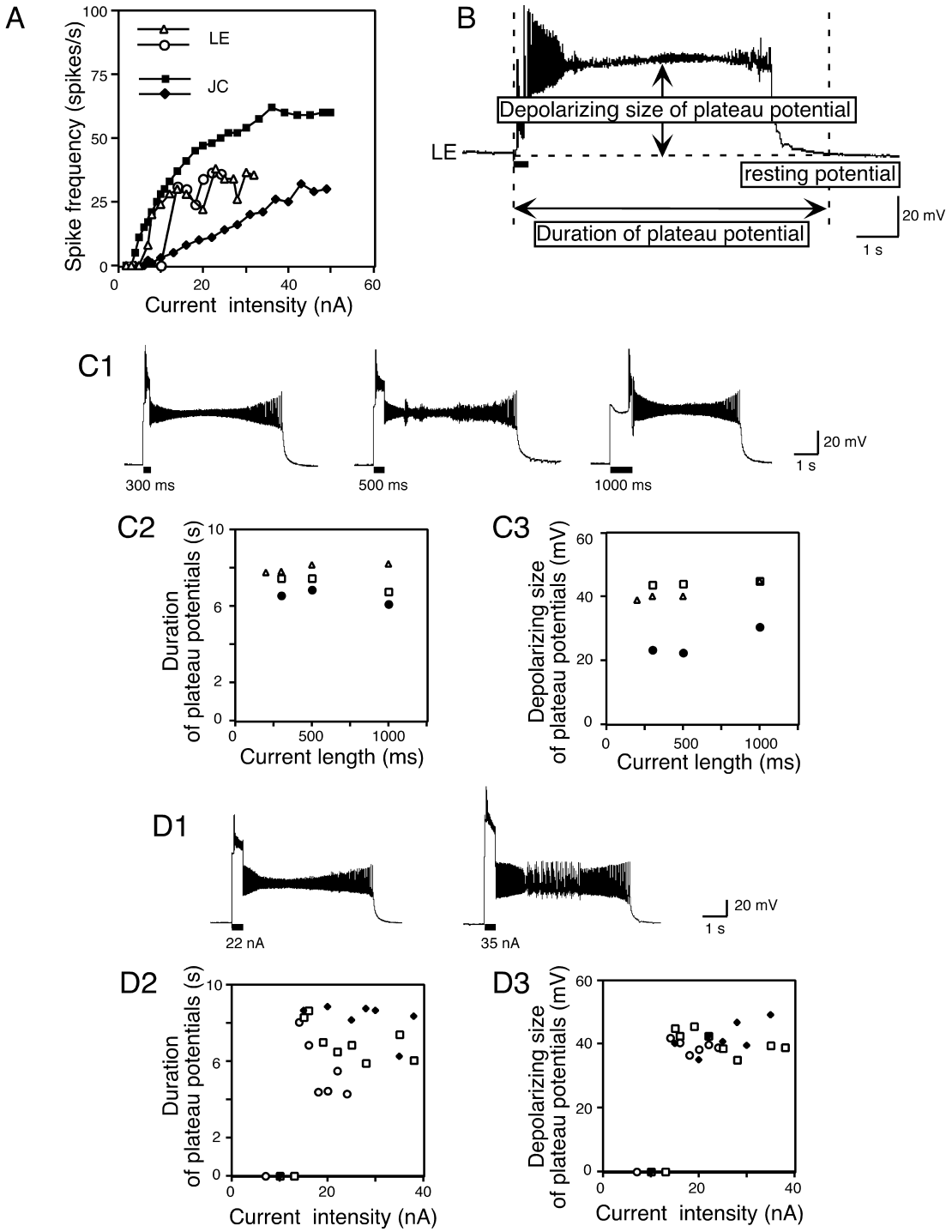

Fig. 4. Generation properties of the plateau potentials. **A)** comparison of the current-frequency relationships in LE and JC. Firing frequencies were measured in the first 500 ms in duplicate and were averaged. The experiments were performed on two preparations for each neuron. **B)** illustration of the duration and depolarizing size of a plateau potential. **C1)** plateau potentials induced by application of depolarizing currents of constant intensity (16 nA) but of three different lengths in the same preparation. **C2)** relationships between current length and duration of the plateau potential in three preparations. **C3)** relationships between current length and depolarizing size in the same preparations as in C2. A pair comprising duration and depolarizing size were obtained from the plateau potentials produced in duplicate for every current condition. The same symbols in C2 and C3 show the paired average data. **D1)** plateau potentials induced by application of depolarizing currents of constant length (500 ms) but of two different intensities in the same preparation. **D2)** relationships between current intensity and duration of the plateau potentials in three preparations. **D3)** relationships between current intensity and depolarizing size in the same preparations as in D2. A pair comprising duration and depolarizing size were obtained from the plateau potentials produced in duplicate for every current condition. The same symbols in D2 and D3 show the paired average data.

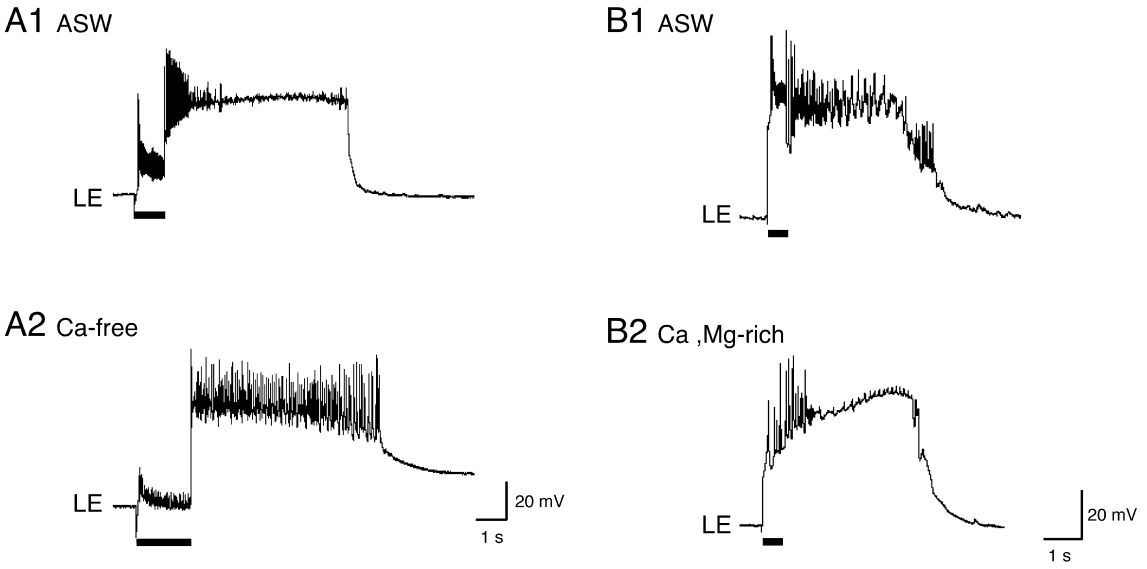

Fig. 5. Effects of external solutions on LE plateau potentials. **A)** sustainment of the LE plateau potential in buccal ganglia bathed in a Ca^{2+}-free solution. **B)** sustainment of the LE plateau potential when buccal ganglia were bathed in a Ca^{2+}, Mg^{2+}-rich solution. Depolarizing currents to induce the plateau potentials were applied at bars.

enous property of LE.

Synaptic connections

Synaptic connections between LE and the buccal neurons (MA and JC) were explored. When a short depolarizing current was applied to LE, it induced a plateau potential in LE and usually induced long-lasting depolarizing potentials in the ipsilateral MA and JC (Fig. 6A1). Large spikes of LE were followed by depolarizing potentials in MA and JC in a one-for-one correspondence (numbered spikes in Fig. 6A2 and spikes 5, 9, and 10 in Fig. 6A3), but smaller spikes were not (spikes 6, 8, 11, and 12 in Fig. 6A3).

To learn whether these connections are monosynaptic or polysynaptic, buccal ganglia were bathed in a Ca^{2+}, Mg^{2+}-rich solution. Even in this solution, depolarizing potentials were induced in MA and JC following current-induced LE spikes in a one-for-one manner (Fig. 6B). We also found that application of hyperpolarizing DC currents to LE induced hyperpolarizing responses in MA and JC (Fig. 6A4), suggesting the existence of electrical connections between LE and both of these neurons. The buccal ganglia were then bathed in a Ca^{2+}-deficient, Mg^{2+}-rich solution, which blocks chemical transmission by lowering Ca^{2+} concentration and raising Mg^{2+} concentration. Depolarization of LE induced a slow depolarizing potential, without fast potentials following the LE spikes, in MA and JC (Fig. 6C). These results suggest that connections of the LE with the ipsilateral MA and JC neurons may be monosynaptic chemical and also electrical, as summarized in Fig. 13.

Conversely, we also explored synaptic inputs to LE from the ipsilateral MA and JC neurons. Firing of the ipsilateral MA induced IPSPs in LE (Fig. 7A). When ganglia were bathed in a Ca^{2+}, Mg^{2+}-rich solution, IPSPs following MA spikes in a one-for-one fashion were induced in LE (Fig. 7B). Also, in a Ca^{2+}-deficient, Mg^{2+}-rich solution, MA firing accompanying depolarization induced only slow depolarization of LE, while hyperpolarization of MA induced slow hyperpolarization of LE (Fig. 7C). These results suggest that the connection of the ipsilateral MA with LE is monosynaptic chemical and electrical (Fig. 13). Injection of a hyperpolarizing current into the ipsilateral JC also induced hyperpolarization of LE (Fig. 8A1, C1). Firing of the ipsilateral JC accompanying depolarization induced fast biphasic potentials following the JC spikes one-for-one in LE (Fig. 8A2, C2), in addition to slow depolarization (data not shown). These fast potentials were induced even when buccal ganglia were bathed in a Ca^{2+}, Mg^{2+}-rich solution (Fig. 8B) or a Ca^{2+}-deficient, Mg^{2+}-rich solution (Fig. 8D). These results suggest that the connection of the ipsilateral JC with LE is only electrical (Fig. 13).

The connection of MA with LE is similar to that of B4/5 with B51 in *Aplysia californica* (Plummer and Kirk, 1990). However, B51 does not produce monosynaptic EPSPs in B4/5 and has only electrical connections with it.

Role of LE in the feeding CPG

Prolonged depolarization of LE with long DC currents produced rhythmic activity in LE plateau potentials (Fig. 9). LE plateau activity was then usually accompanied by JC firing activity and weak MA activity, suggesting that LE may contribute to generation of the firing patterns of these neurons during rhythmic responses.

We therefore explored whether the spike activity of LE affects firing-pattern generation in the ipsilateral MA and JC neurons during the rhythmic feeding response. In the present experiments, electrical stimulation of nerves rather than taste stimulation (Nagahama and Takata, 1990) was used to activate the feeding CPG. Repetitive electrical stimulation of the esophageal nerve produced feeding-like patterned firing activities in MA and JC, in which JC began firing after the maximum frequency of the burst of spikes in MA (Fig. 10A). The MA spikes then often became very small or disappeared at the maximum frequency, probably because of inactivation of ion channels by working at an

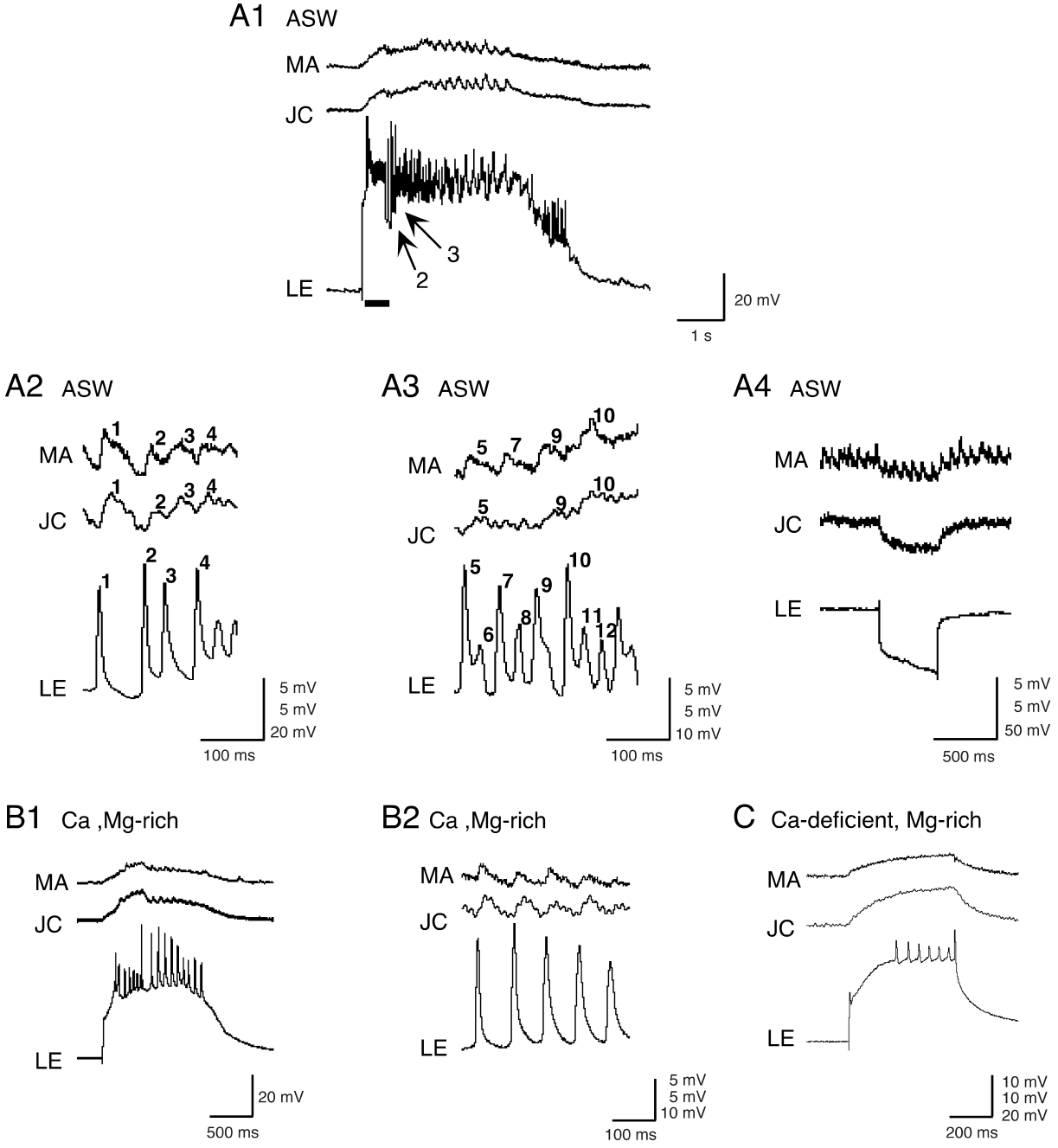

Fig. 6. Synaptic effects of LE on the ipsilateral MA and JC. **A1)** sustained long-lasting depolarization induced in the ipsilateral MA and JC when a plateau potential was produced in LE by application of a brief current (bar). **A2)** a faster-speed recording of the part of A1 indicated by arrow 2, showing that depolarizing potentials following LE spikes one-for-one were always induced in MA and JC. **A3)** a faster-speed recording of the part of A1 indicated by arrow 3, showing that depolarizing potentials induced in MA and JC followed the large LE spikes one-for-one (spikes 5, 9 and 10), but not the small LE spikes (spikes 6, 8, 11, and 12). Spike 7 of LE induced a depolarizing potential in MA but not in JC. **A4)** hyperpolarizing potentials in MA and JC induced by hyperpolarization of the ipsilateral LE with a DC current. **B1)** sustainment of long-lasting depolarization in the ipsilateral MA and JC following LE plateau potentials in ganglia bathed in a Ca^{2+}, Mg^{2+}-rich solution. **B2)** a faster-speed recording of a part of B1, showing that the depolarizing potentials following LE spikes one-for-one were still induced in MA and JC, even when the ganglia were bathed in a Ca^{2+}, Mg^{2+}-rich solution. **C)** a slow depolarizing potential without fast potentials following LE spikes induced in the ipsilateral MA and JC when LE was depolarized in ganglia bathed in a Ca^{2+}-deficient, Mg^{2+}-rich solution.

extremely high frequency. Although depolarization of JC was usually observed in the period of the maximum frequency of MA firing during the food-induced response (Nagahama and Takata, 1990), it was not observed here. This may be due to a large inhibition of JC by MA firing at an extremely high frequency. LE also showed rhythmic depolarization almost simultaneously with MA and JC, and firing on the plateau potentials occurred in the same period

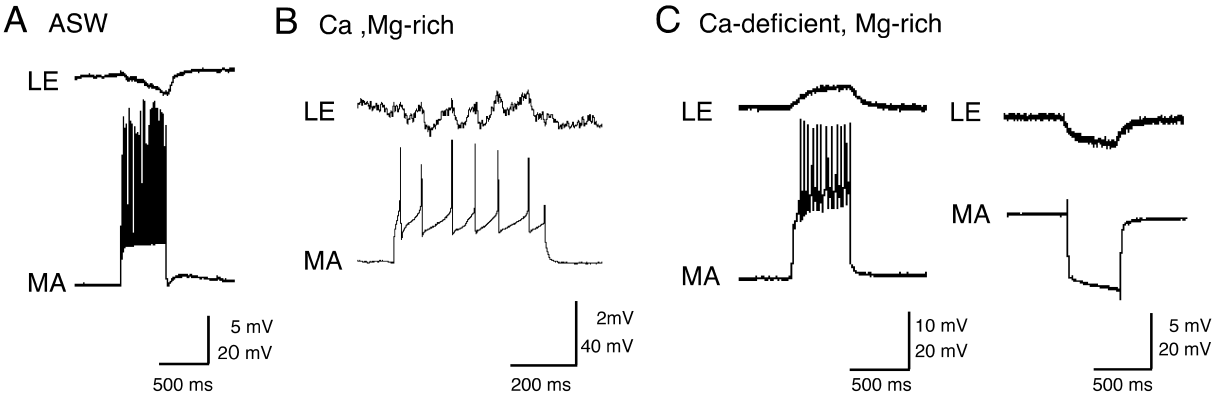

Fig. 7. Synaptic effects of the ipsilateral MA on LE. **A)** sustained inhibitory postsynaptic potentials (IPSPs) induced in LE when the ipsilateral MA was fired with depolarizing current. **B)** IPSPs induced in LE following MA spikes one-for-one when the ipsilateral MA was fired in ganglia bathed in a Ca^{2+}, Mg^{2+}-rich solution. **C)** slow depolarization (left) and slow hyperpolarization (right) induced in LE when the ipsilateral MA was depolarized and hyperpolarized with DC currents, respectively, in ganglia bathed in a Ca^{2+}-deficient, Mg^{2+}-rich solution.

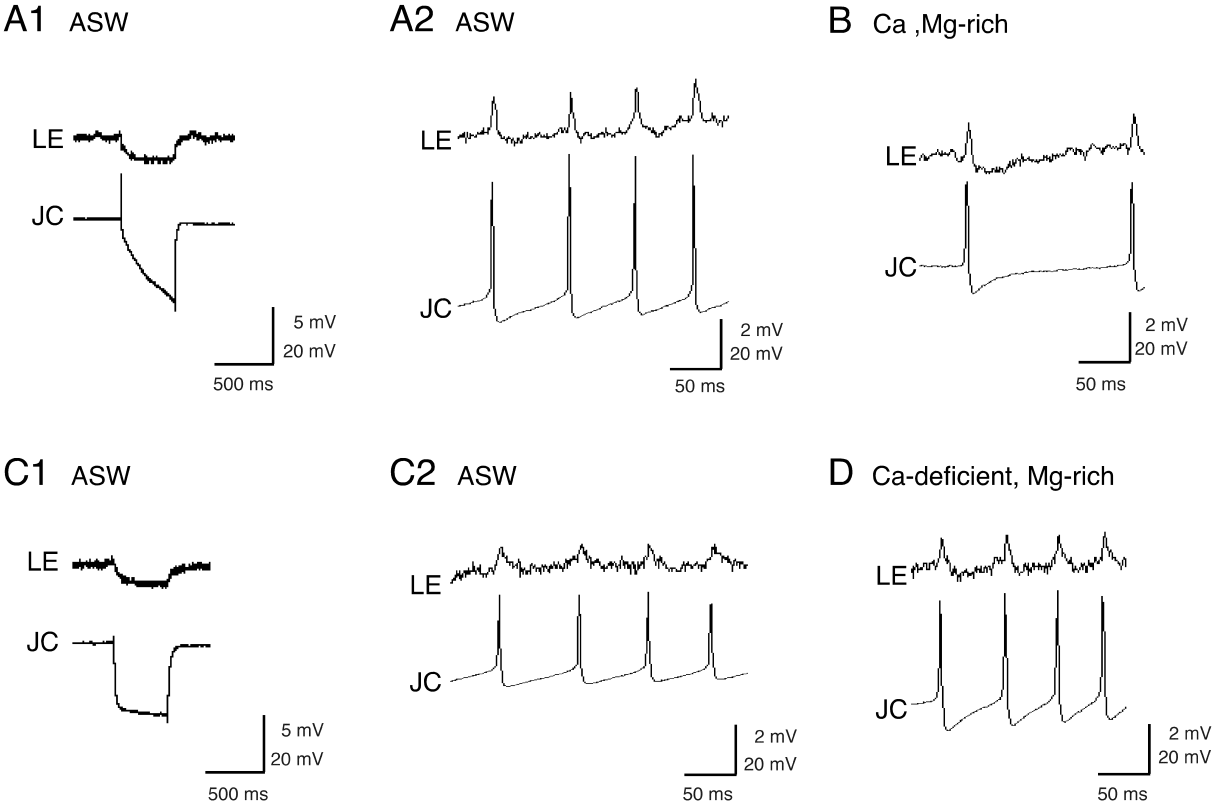

Fig. 8. Synaptic effects of the ipsilateral JC on LE. **A1 and C1)** hyperpolarization of LE induced when the ipsilateral JC was hyperpolarized with DC currents. **A2 and C2)** fast biphasic potentials induced in LE following the ipsilateral JC spikes one-for-one when the ipsilateral JC was fired. At this time, slow depolarization of LE was also induced. **B and D)** fast biphasic potentials induced in LE following the ipsilateral JC spikes one-for-one when JC was fired in ganglia bathed in a Ca^{2+}, Mg^{2+}-rich solution and a Ca^{2+}-deficient, Mg^{2+}-rich solution, respectively.

as JC firing, which is involved in the later phase of radula retraction (Nagahama and Takata, 1988). LE plateau potentials with firing started after the maximum frequency of MA firing, though the onset of LE depolarization corresponded to that of MA depolarization at the maximum frequency of firing. Figs. 10B1 and 10B2 show that when LE fired during plateau potentials at each depolarizing phase, the duration of depolarization of MA and JC increased. Especially, there was no firing activity of JC in the absence of LE plateau potentials.

During the feeding-like response, we inhibited LE spike activity by applying long or short hyperpolarizing currents. When a long hyperpolarizing current was applied to LE, the rhythmic plateau potentials and spikes completely disappeared, though small rhythmic depolarizing potentials were still induced in LE (Figs. 11A and 11D). In addition, a short

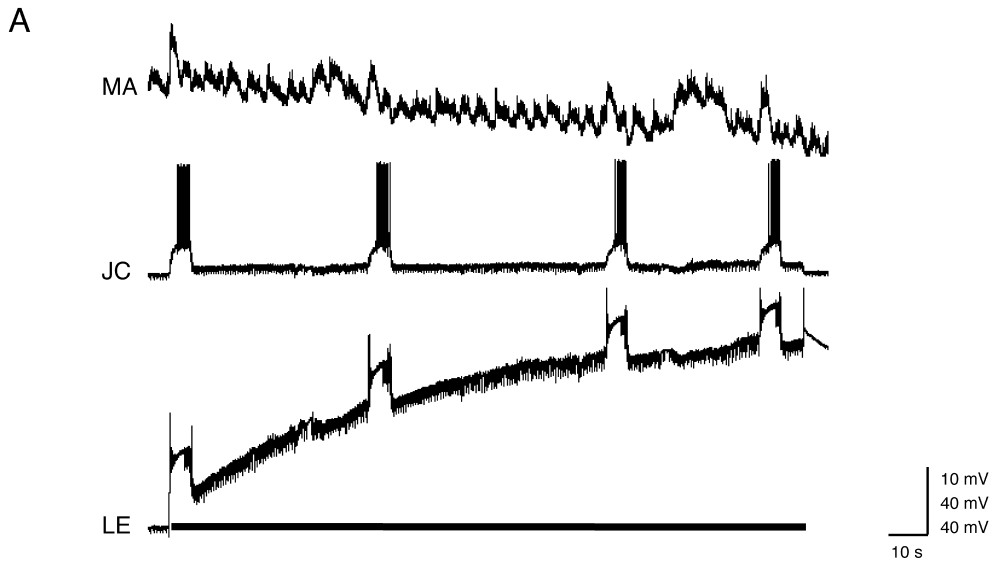

Fig. 9. Rhythmic plateau potentials elicited in LE by its prolonged depolarization with a long DC current (10 nA, 180 s, bar). At this time, LE activity was usually accompanied by activity of JC and weak activity of MA. The large positive change in membrane potential during depolarization resulted from trouble with the bridge balance of the amplifier.

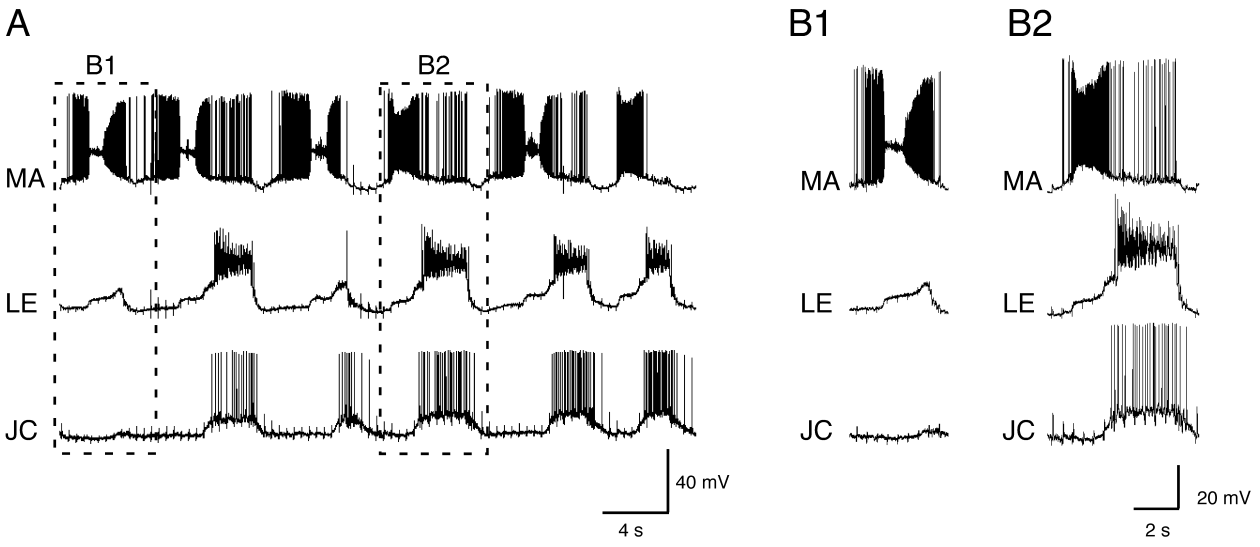

Fig. 10. Rhythmic patterned bursts of spikes in MA, JC, and LE during the feeding-like response. **A)** a simultaneous recording of rhythmic spike activities of MA, JC, and LE during the feeding-like response induced by repetitive electrical stimulation of the esophageal nerve. **B1 and B2)** faster-speed recordings of the depolarizing phases of these neurons, surrounded by rectangles B1 and B2 in A, respectively.

hyperpolarizing current was applied to LE at the expected time of plateau potentials during the rhythmic response (Fig. 12). In both cases, only the later phase of MA depolarization obviously disappeared during hyperpolarization, and firing duration decreased without changing the firing frequency of the earlier phase (Figs. 11B and 12). Then, only the later phase of JC depolarization, which mostly generates JC firing, also disappeared, though LE hyperpolarization tended to hyperpolarize JC largely by the electrical coupling (Figs. 11D and 12). These results suggest that LE may be an element in the feeding CPG circuit, and that the plateau potentials that supply depolarizing outputs of constant duration and intensity to the ipsilateral MA and JC may contribute to generation of the later phase of the depolarization of MA and JC during the feeding response. The small depolarizing inputs to LE remaining during hyperpolarization may come from some presynaptic neuron, such as a rhythm-generating neuron in the CPG circuit, and may contribute to triggering the constant plateau potentials of LE during the feeding response.

In contrast, when a large hyperpolarizing current was applied to LE, the rhythmic activities of LE were completely suppressed, and MA spike activities then disappeared, though the rhythmic small depolarization was still induced in MA (Fig. 11C). When we consider the other result that prolonged depolarization of LE caused the rhythmic activity of

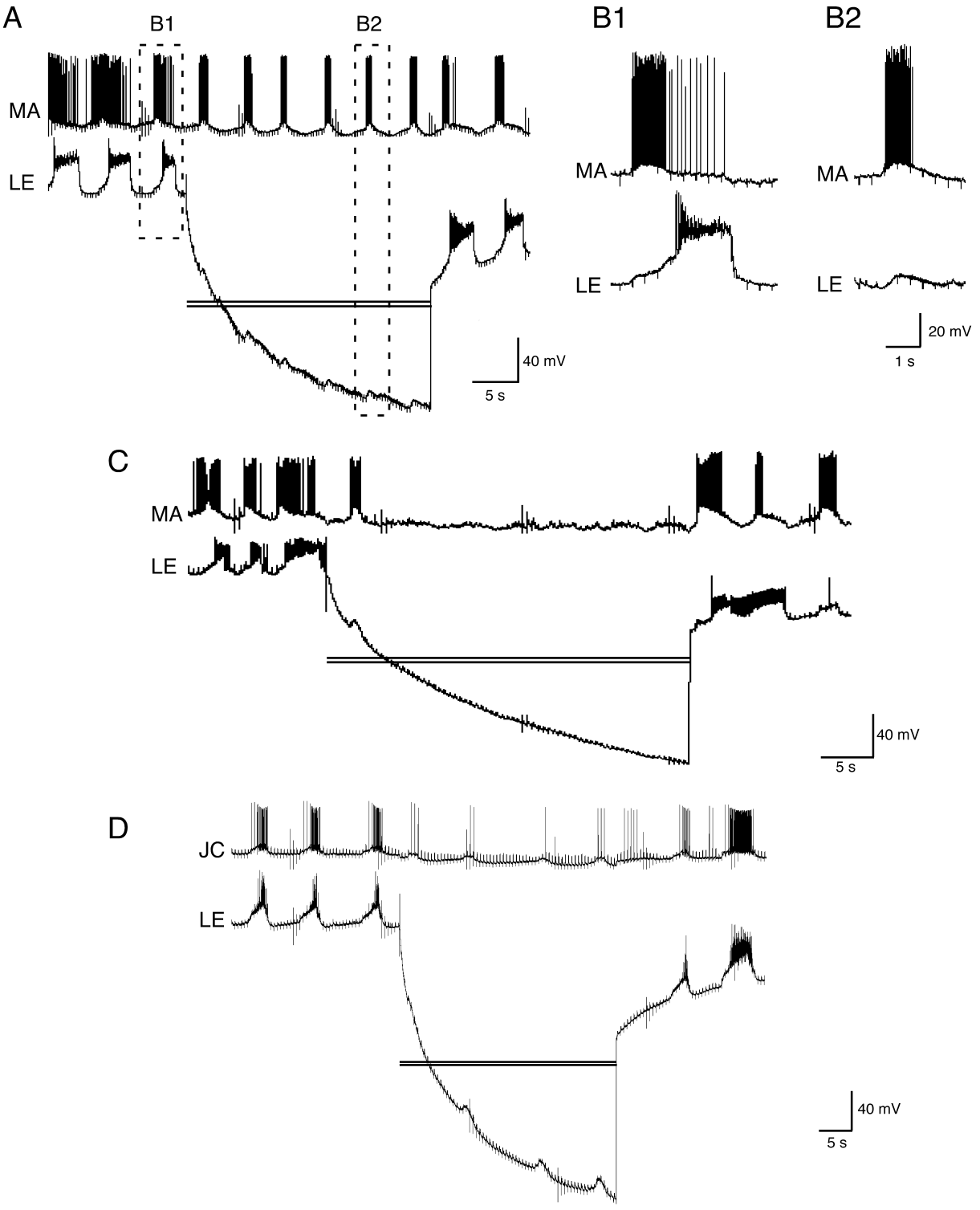

Fig. 11. Effects of inhibition of LE spike activity on rhythmic patterned bursts of spikes in MA and JC during the feeding-like response. **A)** a simultaneous recording of the rhythmic spike activities of MA and LE in the ipsilateral side of the ganglia when a hyperpolarizing current was applied to LE (double line) during a feeding-like response induced by repetitive electrical stimulation of the esophageal nerve. **B1 and B2)** faster-speed recordings of the typical depolarizing phases before and after hyperpolarization of LE, which are surrounded by the rectangles in A. The large negative changes in membrane potential during LE hyperpolarization resulted from trouble with the bridge balance of the amplifier. **C)** a simultaneous recording of rhythmic spike activities of MA and LE in the ipsilateral side of the ganglia when a large hyperpolarizing current was applied to LE (double line). **D)** a simultaneous recording of rhythmic spike activities of JC and LE in the ipsilateral side of the ganglia when a hyperpolarizing current was applied to LE (double line) during the feeding-like response.

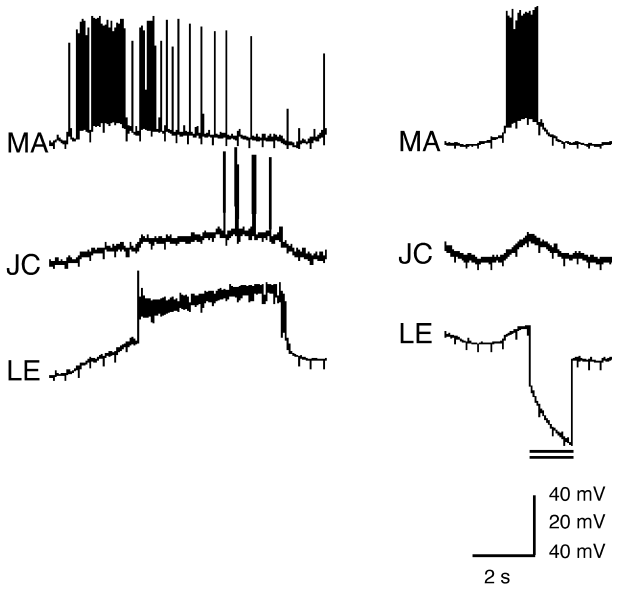

Fig. 12. Effects of short inhibition of LE activity on MA and JC firings during the feeding-like response. Left simultaneous recordings show spike activities of MA, JC, and LE at one depolarizing phase during the rhythmic response. In the right simultaneous recordings, a short hyperpolarizing current was applied to LE at the expected time of the plateau potential (double line).

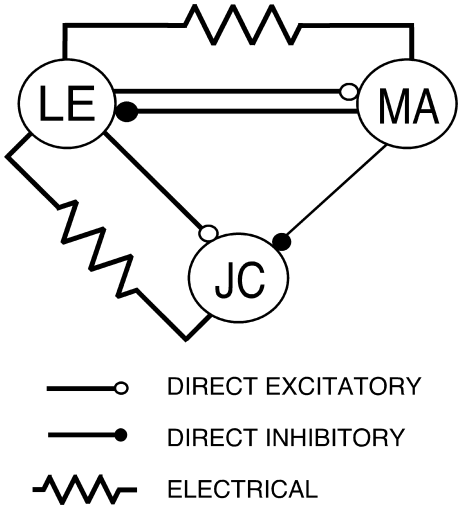

Fig. 13. Diagram summarizing synaptic connections between the neurons discussed in this experiment. Open circles: direct excitation. Filled circles: direct inhibition. Resistors show electrical connections. MA has a monosynaptic inhibitory connection with JC (Nagahama and Takata, 1989).

the plateau potentials (Fig. 9), this result suggests that a large hyperpolarization of LE may inhibit some electrically coupled neuron that has rhythm-generating capability. The small rhythmic depolarization of MA during LE hyperpolarization may be induced by some other rhythm-generating neuron.

DISCUSSION

Both neuronal and non-neuronal excitable cells have been reported to have the ability to generate plateau potentials (Russell and Hartline, 1977, 1978; Llinás and Sugimori, 1980a, b; Noble, 1984; Susswein and Byrne, 1988; Hounsgaard and Kiehn, 1989; Plummer and Kirk, 1990; Hurwitz and Susswein, 1996; Kiehn, 1991). In the present experiments, we found that the LE neuron has the ability to generate plateau potentials lasting for several seconds. LE also showed a step-like current-frequency relationship, a characteristic of the generation of plateau potentials. In most cases, generation of plateau potentials has been reported to require extracellular calcium ions (Tazaki and Cooke, 1979; Schwindt and Crill, 1984; Hounsgaard and Kiehn, 1985; Rekling and Feldman, 1997). However, LE plateau potentials could be generated even when ganglia were bathed in a Ca^{2+}-free solution, suggesting that generation of LE plateau potentials may not require extracellular calcium ions. In *Aplysia californica*, several neurons have been reported to generate plateau potentials, and extracellular calcium ions seem to be unnecessary for B51 and B64 to generate them, though other neurons require calcium ions (Plummer and Kirk, 1990; Hurwitz and Susswein, 1996).

Plateau potentials are classified into two types, endogenous and conditional, depending on the generating mechanism. Endogenous plateau potentials are caused by an intrinsic membrane property, whereas conditional ones are caused by some synaptic input (Dickinson and Nagy, 1983; Nagy and Dickinson, 1983; Israel *et al*., 1990; Hartline and Graubard, 1992; Straub *et al.,* 2002). In *Aplysia californica*, the generation mechanism of plateau potentials in B51 and B64 may be endogenous, while the mechanism in B31/32 may be conditional (Plummer and Kirk, 1990; Hurwitz and Susswein, 1996; Dembrow *et al*., 2004). We therefore explored the mechanism of generation of LE plateau potentials. LE could produce plateau potentials even when the ganglia were bathed in Ca^{2+}, Mg^{2+}-rich solutions instead of Ca^{2+}-free solutions. Ca^{2+}-free and Ca^{2+}, Mg^{2+}-rich solutions block chemical and polysynaptic transmission, respectively. Thus the plateau potentials may be generated by an endogenous property of LE, though we cannot eliminate the possibility that some neuron electrically coupled to LE contributes to the sustained depolarization of LE.

In the present experiments, we explored the generation properties of the long-lasting LE plateau potentials. We showed that LE produces constant plateau potentials of fixed duration and depolarizing size, independently of the length or intensity of the excitatory synaptic inputs, when the length and the intensity are appropriate (current conditions of >200 ms, >20 nA). These constant properties may be explained as a basic property of endogenous plateau potentials (Russell and Hartline, 1982). Some ionic channels, probably excluding calcium channels, may contribute to generation of LE plateau potentials. An ionic mechanism generating a constant potential of duration lasting several seconds is very interesting, and LE plateau potentials will be suitable for future studies of this type of ionic channel.

In order to learn the functional role of LE in the feeding neural circuit, synaptic connections between LE and identified buccal neurons were explored. LE spikes produced monosynaptic EPSPs in the ipsilateral JC and MA, in addition to electrical coupling between LE and both of them. Conversely, the ipsilateral MA produced monosynaptic

IPSPs in LE, though the ipsilateral JC only produced fast electrical potentials in LE. In order to induce the feeding-like response, electrical stimulation of the esophageal nerve rather than taste stimulation was used (Nagahama and Takata, 1990). LE showed rhythmic depolarization almost simultaneously with MA and JC, and LE firing during plateau potentials occurred in the same period as JC firing, which is involved in the later phase of radula retraction (Nagahama and Takata, 1988). When we hyperpolarized LE during the feeding-like response, LE plateau potentials disappeared, and the later phase of depolarization disappeared in MA and JC. The earlier firing phase at high frequency remained in MA, while the small depolarizing phase remained in JC. Therefore, LE may be one of the neurons constituting the feeding CPG circuit and may contribute to generation of the later phase of depolarization in MA and JC. Considering our previous results (Nagahama and Takata, 1990; Nagahama *et al*., 1999), the present results indicate that at least two sequential synaptic inputs may produce JC depolarization. The LE plateau potential may produce the later phase generating firing activity during the ingestive response, while the earlier phase may be masked by inhibitory inputs from MA. In contrast, during the rejective response, a decrease in the inhibitory effects of MA may restore the earlier depolarizing phase and advance JC firing.

A rhythmic small depolarization was still induced in LE during hyperpolarization, suggesting that LE may not contribute to generation of the feeding rhythm. This small depolarization may represent excitatory inputs from some presynaptic neuron that triggers the plateau potential. In addition, we found that LE plateau potentials with firing started after the maximum frequency of MA firing, though the onset of LE depolarization corresponded to that of the MA depolarizing phase that induced the maximum frequency of firing. It is possible that generation of the plateau potentials was delayed by the inhibitory effects of MA in a manner similar to the delay in onset of JC firing (Nagahama and Takata 1989, 1990). In support of this, during LE hyperpolarization the onset of the small JC depolarization almost corresponded to that of the small LE depolarization, suggesting that these neurons may simultaneously receive excitatory inputs in the earlier phase (Fig. 11D). If this is the case, during the rejective response, LE plateau potentials may advance as a result of a decrease in the inhibitory effects of MA. In a future study, we should explore the active phases of LE plateau potentials during the ingestive and the rejective responses induced by the taste application of seaweed extracts. In the present experiments, we found that the long depolarization of LE induced rhythmic plateau potentials in LE and rhythmic firing in JC (Fig. 9). Furthermore, the hyperpolarization of LE with large currents terminated the feeding rhythm completely in LE, but incompletely in the MA (Fig. 11C). These results suggest that some neuron electrically coupled to LE may have a rhythm-generating property that partly contributes to the basic feeding rhythm.

The B51 neuron in *Aplysia californica* projects a major axon into the radular nerves and can produce plateau potentials. In addition, this neuron receives monosynaptic inhibitory inputs from B4/5, equivalent to MA *in Aplysia kurodai*, and fires at the radula-retraction phase during the feeding response (Evans and Cropper, 1998; Hurwitz and Susswein, 1996; Plummer and Kirk, 1990). These properties of B51 are very similar to those of LE in *Aplysia kurodai*, suggesting that LE may be equivalent to B51. However, we also found that LE and B51 had different connections with equivalent buccal neurons in the two species, MA and B4/5, in which B51 only has electrical coupling with the B4/5, whereas LE has both excitatory chemical synaptic connections and electrical coupling with MA. We also found in the two species of *Aplysia* a discrepancy in the number of equivalent MA and B4/5 neurons in the buccal ganglia (Gardner, 1971; Nagahama and Takata, 1989). Our recent study furthermore suggests a discrepancy in the location of the cell bodies of the mechanoafferent ICBM neurons in the two species (Rosen *et al*., 1991; Narusuye and Nagahama, 2002). The peripheral functions of B51 have been studied in detail (Evans and Cropper, 1998). Further study of the peripheral function of LE will be necessary to confirm the equivalency between B51 and LE.

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