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Modulation of a Feeding Neural Circuit by Microinjection of K^+ Channel Expression Genes into a Single Identified Neuron in *Aplysia kurodai*

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ABSTRACT—In *Aplysia* buccal ganglion expression genes for voltage-dependent K^+ channels (AKv1.1a) were injected into one of four electrically coupled multi-action (MA) neurons that directly inhibit jaw-closing (JC) motor neurons and may cooperatively generate their firing pattern during the feeding response. Following the DNA injection, the firing threshold increased and the spike frequency at the same current decreased in the current-induced excitation of the MA neuron; indicating a decrease in excitability of the MA neuron. This procedure also reduced the firing activity of MA neurons during the feeding-like rhythmic responses induced by the electrical nerve stimulation. Moreover, the firing pattern in JC motor neurons was remarkably changed, suggesting the effective contribution of a single MA neuron or electrically coupled MA neurons to the generation of the firing pattern in the JC motor neurons. This method appears useful for exploring the functional roles of specific neurons in complex neural circuits.

Key words: neural circuit, modulation, injection, genes, potassium channels

INTRODUCTION

Knowing the functional roles of one or several specific neurons is very important for the study of invertebrate neural circuits. For this purpose, the lesioning of interesting neurons has been performed by intracellular injection of reagents such as proteolytic enzymes (Parnas and Bowling, 1977; Rosen *et al*., 1989) or dyes (Miller and Selverston, 1979; Eisen and Marder, 1982). In the latter case, neurons are photo-inactivated by shining an intense light after the injection. However, such methods are often insufficient to destroy the whole neuron because they largely depend on diffusion of the reagents and neuronal size and structure; and the killing of the cell may affect surrounding neurons. However, recent molecular biological techniques enable us to reduce the excitability of a specific neuron, keeping it alive by overexpression of ion channels following injection of expression genes into the neuron (Kaang *et al*., 1992; Whim and Kaczmarek, 1998; Chang *et al*., 2000; Lee *et al*., 2000).

K^+ channels are known to contribute to the generation of the resting potential, excitability, and the shape of action potentials (Catterall, 1988; Jan and Jan, 1989). In the marine gastropod *Aplysia californica*, several genes for voltage-dependent K^+ channels have been cloned and their properties investigated (Pfaffinger *et al*., 1991; Quattrocki *et al*., 1994; Zhao *et al*., 1994). Plasmid vectors expressing genes in *Aplysia* neurons (pNEX) have also been constructed and a few types of K^+ channels have been demonstrated to be overexpressed in specific neurons (Kaang *et al*., 1992; Kaang, 1996). The Japanese species, *A. kurodai*, feeds on seaweed with rhythmic patterned movements of the jaws and radula (Nagahama and Shin, 1998). Several neurons for the feeding behavior have been identified in the symmetrical buccal ganglia (Nagahama and Takata, 1987, 1988, 1989). Four electrically coupled multi-action neurons (MA1a-c and MA2) are considered to be components of the central pattern generator circuit for the feeding responses (Nagahama and Takata, 1989, 1990). Their spikes induce monosynaptic inhibitory post-synaptic potentials (IPSPs) in

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the ipsilateral jaw-closing (JC) motor neurons and may cooperatively generate the delay of the firing onset of the JC motor neurons at each depolarizing phase during the rhythmic feeding responses.

In the present experiments, we used the cDNA of a voltage-dependent AKv1.1a channel, cloned from the nervous system of *A. californica*. This channel shows rapid inactivation (Pfaffinger *et al*., 1991; Kaang *et al*., 1992). The present methods could reduce the excitability of a single MA neuron by injection of the channel expression genes and we explored the contribution of the MA neurons to the generation of the firing pattern in the JC motor neurons.

MATERIALS AND METHODS

A. kurodai weighing over 50 g were maintained in an aquaria filled with artificial sea water (ASW) at 14°C. Animals were dissected under Mg^{2+} anesthesia and the isolated preparations comprising the buccal ganglia, the cerebral ganglia and the buccal muscles were used. The cerebral-buccal connectives (CBC) arising from the cerebral ganglia were left intact, but the other cerebral nerves were severed. The buccal mass was removed from the head region and then cut into halves along the midline to separate the paired symmetrical musculature innervated by buccal nerves 2 and 3; the other peripheral buccal nerves were severed. The ganglia and muscles were pinned to the Sylgard surface of the recording chamber filled with ASW containing 470 mM NaCl, 11 mM KCl, 11 mM $CaCl_2$, 25 mM $MgSO_4$, 25 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.8~7.9), and the sheath overlaying the buccal ganglia was surgically removed. Intracellular recordings from individual neurons were performed using conventional electrophysiological techniques. Plasmid DNAs used for microinjection were prepared following the method of Kaan *et al*. (1992). Glass microelectrodes filled with a microinjection solution (10–25 MΩ) were inserted into cell bodies of the neurons for electrophysiological measurement and the injection of pNEX-cDNA. Microinjection solutions contained 1 mg/ml plasmid DNA, 10 mM Tris-HCl (pH 7.3), 100 mM NaCl, and 1.5% fast green (Kaang, 1996). After the excitability experiments, the pipette solution was injected with pressure pulses (40–60 psi, 20 msec, 5 pulses). The nerves were electrically stimulated with a polyethylene suction electrode (diameter 100–200 μm). All experiments were performed at room temperature. The bath solution was then replaced with the culture medium; a mixture of isotonic L15 and *Aplysia* hemolymph in equal volumes. The isotonic L15 contained L15 plus appropriate salts with final concentrations of 400 mM NaCl, 11 mM KCl, 11 mM $CaCl_2$, 27 mM $MgSO_4$, 27 mM $MgCl_2$, and 2 mM $NaHCO_3$. In addition, 6 g/l D(+)-glucose, 0.1 g/l L-glutamate and 50 μg/l penicillin-streptomycin were added to the L15. Preparations were incubated at 20°C for 18 hr and washed several times with fresh ASW. The electrophysiological experiments were repeated.

RESULTS AND DISCUSSION

For a control, we injected the microinjection solution excluding pNEX-AKv1.1a (control solution) into the cell body of the MA neurons, and after incubation compared the excitability of the same neuron before and after injection. The average resting membrane potentials before and after injection of the control solution in the neurons studied were –56.2±3.9 mV and –58.3±2.4 mV (mean±SE, n=6), respectively; there was no significant difference between them (P>0.1, paired t-test). This result suggests that the condition of incubation may be acceptable for the experiments on neuronal excitability. Depolarizing current injection evoked spikes, their frequency increasing with increasing current intensity over the firing threshold. Fig. 1A-2 shows similar relationships between the current intensity and the spike frequency before and after injection of the control solution, suggesting that injection of the control solution does not change the excitability. In other preparations we injected the microinjection solution containing pNEX-AKv1.1a (DNA solution) into the MA cell body. After incubation we compared the excitability before and after injection. The average resting membrane potentials before and after the DNA injection were –57.3±3.0 mV and –56.3±3.4 mV (mean±SE, n=9), respectively; there was no significant difference between

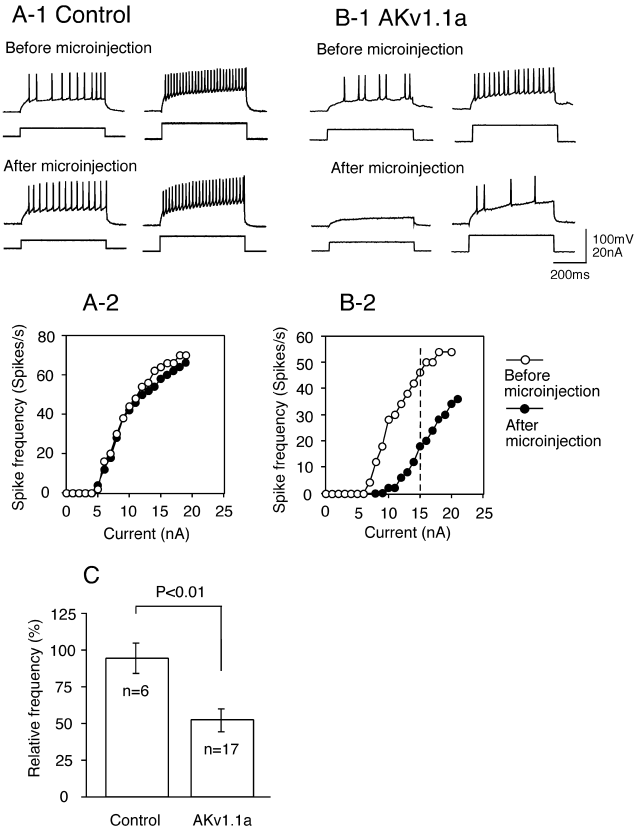

Fig. 1. Changes in excitability of MA neurons on injection of pNEX-AKv1.1a. MA neurons were excited with 500 ms of depolarizing current. A-1, B-1. Current-induced spikes in the same neuron before and after injection of a control solution (A) and a DNA solution (B). Recordings in A and B were obtained from two different preparations. A-2, B-2. Relationships between spike frequency and current intensity before and after injection of a control solution (A) or a DNA solution (B). Spike frequency was obtained from the number of spikes evoked with 500 ms of depolarizing current. For each neuron we explored the spike frequency in 4 series of trials and the averaged values were plotted. C. Comparison of the average relative frequencies for injection of control and DNA solutions. Bars represent ±SE. An example of the current intensity at which the relative frequency is calculated for each preparation is shown as a broken line in B-2. The relative frequencies were averaged for all preparations (n=6 for control solution and n=17 for DNA solution).

them (P>0.1, paired t-test). Further, spike shapes scarcely changed following the DNA injection. An example of the results of excitability in the same neuron before and after the DNA injection is shown in Fig. 1B (n=17). The threshold current intensity for firing increased greatly and the spike frequency at the same current intensity obviously decreased (Fig. 1B-2). To evaluate the effect of the additional DNA on excitability, the relative frequency of the post-injection of control or DNA solutions to the pre-injection was calculated at 2.5-fold the current intensity of the threshold for firing in the pre-injection. Fig. 1C shows a comparison of the average relative frequencies for injections of control and DNA solutions. The value significantly decreased following the DNA injection (P<0.01, two sample t-test), suggesting that the AKv1.1a channels may be overexpressed and the excitability of the MA neurons decreased. Following the DNA injection, depolarization induced by the same current occasionally decreased, probably indicating some current leakage through overexpressed K^+ channels.

Next, we explored whether a DNA injection affects the spike activity of MA neurons during the feeding-like response, which was induced by repetitive electrical stimuli (2 Hz) of the CBC as shown previously (Nagahama and Takata, 1990). Fig. 2A shows an example of the rhythmic firing activity of MA neurons induced by the CBC stimulation before and after injection of the control solution (n=6). No change was observed in any of the MA responses; whereas following a DNA injection the firing activity of the MA neuron at each depolarizing phase always decreased although the rhythmic response was still induced (Fig. 2B, n=19). The size of the depolarization at each rhythmic phase occasionally decreased following the DNA injection (5 in 19 preparations) although in many cases the depolarization was unchanged (Fig. 3B). Figs. 2C and D show comparisons of the average values of the spike frequency and the burst duration of the MA at each depolarizing phase during the feeding-like response for injection of the control and DNA solutions in all preparations. These values significantly decreased following injection of the DNA solution (P<0.001, paired t-test), while they did not change following injection of the control solution (P>0.1). These results suggest that under conditions of the overexpression of AKv1.1a in a single MA neuron, the firing activity of the MA might be especially suppressed in the feeding-related neural circuit composed of many neurons.

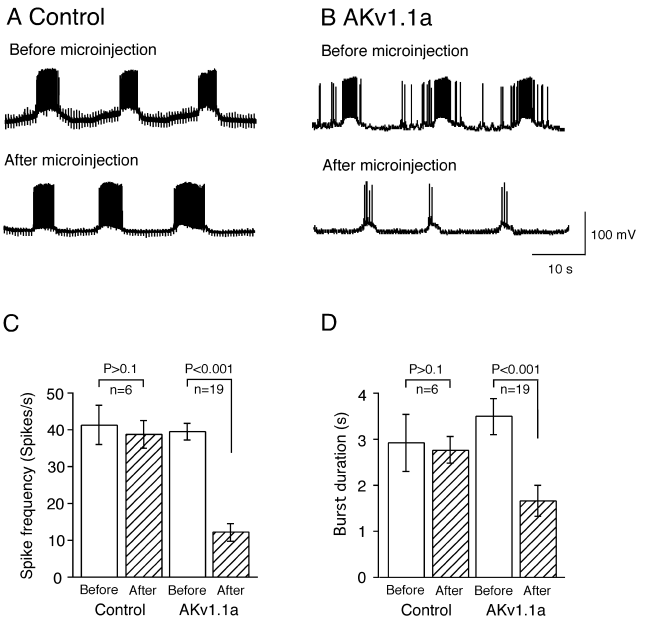

Fig. 2. Changes in rhythmic spike activity in MA neurons during feeding-like responses on injection of pNEX-AKv1.1a. A, B. Rhythmic firing activity in an MA neuron before and after injection of control (A) and DNA (B) solutions. To induce feeding-like responses, the cerebral-buccal connective was electrically stimulated at 2 Hz with a suction electrode. Recordings in A and B were obtained from two different preparations. C. Comparison of average spike frequencies at each depolarizing phase during the feeding-like response before and after injection of the control or DNA solution. D. Comparison of the average burst duration at each depolarizing phase during the feeding-like response before and after injection of the control or DNA solution. For each preparation, the average value was evaluated from several successive bursts of spikes and the obtained values were averaged for all preparations. Bars represent ±SE.

Whether suppression of the excitability of a single MA neuron affects the rhythmic firing pattern in JC motor neurons during the feeding-like response was explored. Fig. 3A shows rhythmic firing activity in MA and JC neurons before and after DNA injection. In the control recording, both neurons underwent an almost synchronous depolarization but the JC motor neuron started firing after the MA neuron at high frequency, suggesting that the MA firing suppressed the JC firing (Figs. 3A-1, 3B-1). The next DNA injection into the same MA neuron obviously advanced the onset of the JC firing and increased its activity at each depolarizing phase (Figs. 3A-2, B-2). The MA depolarization was changed little following the DNA injection. A comparison of the average delay times of the JC firing before and after DNA injection for all preparations is shown in Fig. 3C. The delay significantly decreased following DNA injection into the MA neurons (P<0.002, n=9, paired t-test). These results suggest that inactivation of a single MA neuron greatly affects the firing patterns in JC motor neurons during the feeding-like response and the MA neuron may markedly contribute to the generation of the firing delay of the JC motor neurons at each depolarizing phase.

The present study demonstrated that overexpression of the K^+ channels in a specific MA neuron could reduce the excitability of the neuron without really changing the spike shape. Kaang *et al*. (1992) reported that overexpression of the same channels obviously changed the spike shape in some neurons of *A. californica*. The different effects on the spike shape may be caused by the types of channels existing in the membrane of individual neurons. The spike duration of the MA is essentially short and we may be not able to know the change in the spike shape. The present procedure could also demonstrate the large contribution of the neuron to the firing pattern of the JC motor neurons. In this

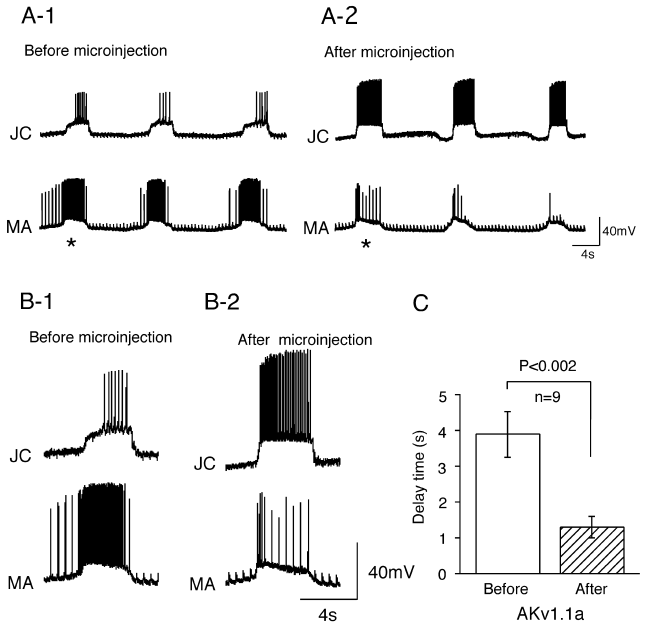

Fig. 3. Changes in firing patterns in JC motor neurons following suppression of the excitability of a single MA neuron by injection of pNEX-AKv1.1a. A. Simultaneous recordings of rhythmic spike activities in an MA neuron and a JC motor neuron during the feeding-like responses before (1) and after (2) DNA injection in the same preparation. Enlarged representations of the firing patterns in these neurons at the first depolarizing phase in A-1 and A-2 (asterisks) are also shown in B-1 and B-2. C. Comparison of the average delay time of the JC firing onset before and after DNA injection. The delay time of the onset of JC firing from the onset of each MA depolarization was evaluated from several successive bursts of spikes for each preparation, and the obtained values were averaged for all preparations. Bars represent ±SE.

case, the marked effects of inactivation of a single neuron on the firing patterns in JC motor neurons may result from the fact that four MA neurons are electrically coupled and the results may suggest the contribution of the electrically coupled MA neurons. However, we found that the overexpression of the AKv1.1a channels greatly affected spike generation without obviously reducing depolarization. Therefore, the present results suggest that the depolarization and firing activity of the other electrically coupled MA neurons are not affected because the AKv1.1a channels will be expected to be overexpressed in the single MA neuron alone. Then the method shown here may be useful for the investigation of the functional roles of a single neuron in complex neural circuits. It will be necessary to further explore the influence of AKv1.1a overexpression in a single MA neuron on the excitability of the other electrically coupled MA neurons.

It has been reported that neuronal expression genes cloned from other species of animals can be overexpressed in *Aplysia* neurons (Lee *et al*., 2000). Therefore, the excitability of a single neuron can be changed at will when the appropriate ion channel is chosen. Further, specific synaptic inputs to a single neuron can be modulated when an appropriate synaptic receptor is chosen. In future we can use this method to ascertain the functional roles of a single or a few neurons in neural circuits for food preference behavior in *Aplysia* (Nagahama and Shin, 1998; Nagahama *et al*., 1999).

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