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Ionic Dependence of the Axotomy-Induced Long-Lasting Firing in an Identified Crayfish Motoneuron

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ABSTRACT—I have reported previously that axotomy of an identifiable anal motoneuron of crayfish *Procambarus clarkii* induces a long-lasting firing and that a prolonged depolarizing pulse to its cut end can induce a similar response. In this study, I confirmed that this stimulus is comparable to axotomy; the frequency of stimulus-induced firing increases linearly with the stimulus intensity and its firing pattern is the same as that following axotomy. Then, when the cut end was bathed for more than 1 hr in test solutions, it was examined whether the stimulus to the cut end induces or blocks the response. Na^+-free saline ($Tris^+$ replaced Na^+) or TTX (3×10^{-7} M) reversibly blocked the response within 30 min. By contrast, Mn^{2+} saline (40 mM Mn^{2+} replaced Ca^{2+}) or Ca^{2+}-free salines (Mg^{2+} or 1 mM EDTA replaced Ca^{2+}) cannot block the response, but instead increased the firing frequency. These results obtained with stimulus were confirmed also by those with axotomy. I concluded that axotomy-induced firing, which occurs locally at its cut region, is primarily responsible for voltage-dependent Na^+ conductances, but not for Ca^{2+} ones.

INTRODUCTION

Axotomy or local application of colchicine (for insects, axotomy was performed by colchicine treatment) converts the normally non-spiking somata of crayfish (Kuwada and Wine, 1981), cockroach (Pitman *et al.*, 1972) and locust (Goodman and Heitler, 1979) motoneurons and interneurons into spiking somata within 4–7 days. Such axotomy-induced soma excitability has also been shown in some vertebrate neurons (for review, Titmus and Faber, 1990). The ionic basis of axotomy-induced soma excitability has been examined directly, using pharmacological manipulations of ionic conductances in these vertebrate (Titmus and Faber, 1990) and invertebrate neurons (Goodman and Heitler, 1979; Pitman, 1979; Kuwada, 1981). In all cases, the enhanced excitability appeared to depend on voltage-dependent Na^+ conductances.

On the other hand, I have reported previously in *P. clarkii* that an identifiable anal contractor motoneuron is capable of exhibiting prolonged firing ($\leq$ 67 min) following axotomy and that application of a depolarizing pulse to its cut end induces a similar firing response (Muramoto, 1993). From the fact that axotomy-induced firing in this motoneuron is blocked by a hyperpolarizing pulse to the end, it was assumed that the axotomy-induced firing occurs peripherally at the cut end (Muramoto, 1993). However, which ions are involved in producing this peripheral firing or whether there is a difference in ionic dependence between the peripheral excitability and soma excitability remains to be clarified.

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The aim of the present study is to explore the ionic dependence of axotomy-induced peripheral firing in this motoneuron. Depending on whether not only axotomy of the bathed end, but also application of the stimulus to the end induces or blocks the firing response when the transected end was bathed in various ionic solutions, I report that the axotomy-induced firing is dependent on Na^+ influx, and its enhancement is not affected by external Ca^{2+}-depletion. There is little information about such peripheral firing in motoneurons following axotomy except the present motoneuron (Kuwada and Wine, 1981; Muramoto, 1993; Titmus and Faber, 1990; Moffett, 1996). I discuss the enhancement of prolonged peripheral excitability following axotomy in this motoneuron and its possible function.

MATERIALS AND METHODS

Preparation

The anal motoneuron L (AML) of the crayfish *Procambarus clarkii*, which is capable of driving rhythmic anal contractions, appears to be T-shaped unipolar: its soma is located in the 6th abdominal ganglion (A6), one axonal process innervates the anal musculature via the posterior intestinal nerve (PIN) and the other process ascends the nerve cord to end in the first abdominal ganglion (A1) (Muramoto, 1993). Then, experiments were performed on the abdominal nerve cord preparation dissected from *P. clarkii*, which was obtained from a commercial source. This preparation is composed of a chain of 6 abdominal ganglia (A1–A6) linked by 5 inter-ganglionic connectives and PIN.

The preparation was placed in a small chamber filled with normal saline consisting of 208 mM NaCl, 5.4 mM KCl, 13.3 mM $CaCl_2$ and 2.6 mM $MgCl_2$, buffered with 10 mM Tris at pH 7.5, and kept at 10°–11°C by a bath temperature control system (DTC-200, Daiya

Medical Co., Japan). The chamber consisted of three compartments separated by a bank of grease (silicone lubricant); the partition between the first two adjacent compartments was usually placed between A2 and A3 or A3 and A4, while that between the last two adjacent ones was always placed between A5 and A6 (see Figs. 1, 5 and 6, inset). In the first compartment, the nerve cord was transected with scissors and its cut end was bathed in test solutions. The first and the middle compartments were used for application of stimulation without disturbing recording of PIN activity, as described below. The PIN activity was monitored in the end of the compartments.

Stimulation

The method for stimulation was the same as that described previously (Muramoto, 1993). The first two adjacent compartments were equipped with Ag-AgCl electrodes (S1 and S2 in Figs. 1, 5 and 6, inset), through which polarizing current were passed. In this study, the electrodes were bent like a ring, which allows them to contact well with the cord. Effects of test solutions or electrical stimulation on the cut end of AML can be examined by application of these stimuli to the nerve cord transected at various levels below A1 along its long axis, because AML runs through the nerve cord without bifurcation (Muramoto, 1993). However, in this study, these stimuli were usually applied to the stump of the connectives of A2 or A3. Namely, the one electrode was placed on this stump in the first compartment, while the other electrode was placed on the connectives rostral to A3 or A4 in the middle compartment (Figs. 5 and 6, inset). A battery-operated (1.5 V) stimulator with microammeter to monitor the current strength was connected to the electrodes. Either hyperpolarizing or depolarizing current (pulse) was applied to the stump of the cord through these electrodes, by changing the polarity by means of a switch placed in the circuit (for details, see Fig. 6 in Muramoto, 1993).

Bath application and test solutions

The normal saline in the first compartment was replaced by test solutions. Na^+-free solution was made up by replacing NaCl of the normal saline with Tris-HCl. In Ca^{2+}-free salines, $CaCl_2$ was replaced by $MgCl_2$ or 1 mM EDTA. In this study, these solutions were referred to as Mg^{2+} and EDTA solution, respectively. In TTX solution, a concentration of 3×10^{-7} M tetrodotoxin (TTX) was dissolved in the normal saline. In Mn^{2+} solution, 40 mM $MnCl_2$ replaced $CaCl_2$. All of these solutions were buffered with Tris at pH 7.5, except Mg^{2+} and TTX solutions that were pH 7.4–7.5.

Identification and recording

AML can be easily identified by its largest amplitude among units recorded from PIN (Muramoto, 1993). The discharge activity of AML was recorded extracellularly with a suction electrode from a distal branch of PIN as described previously (Muramoto, 1993). The AML activity was displayed on a pen recorder via an amplifier and stored on magnetic tape. Spike intervals were calculated with a signal processor (7TOTA, San-ei Instr. Co., Japan).

Expression of the results

Data, when quantified, were expressed as means ± SE and compared, when necessary with the Mann-Whitney U-test.

RESULTS

Axotomy- and stimulus-induced LLFs

The previous study (Muramoto, 1993) showed that transection (axotomy) of the nerve cord at any level below A1, or a more distal position, PIN can always induce a long lasting firing (LLF) in AML and a prolonged depolarizing pulse to the cut end of the nerve cord or to its intact one, is capable of inducing a similar firing response during the stimulation.

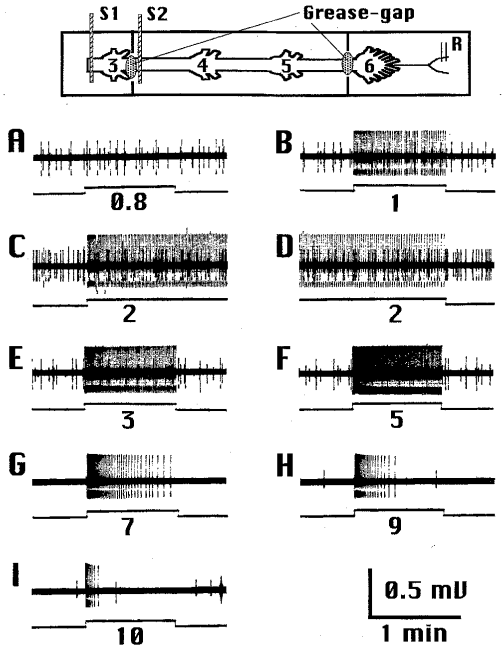

Fig. 1. Response of AML to depolarizing pulse. Upper inset represents schematic arrangement of the preparation composed of A3 (3) to A6 (6) with PIN, the stimulation electrodes (S1, S2) and the recording electrode (R). Note that the firing rate and the firing duration of an LLF in AML change with an increase in the intensity of the depolarizing pulse. Upper traces represent discharge activities (AML spikes are the largest). The upward displacement of lower traces indicates the duration of a depolarizing pulse (S1 is negative with respect to S2), which applied to the cut end of the connectives of A3 and the figure indicates the stimulus intensity (μA). **A-I**, other than **C** and **D**, are the same preparation. **C** and **D** are the initial and the last parts of an LLF, which lasted throughout the stimulation for 6 min.

The present study also shows that axotomy of the nerve cord (for example, Figs. 5E and 6D) or stimulation to its stump (Fig. 1) can elicit the LLF response. To determine whether there is any difference between these axotomy- and stimulus-induced LLFs or not, frequency and firing pattern of stimulus-induced LLFs were examined.

The stimulus to the stump of the nerve cord could induce a LLF in quiescent AML (Fig. 1B-I), and the stimulus applied during LLF could raise its firing rate (Figs. 5A and 7A, B). Less than 1 μA of stimulus failed to induce a firing response in AML in all cases (N = 6) (Fig. 1A). In the range of 1 to 6 μA, stimulation could always produce a tonic firing in quiescent AML, which lasted throughout the stimulation (Fig. 1B-F). AML showed a high-frequency firing at the beginning of the stimulation and then a progressive decline. The longest period of the stimulation examined in this study was 6 min, during which AML could actually retain a typical firing (Fig. 1C, D). At a stimulus of 7 μA, in the 3 cases out of 5 the firing response lasted throughout the stimulation (not shown), but in the others it failed to last during the stimulation as shown in Fig. 1G. Above 7 μA, there were no responses in 3 out of 5 cases, or even if AML fired in 2 out of 5 cases, the response did not last during the stimulation and the firing frequency to stimulus decreased (Fig. 1G-H). Then it was found that the range of stimulus for LLF generation throughout the stimulation was

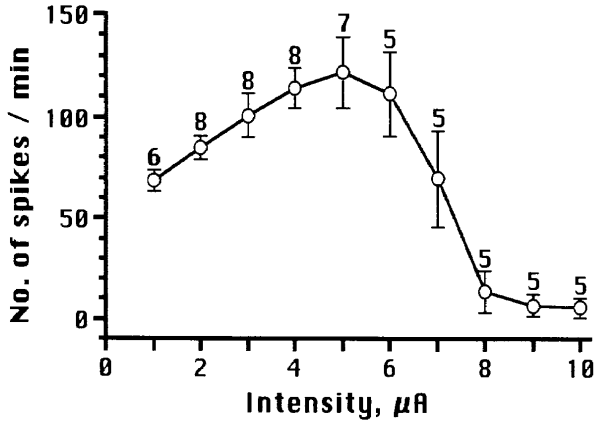

Fig. 2. Relationship between firing frequency of a stimulus-induced LLF in AML and stimulus intensity. Data (means ± SE) were plotted as the number of AML spikes during an LLF induced by depolarizing pulses (at 1 to 10 μA for 1 min), which applied to the stump of the connectives of A2 or A3. The figures above the bar SE indicate the number of the preparations. Note that in the range of 1 to 6 μA, frequency increases with an increase in the intensity (see Fig. 1 and the text).

among 1 and 6 μA.

Figure 2 depicts the relationship between the firing frequency and stimulus intensity. The firing rate of AML was calculated on the basis of the number of AML spikes in an LLF produced by various intensities of stimulus (at 1 to 10 μA, for 1 min). The mean frequency with SE at 1 μA of the stimulus was 1.1 ± 0.09 Hz (N = 6) and showed a largest value at 5 μA (2.0 ± 0.29 Hz, N = 7). Figure 2 shows that within the stimulus, 1–6 μA, the frequency increases linearly with an increase in the stimulus intensity (r = 0.90).

A stimulus-induced LLF has a characteristic firing pattern. The interspike intervals during an LLF were found to increase exponentially at first and to reach a steady state (regular interval) within 30 sec to 1 min, which was maintained during 1 to 6 min, for stimulus intensities among 1 and 6 μA (Fig. 3). Figure 4 shows the time course of changes in firing frequency during an LLF. A similar yet somewhat different time course was found depending on the intensity of the stimuli.

Stimulus-induced LLF was thus characterized by an initial high-frequency firing with an exponential increase in interspike intervals during the first minute (Fig. 3), which was followed by a further slow decline in frequency over the successive time course (Fig. 4). The characteristics of the firing pattern for the stimulus-induced response were found to be similar to the characteristics previously reported for an axotomy-induced response (Muramoto, 1993).

These results indicate that stimulus to the cut end of the nerve cord can induce the similar firing activity in AML to that following axotomy, supporting the previous idea that the prolonged firing of AML following axotomy is based on prolonged depolarization that occurs at its cut end (Muramoto, 1993).

Ionic dependence of axotomy-induced LLF

In the axotomy experiment, the cut end must be repeatedly sectioned, but the stimulation experiment permits pro-

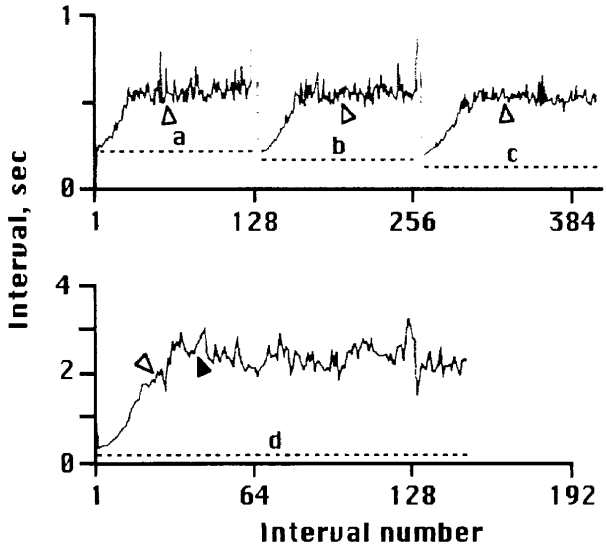

Fig. 3. The plot of sequential interspike intervals against interval number during a stimulus-induced LLF in AML. Dotted lines indicate the duration of the applied depolarizing pulse. In **a-c**, the stimulus was applied for 1 min every 1 min to the cut end of the connectives of A2 and its intensity was as follows: **a**) 1 μA, **b**) 2 μA, **c**) 3 μA. In **d**, the stimulus (6 μA) was applied for 6 min to the cut end of the connectives of A3. The interspike intervals were calculated with a signal processor; clock, 1 msec. White and black triangles represent 30 sec and 1 min from the beginning of the LLF, respectively. Note that intervals reach almost a constant level within 1 min.

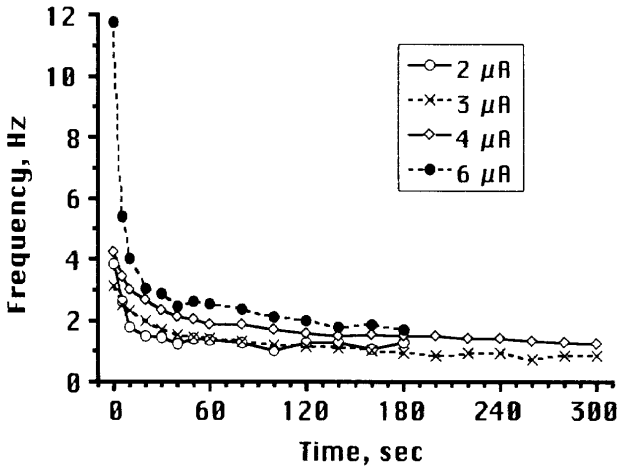

Fig. 4. Time course of the changes in firing frequency during a stimulus-induced LLF in AML. Frequency of 4 preparations obtained by various intensities of stimulus (at 2, 3, 4 or 6 μA for 180 sec or 300 sec) applied to the cut end of the connectives of A3 was calculated by measuring interspike intervals at 10- to 20-sec intervals during a period of the stimulation. Note the high-frequency firing during the first minute of the LLFs.

longed (≥ 1 hr) observation of test solutions to the same cut region of AML and quantitative analysis. The ionic dependence of an axotomy-induced LLF was elucidated by both axotomy and stimulation experiments, but quantitative differences in the firing activity to various test solutions were exclusively examined by the stimulation experiment. In both experiments,

after exposures of the cut end of the nerve cord to test solutions for 60 min or more, the examination was made as to whether transection of the cut end or application of depolarizing pulses (at 3 to 5 μA for 20 to 60 sec) to this end can induce or block an LLF response.

Effect of sodium ions

A stimulus-induced LLF was reversibly blocked when the cut end of the nerve cord was bathed in sodium free solution ($Tris^+$ replaced Na^+). This was illustrated in Fig. 5, where the solution was applied during an AML firing following axotomy. In normal saline (Fig. 5A), AML always showed high-frequency firing in response to a depolarizing pulse, but after the bath application its discharge activity soon stopped and its firing frequency to stimulus rapidly declined (Fig. 5B). After bathing the preparation for 20 min (Fig. 5C), the response to stimulus was no longer visible. In all cases (N = 6), the stimulus-induced LLFs were completely blocked within 20 min of the bath application (14.8 ± 1.74 min). After returning to normal saline, AML resumed its firing response to stimulus in 12.7 ± 1.20 min (in the range of 10 to 16 min, N = 6) (Fig. 5D). Such blockage of an AML firing to sodium-free saline was also confirmed in an axotomy-induced LLF. When the cut end of AML bathed in sodium-free saline was transected, the axotomy-induced LLFs were reversibly blocked (Fig. 5E-G). It took 18 ± 7.3 min (in the range of 5 to 30 min, N = 3) to block the firing response. After return to the normal saline, axotomy was able to restore the response (Fig. 5G).

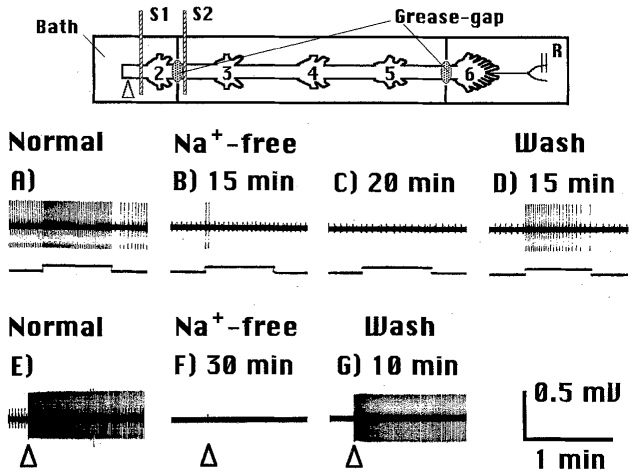

Fig. 5. Effect of Na^+-free saline on AML activity. Upper inset represents schematic arrangement of the preparation composed of A2 (2) to A6 (6) with PIN, the bath (Bath) of which solution was replaced, the position of axotomy (Δ), the stimulation electrodes (S1, S2) and the recording electrodes (R). **A-D** and **E-G** are stimulus- and axotomy-induced characteristics, respectively. (**A**) High frequency firing in AML to a depolarizing pulse applied to the stump of the connectives of A2, in which the stump was bathed in normal saline and the stimulus was applied during an AML activity following axotomy. (**B**) Diminution in the frequency of AML activity 15 min and (**C**) its blockage 20 min after bath application of Na^+-free solution ($Tris^+$ replaced Na^+ of normal saline). (**D**) Restoration of stimulus-induced AML response 15 min after the bath was washed with normal saline. (**E**) Axotomy-induced AML response (largest spikes) when the stump was bathed in normal saline. (**F**) Blockage of axotomy-induced AML response 30 min after bath application of Na^+-free solution. (**G**) Restoration of axotomy-induced AML response 10 min after the bath was washed with normal saline. **A-D** and **E-G** are different preparations. In **A-D**, upper traces represent discharge activities (AML spikes are the largest) and the upward displacement of lower traces indicates the duration of a depolarizing pulse (4 μA, S1 is negative with respect to S2). In **E-G**, the bathed stump of the connectives of A2 was transected at triangles repeatedly (at Δ in upper inset).

TTX, as well as Na^+-free solution, can reversibly block stimulus-induced LLFs (Fig. 6A-C). Stimulation induced a high frequency firing in quiescent AML in normal saline (Fig. 6A), but the response to stimulus was blocked after the application of TTX (Fig. 6B). After return to normal saline, AML resumed its firing response to stimulus (Fig. 6C). In all cases (N = 6), the stimulus-induced LLFs were blocked 23 ± 3.5 min (in the range of 13 to 35 min) after exposure to TTX and recovered 17.3 ± 2.47 min (in the range of 10 to 25 min) after returning to the normal saline. Such effects of TTX observed on the stimulus-induced LLFs were also seen in the axotomy-induced LLFs (Fig. 6D-F). In normal saline (Fig. 6D), axotomy induced an LLF in AML, but after bath application of TTX, where when the solution was applied during an axotomy-induced LLF, its firing rate declined and its discharge soon ceased completely (not shown), and thereafter the response to axotomy was no longer visible (Fig. 6E). After returning to normal saline,

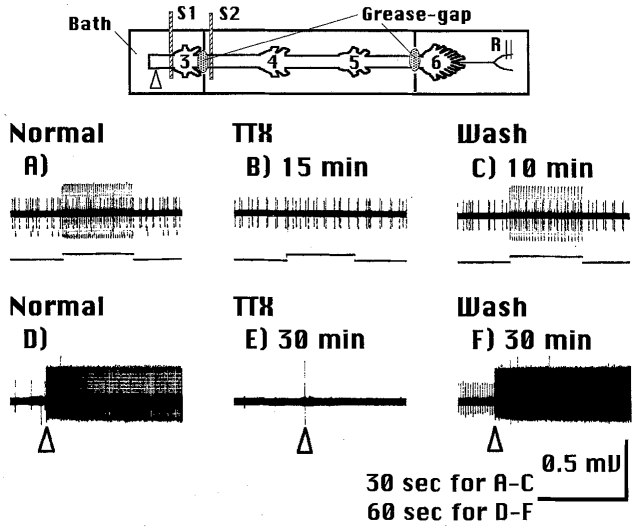

Fig. 6. Effect of TTX on AML activity. Upper inset represents schematic arrangement used for the experiments. For illustration, see Figs. 1 and 5. **A-C** and **D-F** are stimulus- and axotomy-induced characteristics, respectively. (**A**) Response of AML to a depolarizing pulse applied to the stump of the connectives of A3, in which the stump was bathed in normal saline. (**B**) Blockage of stimulus-induced AML response 15 min after bath application of TTX (3×10^{-7} M). (**C**) Restoration of stimulus-induced AML response 10 min after the bath was washed with normal saline. (**D**) Axotomy-induced AML response (largest spikes) when the stump was bathed in normal saline. (**E**) Blockage of axotomy-induced AML response 30 min after bath application of TTX. (**F**) Restoration of axotomy-induced AML response 10 min after the bath was washed with normal saline. **A-C** and **D-F** are different preparations. In **A-C**, upper traces represent discharge activities (AML spikes are the largest) and the upward displacement of lower traces indicates the duration of the applied current pulse (4 μA). In **D-F**, the bathed stump of the connectives of A3 was transected at triangles repeatedly (at Δ in upper inset).

axotomy restored the response (Fig. 6F). In all cases (N = 6), the axotomy-induced LLFs were blocked 17 ± 4.1 min (in the range of 5 to 30 min) after exposure to TTX and recovered 14 ± 5.5 min (in the range of 2.3 to 30 min) after returning to the normal saline. No significant difference was found in the time required for blocking or recover (Mann-Whitney U-test; P = 0.42 or P = 0.52, respectively) between stimulus- and axotomy-induced LLFs.

These results indicate that the inward current of LLF is carried by sodium ions.

Effect of calcium ions

On the other hand, Mg^{2+} solution (Mg^{2+} replaced Ca^{2+}) failed to block stimulus-induced LLF in all experiments (N = 4). In some cases, even after a 120-min exposure to this solution, a depolarizing pulse to the cut end of AML was capable of eliciting an LLF in AML and a hyperpolarizing pulse suppressed the response during the stimulus in the same manner as in the normal solution (Fig. 7A, B). This indicates that the response occurs peripherally at the cut end. The AML activity seems to be rather activated by exposure to Mg^{2+} solution, because during a 60- to 150-min exposure to this solution AML fired continuously in 2 out of 4 cases (Fig. 7B) and began to fire spontaneously in the other 2 cases (not shown). Such effects of Mg^{2+} solution observed on the stimulus-induced LLFs were also the case in the axotomy-induced LLFs. In all cases (N = 5), after 60 min of exposure to this solution, axotomy could always induce LLF (Table 1). Figure 7C represents that axotomy can induce a typical LLF in AML even after 130 min of exposure to Mg^{2+} solution.

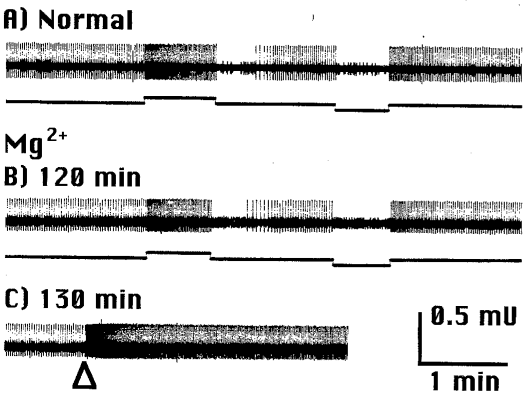

Fig. 7. Effect of Ca^{2+}-free saline on AML activity. Schematic arrangement used for the experiments is the same as that of upper inset in Fig. 6. (**A**) Response of AML to depolarizing or hyperpolarizing pulse (3 μA) applied to the stump of the connectives of A3, in which the stump was bathed in normal saline and a pulse was applied during an AML activity following axotomy. (**B**) Response of AML 120 min after bath application of Mg^{2+} solution (Mg^{2+} replaced Ca^{2+} of normal saline). (**C**) Response of AML to axotomy (the bathed stump of the connectives of A3 was transected at a triangle) 130 min after bath application of Mg^{2+} solution. Note that AML fires continuously in this solution. The upward displacement of the lower trace in **A** and **B** indicates the duration of a depolarizing pulse (S1 is negative in respect to S2) and the downward displacement that of a hyperpolarizing one (S1 is positive with respect to S2). **A** to **C** are the same preparation.

The response characteristic of AML activity to the application of EDTA solution (not shown), where EDTA is known to be a calcium chelating agent, was similar to that to Mg^{2+} solution shown in Fig. 7. In this solution, AML tended to fire continuously; AML lasted to fire during a 60-min exposure in all cases (N = 4 for stimulation experiment, N = 5 for axotomy experiment). In some cases, even after 100 min of exposure, AML continued to fire, which was blocked by a hyperpolarizing pulse to the cut end (not shown), in the same manner as that observed for Mg^{2+} solution (Fig. 7B). This indicates that a prolonged firing observed in the presence of EDTA is retained at the cut end. However, the firing frequency was higher than that with Mg^{2+} solution, as is described in the next section.

Next, the effect of Mn^{2+} solution (40 mM Mn^{2+} adds to 0-Ca^{2+} saline) was examined on the 6 preparations. In this solution, manganese ions are known to suppress the action potentials in which calcium is the major entrant cation (Hagiwara and Nakajima, 1966). In all cases, Mn^{2+} solution can not block stimulus-induced LLF during a 60-min exposure (Table 1). When the immersed end was transected after these stimula-

Table 1. Effects of test solutions on the enhancement of an LLF in AML by axotomy or electrical stimulation

Test solutions	Axotomy-induced LLFs	Stimulus-induced LLFs
Na^{+}-free	– (3)	– (6)
TTX	– (6)	– (6)
EDTA	+ (5)	+ (4)
Mg^{2+}	+ (5)	+ (4)
Mn^{2+}		+ (6)

When the bathed end of the connectives of A2 or A3 was transected, or the stimulus (at 2 to 5 μA for 20 to 60 sec) was applied to this end, whether the LLFs are induced (+) or not (–) was tested during a 60-min bath application of test solutions. The number of preparations is in parentheses.

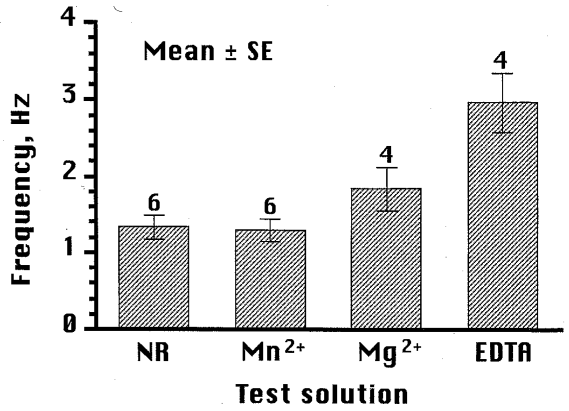

Fig. 8. Effect of test solutions on firing frequency of stimulus-induced an LLF in AML. Mean frequencies with SE were calculated from LLFs induced by depolarizing pulses (at 4 μA for 20 to 30 sec) to the stump of the connectives of A3 after the stump was bathed in each solution for 60 min. The figure above the bar SE indicates the number of the preparations from which the frequency data were obtained with SE. NR, normal saline; Mn^{2+}, Mn^{2+} saline; Mg^{2+}, Mg^{2+} saline; EDTA, EDTA saline.

tion experiments, the axotomy could always induce LLF (not shown), like as in the Ca^{2+}-free solutions. On the other hand, in this solution AML did not sustain its spontaneous activity as it did in the Ca^{2+}-free solutions.

The effect of test solutions during a 60-min exposure is summarized in Table 1. These results indicate that the inward current of AML spikes during LLF is primarily caused by sodium ion, but not calcium ion.

Effect of test solutions on frequencies of stimulus-induced LLFs

As described above, in Mg^{2+}, EDTA and Mn^{2+} solutions, stimulus-induced LLFs were found to be retained even after a 60-min exposure to these solutions. Then, to know the difference in AML excitability to these 3 solutions, their frequencies observed after a 60-min exposure to each solution were compared with that observed after a 60-min adaptation to normal saline (NR). In these cases, the frequency was calculated on the basis of the number of AML spikes in an LLF induced by the same stimulus (at 4.0 μA, for 20 to 30 sec) to the stump of the connectives of A3. Figure 8 shows these results. No significant difference (Mann-Whitney U-test, $P = 0.69$) was found in the frequencies between Mn^{2+} solution (1.30 ± 0.14 Hz, $N = 6$) and NR (1.33 ± 0.15 Hz, $N = 6$). The value for Mg^{2+} solution (1.85 ± 0.29 Hz, $N = 4$) or EDTA solution (2.97 ± 0.38, $N = 4$) is larger than that for NR. In particular, the value for EDTA is significantly larger than that for NR (Mann-Whitney U-test, $P < 0.01$).

These results support the idea that the LLFs are primarily caused by the Na^+ inward current, but not by Ca^{2+} current.

DISCUSSION

Comparison of axotomy- and stimulus-induced LLFs

The present observation revealed no remarkable differences in the characteristics in the two firing patterns of stimulus- and axotomy-induced LLFs: an initial high-frequency firing with exponential increase in interspike intervals during the first minute and gradual decay of firing frequency for stimulus-induced LLFs, which are exactly as reported previously for axotomy-induced LLFs (Muramoto, 1993). Therefore, it is assumed that the stimulus to the cut end of the nerve cord can induce the similar firing activity in AML to that following axotomy. This similarity supports the previous idea that the prolonged firing following axotomy is due to a prolonged depolarization at the cut region of AML (Muramoto, 1993). The other evidence for this idea is as follows. First, the firing response could be seen throughout a depolarizing pulse (1–6 μA) which was correlated positively with the magnitude of the stimulus ($r = 0.90$ in Fig. 2) to the cut end. However, the reason for a decrease in the firing response to a larger pulse (≥ 7 μA) is unknown. Second, the LLF response produced by axotomy could be blocked during a prolonged hyperpolarizing pulse to the cut end. From these facts it is concluded that axotomy of AML axon is comparable to application of a prolonged depolarizing pulse to its end, and its discharge activity following axotomy is generated at its axotomized site.

On the other hand, activity for axotomy-induced LLFs seems to be higher than that for stimulus-induced LLFs (Figs. 5 and 6). As Fig. 2 shows, the frequency for stimulus-induced LLFs, even if it is compared with its largest value at a stimulus of 5 μA (2.0 ± 0.29 Hz, $N = 7$), is smaller than those for axotomy-induced LLFs which were obtained by cutting AML axon at the position of the connectives between A1 and A2 (3.3 ± 0.19 Hz, $N = 17$) or A2 and A3 (2.6 ± 0.17 Hz, $N = 14$) in my previous study (Muramoto, 1993). This difference suggests that the depolarization underlying axotomy-induced LLFs is larger than that for stimulus-induced ones. The membrane depolarization following axotomy has been reported in vertebrate and invertebrate neurons. In earthworm medial giant axons (Krause *et al.*, 1994), *Aplysia* axons in tissue culture (Spira *et al.*, 1993), cockroach giant axons (Yawo and Kuno, 1984) and lamprey giant axons (Strautman *et al.*, 1990), axotomy produces membrane depolarization at their severed sites.

As is discussed in the next section, the depolarization following axotomy of AML is due to an increase in voltage-sensitive Na^+ current at its cut end, which suggests an increase in numbers of Na^+ channels at the end. For the axotomy-induced soma excitability in locust and crayfish motoneurons (Goodman and Heitler, 1979; Kuwada, 1981), the enhanced excitability appears to result from increased numbers of voltage-sensitive Na^+ channels in the soma-dendrite membrane (Titmus and Faber, 1990). Moreover, for the abnormal axonal excitability that often develops at the tips of injured axons in goldfish nerve, England *et al.* (1994) report that a focal accumulation of Na^+ channels within these tips may be responsible for this hyperexcitability. Similarly, the present hyperexcitability following axotomy may be explained by a larger increase in Na^+ channel density in the cut end of AML compared with that for stimulation, a possibility that awaits further investigation.

Ionic mechanism underlying AML spikes during LLF

Axotomy-induced soma excitability has been reported on crayfish (Kuwada and Wine, 1981), cockroach (Pitman *et al.*, 1972), and locust (Goodman and Heitler, 1979) motoneurons and interneurons. Such soma excitability has been also reported in some vertebrate neurons (Titmus and Faber, 1990). In all cases, the enhanced excitability appeared to be due to increased voltage-dependent Na^+ conductances. Such Na^+ dependence of increased excitability following axotomy in invertebrate neurons (Pitman, 1975; Goodman and Heitler, 1979; Kuwada, 1981) and in vertebrate neurons (Titmus and Faber, 1990) was established by the results that these spikes were blocked by removing extracellular Na^+ or by adding TTX.

Similarly, for the axotomy-induced LLF in AML, Na^+ was found to be the major carrier of inward current of the LLFs, since they were reversibly blocked by the removal of Na^+ from the bath solution or the addition of TTX to this bath. A voltage-dependency of such Na^+ conductances is also suggested by the observations that a prolonged depolarizing pulse to the

cut end can evoke LLF throughout the stimulus of 1–6 μA and that there is a positive correlation ($r = 0.90$) between the magnitude of depolarization and the firing rate (Fig. 2). It is then assumed that a large increase in the voltage-sensitive Na^+ current might occur at the cut end of AML following axotomy.

On the other hand, the presence of the inward current ("injury current") at the tips of transected peripheral nerve axons has been demonstrated in vertebrates (Borgens *et al.*, 1980) and invertebrates (Krause *et al.*, 1994). Borgens *et al.* (1980) measured "injury currents" consisting mainly of Na^+ and Ca^{2+}, which entered the acutely transected ends of lamprey spinal axons. Moreover, in axotomy-evoked soma spiking for insect motoneurons (Pitman, 1975), Ca^{2+} conductances, in addition to Na^+ conductances, appear to make some contribution to this axotomy-induced excitability. However, such Ca^{2+} contribution to the LLFs is very unlikely for two reasons. First, removal of Ca^{2+} or uses of Ca^{2+} blockers can not block the LLFs, but instead Ca^{2+}-free salines (Mg^{2+} and EDTA solutions) appear rather to increase the firing rate (Fig. 8). The reason for the significantly higher firing value for EDTA than that for Mg^{2+} (in Fig. 8; Mann-Whitney U-test, $P < 0.05$) is, however unknown. Second, manganese ions are known to reduce the influx of calcium ions during action potentials and so to suppress action potentials in which calcium is the major entrant cation (Hagiwara and Nakajima, 1966). However, frequency of AML activity was little affected by saline containing 40 mM Mn^{2+}, since there observed no significant difference (Mann-Whitney U-test, $p = 0.69$) in the frequency of stimulus-induced LLFs between Mn^{2+} solution and NR.

It was then concluded that the generation of a prolonged firing in AML following axotomy depends primarily on voltage-dependent Na^+ conductances, but not on Ca^{2+} conductances.

Functional significance of the axotomy-induced LLF

Effects of axotomy on neural excitability have been investigated in motoneurons and interneurons of crayfish (Kuwada and Wine, 1981), locust (Goodman and Heitler, 1979), cockroach (Pitman *et al.*, 1972), and cricket (Roederer and Cohen, 1983), yet none of them have shown a prolonged firing following axotomy (Titmus and Faber, 1990), except AML (Muramoto, 1993). The present study showed that not only axotomy, but also application of a prolonged depolarizing pulse can elicit an LLF in AML. AML appears to be unique in exhibiting a prolonged peripheral firing following axotomy.

What is the functional significance of such an LLF response? From the fact that the LLF response is generated and retained peripherally at the cut site following axotomy, it is expected that it might be concerned with morphological changes following axotomy such as repair or regeneration. This possibility appears to be in agreement with the facts; following transection of a nerve fiber, the nerve membrane at the cut end is resealed within 30 min in cockroach (Yawo and Kuno, 1984) and crayfish (Eddleman *et al.*, 1997), imposed electric fields (DC fields) promote recovery of acutely injured spinal cord axons in rat (Fehling and Tator, 1992), and in the earthworm, DC fields promote reconnection of axonal stumps (Todorov *et al.*, 1992). However, the possibility is unlikely for the following reasons. In invertebrate neurons, it is known that repair of disrupted axonal membranes or axonal regeneration after axotomy requires external calcium (Yawo and Kuno, 1984; Krause *et al.*, 1994; Steinhardt *et al.*, 1994; for review, Moffett, 1996). Recently, Eddleman and others (1997) report in the crayfish, *P. clarkii* medial giant axons that when they were transected in Ca^{2+}-free saline, their cut ends do not reseal. On the other hand, in the present study, external calcium was found to be unessential for enhancement of the LLFs, since AML excitability was unchanged or rather elevated in Ca^{2+}-free saline or saline containing Ca^{2+} blockers.

It was then suggested that the axotomy-induced LLF might not be involved in repair or regeneration. At present, why AML has such a specific firing property following axotomy is still open to question.

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