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Spontaneous Firing in the Isolated Anucleate Axonal Segment of an Identified Crayfish Motoneuron

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ABSTRACT—The aim of this study was to present evidence for prolonged spontaneous firing in an anucleate axonal segment of an identifiable crayfish anal motoneuron L (AML) and to determine the initiation site of this firing. AML has its soma in the 6th abdominal ganglion (A6). By separating a nerve with the AML axon from A6 and the target muscle, various lengths of an anucleate AML axonal segment were procured. Then, AML activity was recorded extracellularly for 14–26 hr from the distal end of this axonal segment. This segment (n=19) exhibited spontaneous firing, which occurred without any stimulation 0.03–5.13 hr after the A6-cut and persisted tonically for 0.20–19.98 hr. During firing, the frequency augmented gradually, whereas the amplitude decreased gradually. There was no significant correlation between latency and duration of the firings. No correlation was noted between latency and length of the axonal segment or its size, or between duration and length or size. These results revealed that the anucleate AML axon itself can inherently generate prolonged firing. The delay in the appearance of AML impulses between the proximal and distal regions at the same axonal segment proved that the firing occurred proximally. There was no significant difference in delays between firing following the A6-cut and the spontaneous firing observed after the A6-cut. This suggests that the initiation site of the spontaneous firing is at the proximal end of the AML axonal segment, since the AML firing following the A6-cut occurs at its cut end.

INTRODUCTION

In contrast to mammalian axons which undergo rapid degeneration once severed from their somata, crayfish and lobster axons can survive, conduct action potentials and release transmitters for months after being separated from their somata (Hoy *et al.*, 1967; Wine, 1973; Bittner and Johnson, 1974; Atwood *et al.*, 1989; Blundon *et al.*, 1990; Parnas *et al.*, 1991). For such anucleate axons, excitability appears to be an important factor regulating their growth and survival. In crayfish motoneurons, the growth rate of phasic and tonic neurons in explant cultures is correlated with levels of discharge activity (Egid and Lnenicka, 1993), in *Aplysia* sensory neurons, prolonged discharge activity can release serotonin which promotes growth (Glanzman *et al.*, 1989), and in many types of embryonic neurons, depolarization enhances their survival (Scott, 1977; Collins and Lile, 1989). However, no spontaneous firing has yet been reported in severed anucleate axons (Titmus and Faber, 1990; Bittner, 1991).

This paper on an isolated anucleate axon of an identifiable anal contractor motoneuron of the crayfish, *Procambarus clarkii*, presents the first evidence that this anucleate axon can exhibit prolonged spontaneous firing without stimulus. A

previous study revealed that transection (axotomy) of any level of this axon can elicit prolonged firing (≤ 67 min) at its axotomized end (Muramoto, 1993), but spontaneous firing after cessation of such axotomy-induced firing has not been clarified. I reported previously that this contractor motoneuron has its soma in the terminal abdominal ganglion and that the axon extends to the anal musculature out from the ganglion (Muramoto, 1977). In this study, various lengths of an isolated anucleate axonal segment of this motoneuron were obtained. Long-term recording (≥ 14 hr) of this isolated segment revealed a prolonged firing (< 20 hr), which begins a few hr after the separation.

It is possible that the spontaneous firing observed in the present axonal segment is dependent on various factors, such as a) the length of the axonal segment, b) the presence of other axons, and c) the presence of some other structures. To address this possibility, the relationship between latency or duration of the spontaneous firing and length or size of the axonal segment was examined. The results demonstrated that there was no relationship between them, suggesting that the anucleate axonal segment itself could exhibit prolonged spontaneous firing.

It has been accepted that in ordinary motoneurons, action potentials are initiated at the initial segment (Eccles, 1964; Stuart and Sakmann, 1994) and in some invertebrate neurons they are initiated at separate regions of their neu-

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ronal arborization (Gu *et al.*, 1991; Zec̄ević, 1996). Of interest is whether such restricted regions were also responsible for the present spontaneous firing. To answer this question, the propagation delay between the proximal and distal regions of the same axonal segment was recorded and compared to those in the firing immediately after the axotomy, because the firing after the axotomy occurs at the axotomized end of the motoneuron being studied (Muramoto, 1993). These results provide evidence that the spontaneous firing occurs at the proximal end of the isolated axonal segment.

MATERIALS AND METHODS

Preparation

A crayfish, *Procambarus clarkii*, which was procured from a commercial source, was used throughout the experiment. The soma of the anal motoneuron L (AML), which is capable of driving rhythmic anal contractions of the crayfish, is located in the 6th abdominal ganglion (A6) (Muramoto, 1977). One unpaired intestinal nerve (IN) originates from A6 and it splits further into 3 more branches, the pair of the anterior intestinal nerves (AINs) and the posterior intestinal nerve (PIN). An axonal process of AML runs down via IN and PIN, where PIN bifurcates into two branches (B in Fig. 1, inset) before innervating to the anal musculature (Muramoto, 1977, 1993). Then, A6 with IN, PIN and AINs, in which IN was left intact attaching A6 while AINs were trimmed off, were dissected from the animal.

This dissected nerve preparation was placed in a small chamber filled with normal saline consisting of 208 mM NaCl, 5.4 mM KCl, 13.3 mM CaCl₂ and 2.6 mM MgCl₂, buffered with 10 mM Tris at pH 7.5, and kept initially (≤ 14 hr) at 10°–11°C using a bath temperature control system (DTC-200, Daiya Medical Co., Japan), but was

allowed to warm to room temperature during the experiment (14–26 hr), when the bath temperature was 10°–17°C.

As the inset of Fig. 1 shows, the nerve preparation was immobilized by fixing both distal ends of the AINs with silicon grease and the discharge activity was recorded extracellularly with a suction electrode from either of the cut ends of the PIN distal branches and a reference electrode was located in the bath fluid, as described previously (Muramoto, 1993). By transecting the nerve with scissors at various positions between A6 and the electrode, various lengths of an isolated anucleate segment of the AML axon (axonal segment preparation) were obtained. The severed portion with A6 was removed from the chamber to avoid any effect of trophic factors derived from this portion. To detect spontaneous firing of the anucleate AML segment, long-term (≥ 14 hr) recording of AML activity was made before, during and after the transection.

Identification and recording

The AML activity can be easily identified by its largest amplitude among units recorded from PIN (Muramoto, 1977; 1993). The activity was displayed on a pen recorder via an amplifier and stored on the hard disk of a Macintosh computer as a CSV type file for 30 sec every 10 or 15 min. The file was then imported into KaleidaGraph data analysis and graphic presentation software (Synergy Software, USA, PA), and AML action potentials during a 30 sec and amplitude (foot to peak) of an initial spike were measured using functions on KaleidaGraph.

Measurement of the delay of AML action potentials

The delay between a peak (P) to a peak (P') of AML action potentials was measured by simultaneous recordings from the proximal and distal electrodes on the same preparation; the former electrode was located on the proximal stump of the axonal segment or on the more distal section to this stump as shown in the inset of Fig. 1, and the latter electrode was always located on the distal end of the

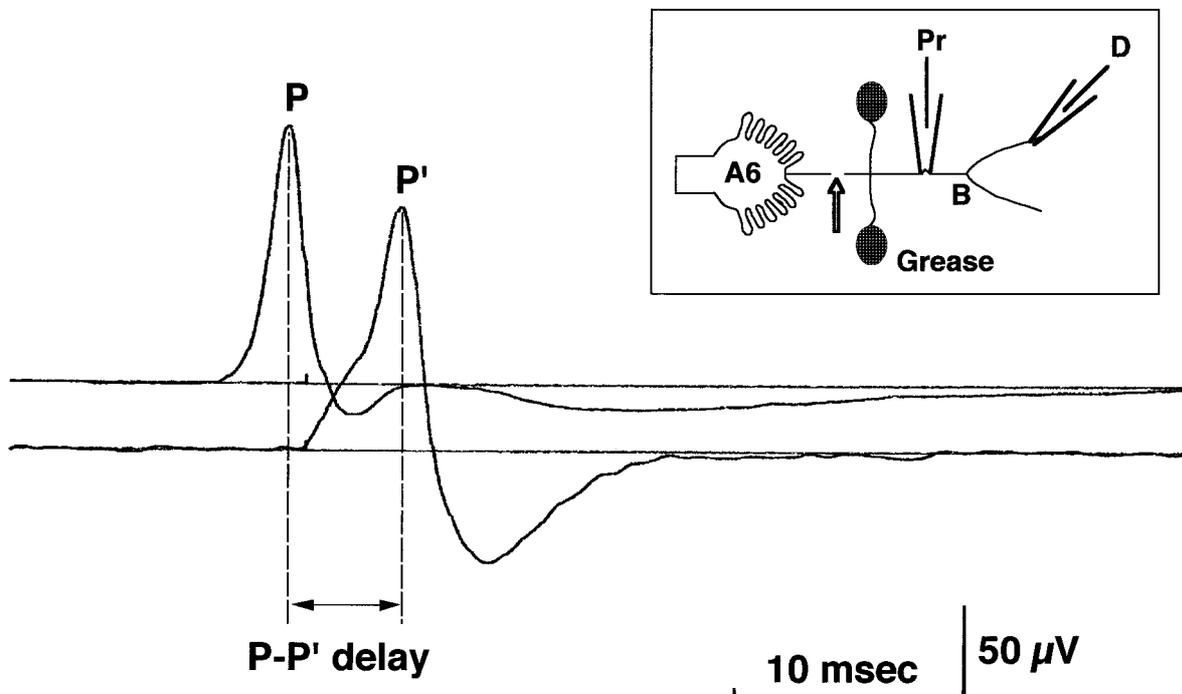


Fig. 1. A scheme of preparation and arrangement for recording electrodes. A6, the 6th abdominal ganglion in which the AML soma is located; B, a bifurcation of the AML axon; arrow, position of the separation from A6; Grease, silicon lubricant for fixing the preparation. Measurement of the P–P' delay in the simultaneous recording of SF activity. The upper trace represents a spontaneous AML action potential (averaged for 50 samples) recorded by the proximal electrode (Pr) and by the distal one (D). P–P' delay: between peak (P) to peak (P') of the action potentials.

axonal segment. Simultaneous recordings of AML action potentials were made between these electrodes and the reference electrode immersed in the bath fluid. Figure 1 represents a schematic arrangement for the measurement of the P-P' delay. These data were stored on magnetic tape and analyzed on a signal processor (7TOTA, Sanei Inst. Co., Japan), where the P-P' delay was measured from the average of 50 sweeps every 10 or 15 min during AML spontaneous firing activity (Fig. 1).

The distance between the proximal and distal electrodes was determined from a microscopic photograph of the arrangement of the preparation. The conduction velocity was determined by dividing this distance by the initial P-P' delay (at 0 hr in Fig. 7).

Measurement of length or size

After physiological experiments were finished, the used axonal segment preparation was put on a slide glass and fixed by drying for 1 hr at room temperature. Then, the preparation was stained with Giemsa's solution. This stained preparation was scanned as a PICT type graphic file using a color image scanner (GT-6500, EPSON, Japan). The analysis of length and size of the scanned image was performed on a Macintosh computer using the NIH Image program (written by Wayne Rasband at the U.S. NIH). The inset of Fig. 5 is an example of these scanned images, where the length from the proximal (P) to distal end (D) of the axonal preparation, along the side of the axonal segment at which the recording electrode was located (asterisk), was measured. For size, the whole surface area of the axonal preparation (black region of the scanned image) was measured.

Expression of the results

Data were expressed as mean±SE. Regression line slopes and elevations were compared using analysis of correlation/covariance. Two group comparisons were performed with a *t*-test, while multiple comparisons were made by analysis of variance (ANOVA). The dif-

ferences between these means were considered significant at a level of $P < 0.05$.

RESULTS

Long-term recording of spontaneous firing

A previous study (Muramoto, 1993) showed that the axotomy of an AML axon is always capable of eliciting prolonged firing in otherwise quiescent AML. This was also the case in this study; when A6 (the soma) was separated from the axonal segment ($n=19$). The separation always produced prolonged firing in AML. This axotomy-induced firing (AIF) following the A6-cut persisted for 8 sec to 26 min (mean±SE, 0.11 ± 0.03 hr, $n=19$), and then ceased.

After the AIFs stopped, AML activity was further recorded for 14 to 26 hr (19.65 ± 0.77 hr, $n=19$). AML was found to fire spontaneously without any stimulation. As Fig. 2 shows, once AML begins to fire spontaneously, spontaneous firing (SF) continues tonically for a while, though it stops sometimes for a few minutes during a SF period. In this case, the SF continued for about 7.6 hr. In most cases ($16/19=84\%$), such SF activity did not occur repetitively during the present observations. However, in some cases ($3/19=16\%$), it occurred again during this period; the second SF activity occurred 3.03 to 6.58 hr (4.48 ± 1.07 hr, $n=3$) after cessation of the first SF activity, and persisted for 2.22 to 16.42 hr (7.38 ± 4.54 hr, $n=3$). Next, the latency or duration of the first SF activity was analyzed and is depicted in Fig. 3. The SF activity was found to occur at various time latencies after the A6-cut (1.91 ± 0.35 hr,

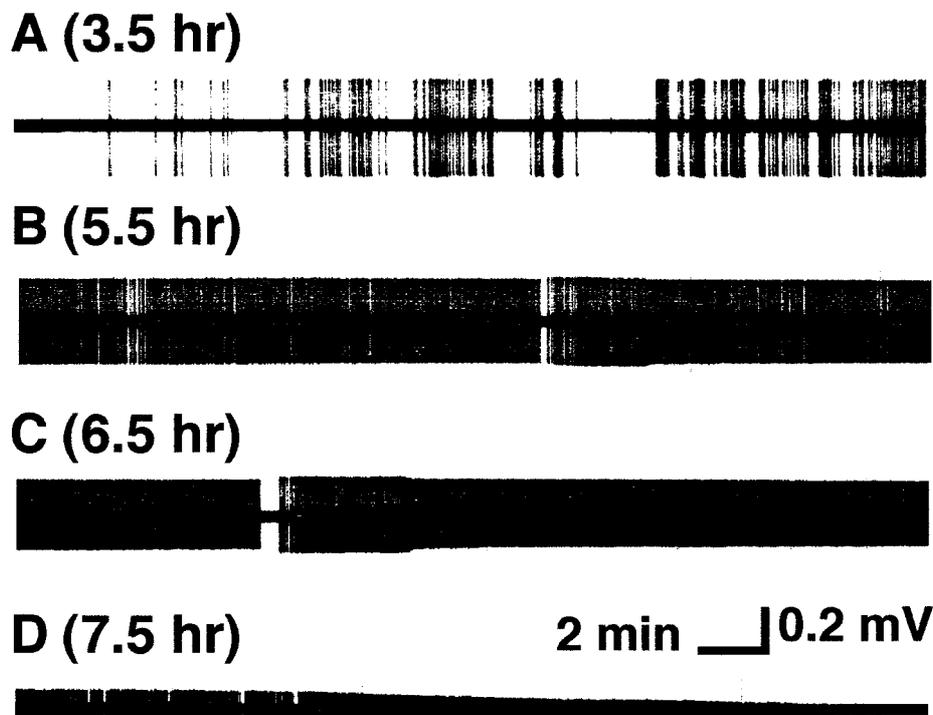


Fig. 2. SF recorded from an isolated axonal segment of AML. AML began to fire spontaneously 3.5 hr after the separation from A6 and continued to fire tonically for about 7.6 hr (A to D). The figures in parentheses indicate the time lapse after the separation. Note a gradual augmentation in firing frequency and a gradual decrease in amplitude.

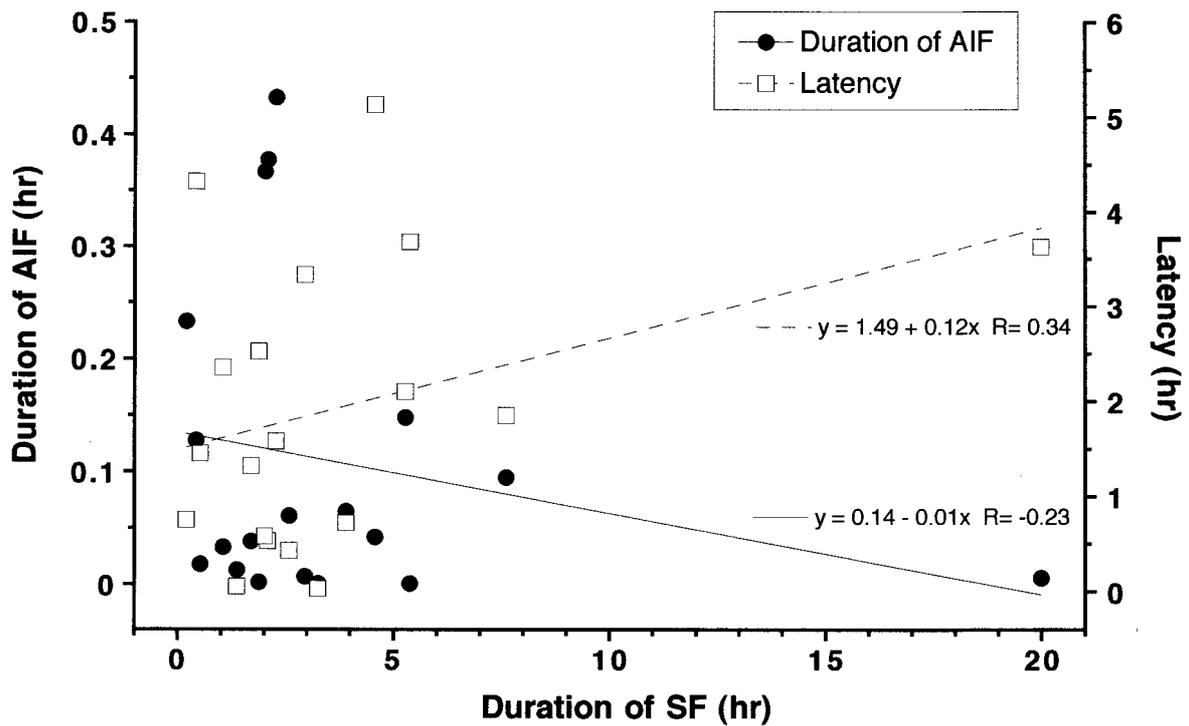


Fig. 3. No correlation between duration of AIFs and SFs or latency and duration of SFs ($n=19$). Dotted line, regression of latency versus duration of SFs. Solid line, regression of duration of AIFs versus that of the SFs.

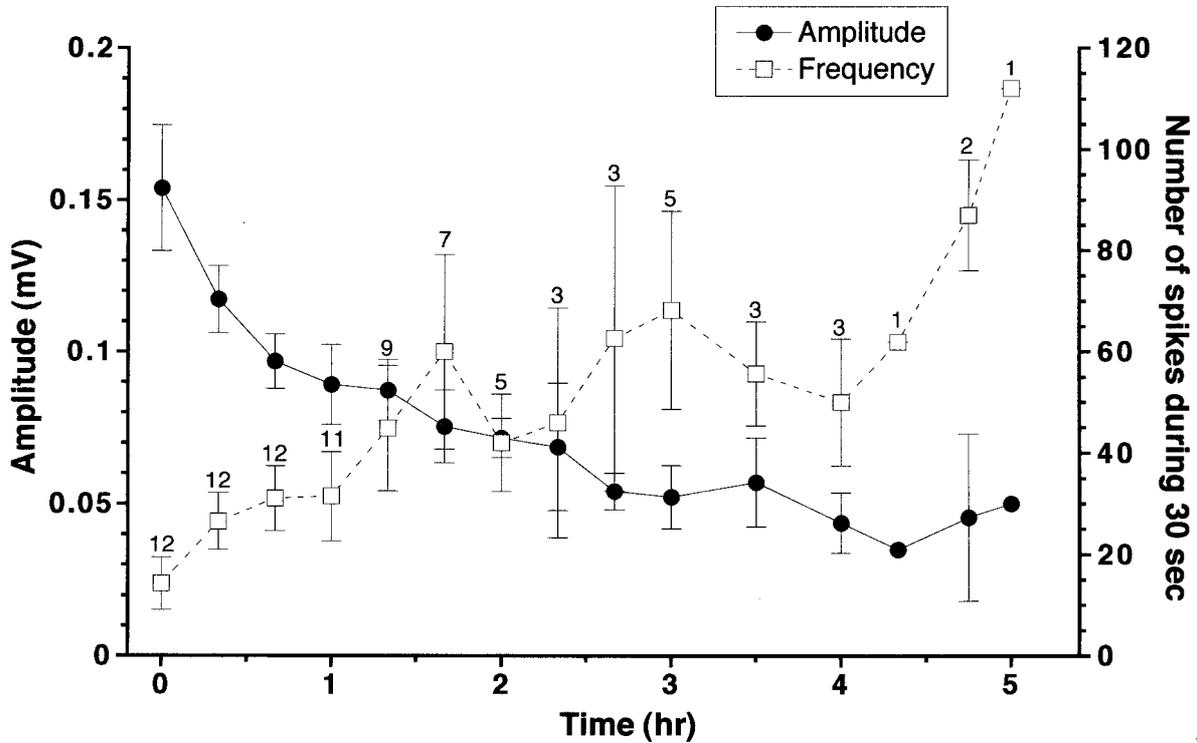


Fig. 4. Time course of amplitude and firing frequency during SF in an isolated AML axonal segment. Data (mean \pm SE) were plotted at 20-min or 30-min intervals during a period of 5.25 hr after the beginning (0 hr) of SF. The amplitude was the foot to peak height of the spike, while the frequency was the number of spikes during 30 sec. The SFs ranged from 0.95 to over 5.25 hr. The figures above the bar SE indicate the number of preparations.

0.03 to 5.13 hr, $n=19$) and to persist for various durations (3.63 ± 1.01 hr, 0.20 to 19.98 hr, $n=19$). Figure 3 illustrates the plots for duration of the AIFs and SFs, and for SF latency. Figure 3 shows that no significant correlation is observed between the durations of the AIFs and the SFs ($r=-0.23$, $n=19$, $P=0.36$), and this is also the case between the latency and duration of the SFs ($r=0.34$, $n=19$, $P=0.16$). Moreover, there was no significant correlation between the duration of the AIFs and latency ($r=-0.30$, $n=19$, $P=0.21$). These results indicate that SF activity is not concerned with the AIF response.

Figure 2 clearly shows that during a SF period, firing frequency tends to increase with time, while amplitude tends to decrease with time. Also, in 12 cases, the time course of changes in frequency and amplitude during a SF period was examined by counting AML spikes during a 30 sec period and its initial amplitude (foot to peak) every per 20 min over 5 hr. Figure 4 represents these results. There was a significant correlation between amplitude and time ($r=-0.89$, $P<0.0001$), and between frequency and time ($r=0.87$, $P<0.0001$). SF was thus characterized by an initial low frequency firing, followed by a progressively higher frequency over successive time courses and by an initial high amplitude followed by progressively lower ones.

These results indicate that a peripheral axonal segment of AML can exhibit SF activity, which persists for a long time and has a characteristic firing pattern.

Effect of length or size of an axonal segment on SF activity

To investigate the effect of length or size of an axonal segment preparation on SF activity, the length or size was

calculated on the 9 preparations after measuring the duration and latency of SFs. It was found that, on average, length was 3.60 ± 0.43 mm (between 1.97 to 5.24 mm), size was 2.13 ± 0.31 mm² (0.57 to 3.88 mm²), latency was 3.66 ± 0.27 hr (2.10 to 5.03 hr), and the duration was 6.08 ± 1.84 hr (0.42 to 19.75 hr). The relationship between latency and duration observed here showed no significant correlation ($r=-0.14$, $P=0.73$), similar to that shown in Fig. 3.

When length or size was plotted against duration of the SFs (Fig. 5A), the plots appear to decrease with an augment in duration, but no marked correlation was found statistically between length and duration ($r=-0.38$, $n=9$, $P=0.33$) or between size and duration ($r=-0.43$, $n=9$, $P=0.26$). This was also the case for a plot of length or size against latency (Fig. 5B). No statistically significant correlation was observed between length and latency ($r=0.66$, $n=9$, $P=0.05$) or between size and latency ($r=0.53$, $n=9$, $P=0.15$), though length or size tended to increase with an augment in latency (Fig. 5B). These results show that SF activity is not dependent on the length or size of the axonal segment.

Simultaneous recording of SF activity

To determine the site of initiation of SF activity, simultaneous recordings were made from the proximal and distal regions of the same axonal segment preparation. Figure 6 shows a typical example of these recordings, where AML impulses are initiated first in the proximal region (upper trace) and then propagate to the distal region (lower trace), indicating that the site of origin of the impulses may be at or near the proximal recording location (proximal electrode). Here, the SF activity continued for 1.57 hr, and amplitude tended to decrease with

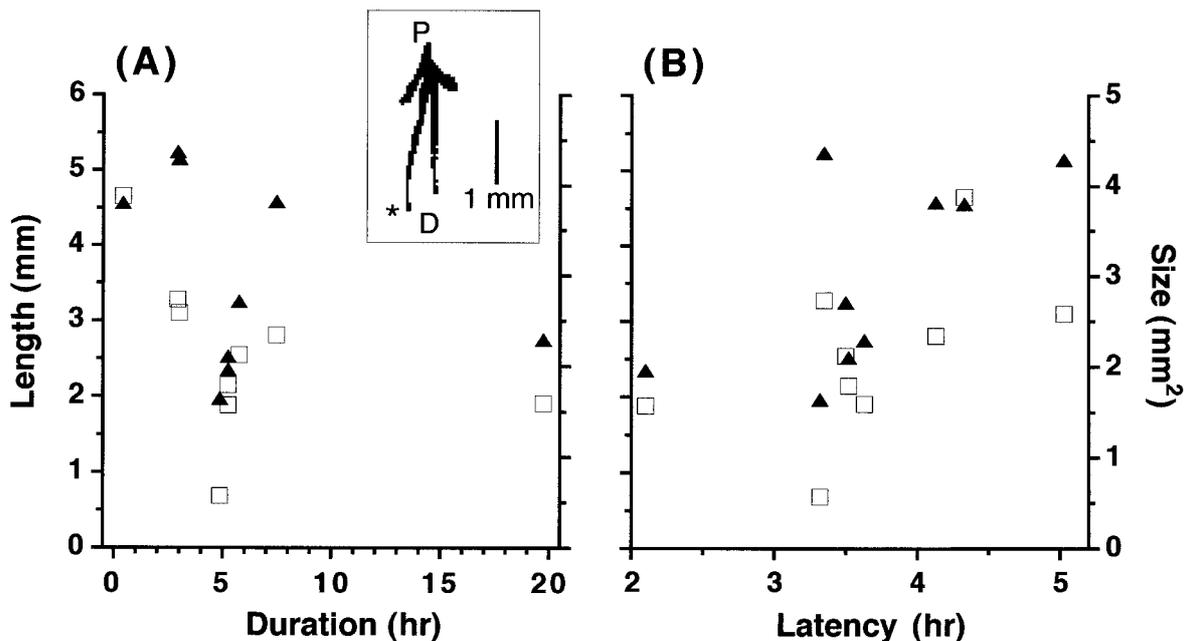


Fig. 5. Effect of length (filled triangles) or size (open squares) of an axonal segment on duration (A) or latency (B) of SF activity ($n=9$). Inset; a scanned image of the axonal segment employed to determine its length and size. Asterisk indicates the recording side of SF activity. P and D, the proximal and distal end of the axonal segment.

time, while frequency tended to increase with time. Such changes in amplitude and frequency were the same as those observed in Figs. 2–4. Figure 6 clearly shows that fluctuation or diminution in amplitude of the action potentials recorded proximally (upper trace) is more predominant compared with that recorded distally (lower trace). These results support the

idea that the action potentials occur at or near the proximal electrode and then actively propagate in a proximal to a distal direction along the axonal segment.

Next, the time-course change in delay (P–P' delay) of the AML spikes was examined on the 10 preparations during a SF period. The P–P' delay was calculated every 10 min over

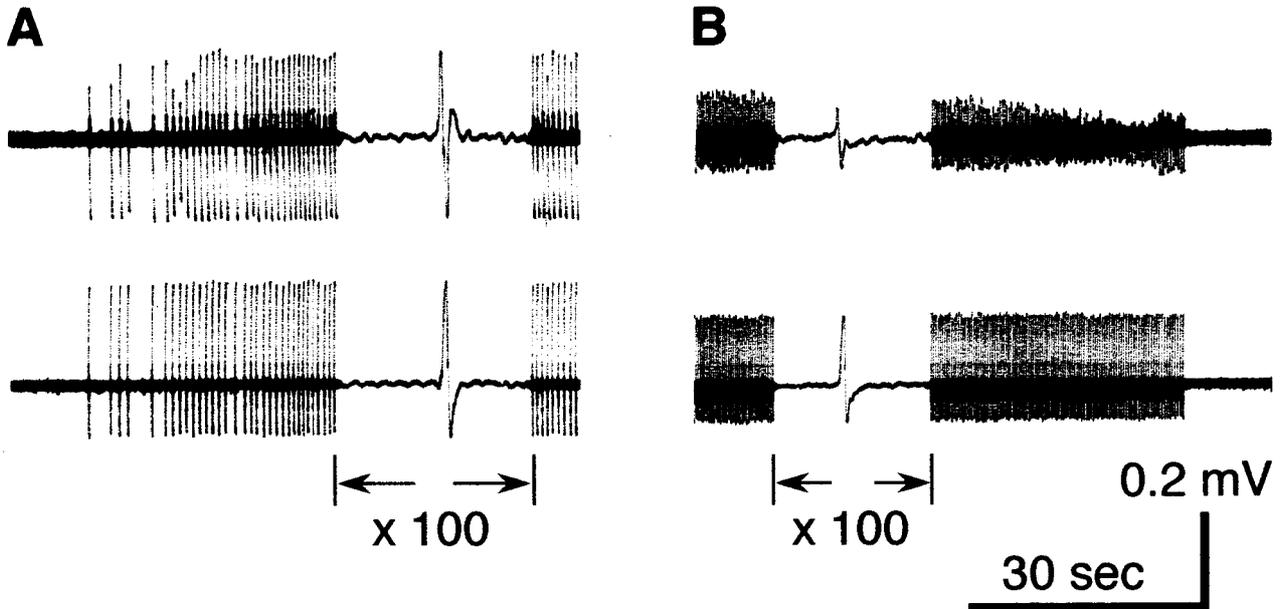


Fig. 6. Simultaneous recording of SF activity made from the proximal (upper trace) and distal region (lower trace) of the same AML axonal segment. The initial (A) and the last parts (B) of SF activity, which continued for 1.57 hr, are shown.

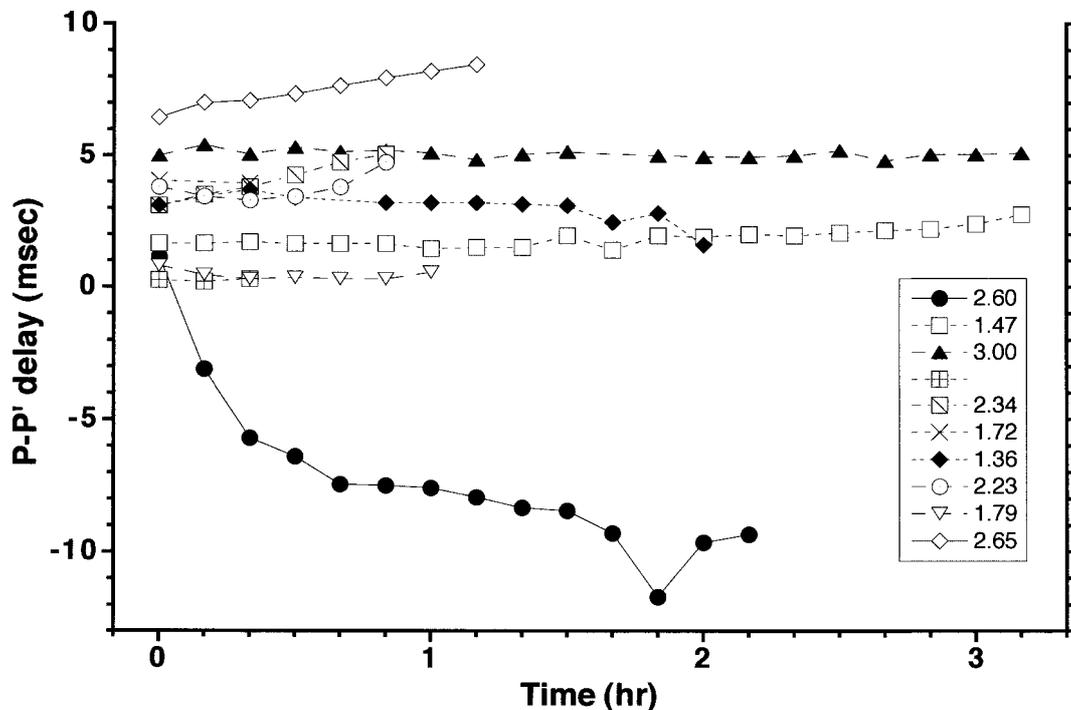


Fig. 7. Time course of P–P' delay during SF. Data from 10 isolated axonal segment preparations are plotted as the P–P' delay at 10-min intervals during a period of 3.33 hr after the beginning (0 hr) of SF. The SFs ranged from 0.52 to 3.33 hr. The figures in the right square indicate the distance (mm) between the proximal and distal electrodes.

3 hr, and the results are depicted in Fig. 7; when P precedes P' as in Fig. 1, the P-P' delay was expressed as a positive value, whereas when P' precedes P (not shown), it was expressed as a negative value. In these experiments, the distance between the proximal and distal electrodes was measured in the 9 cases out of 10 and the values are shown in Fig. 7. The distance ranged 1.36 to 3.00 mm (2.13 ± 0.19 mm). The mean conduction velocity was 0.96 ± 0.25 m/sec (0.41 to 2.26 m/sec, $n=9$). Variability in the conduction velocity may be reflected as uncertainty in the determination of the distance between the two electrodes.

Figure 7 shows that in one case (1/10=10%), the P-P' delay was positive during the initial period of a SF period, but became negative during its progressive period, in which an exponential decay with time was noted (the line with filled circles, in Fig. 7). Here, even after the AML discharge activity recorded proximally disappeared, it lasted and was recorded distally for a while (not shown). These results suggest that the initiation site of SF activity may shift exponentially in a proximal to distal direction. On the other hand, in all cases except one (9/10=90%), P-P' delay was always positive during a long period of SF; in some cases (6/10=60%), the P-P' delays remained almost constant during a long period of SF, and in others (3/10=30%), they tended to increase slightly with time (Fig. 7).

The above results suggest that SF activity is in most cases initiated proximally during a SF period. Then, the P-P' delay in the AIF following the A6-cut was compared with that in the SF after the A6-cut on the same preparations ($n=4$), in which the positions of the proximal and distal electrodes remained unchanged during the experiment, because the AIF following the A6-cut is thought to occur at the proximal end of an isolated AML axon (Muramoto, 1993). P-P' delays for AIFs and

SFs were calculated from the initial (Initial), the last (Last) or the total (Total) parts of an AIF or a SF. Figure 8 represents these results; the mean \pm SE for the AIFs was 3.64 ± 0.38 msec for the Initial, 2.96 ± 0.39 msec for the Last and 3.49 ± 0.27 msec for the Total, and results for the SFs were 4.09 ± 0.48 msec for the Initial, 4.28 ± 1.00 msec for the Last and 4.08 ± 0.56 msec for the Total. There was no interaction effect ($P=0.40$, from a two-way repeated measures ANOVA) between the firing response (AIF and SF) and the parts of recording (Initial and Last). There was no significant discrepancy ($P=0.38$, by unpaired *t*-test) between the values for the Total of the AIFs and the SFs. These results indicate that the two firing responses do not differ significantly ($P>0.05$) in P-P' delay.

It was concluded that the initiation site of the spontaneous firing observed in the anucleate axonal segment of AML is at its proximal end.

DISCUSSION

Spontaneous firing in an anucleate AML axonal segment

The present study on an identified motoneuron, AML, of the crayfish revealed that its peripheral axonal segment separated from its soma and its target muscle exhibited a long-lasting spontaneous firing (SF). In various lengths of this anucleate axonal segment, SF activity actually occurred without any stimulation and persisted for a long time (3.67 ± 1.01 hr, $n=19$), in which duration of the SFs was various, but characteristics in firing patterns were similar. This strongly supports the idea that the anucleate peripheral axon of AML itself can inherently generate prolonged firing.

Such an ability seems to be unique to AML and is not generalized to other neurons. The present axonal segment preparation contained other units than AML (Muramoto, 1977, 1993), but they did not exhibit SF in this study. In crayfish and lobsters, axons can survive, conduct action potentials and release transmitters for months after being separated from their somata (Hoy *et al.*, 1967; Wine, 1973; Bittner and Johnson, 1974; Atwood *et al.*, 1989; Blundon *et al.*, 1990; Parnas *et al.*, 1991), yet none of them have shown prolonged spontaneous firing (Titmus and Faber, 1990; Bittner, 1991).

Although the AML axon can show a prolonged firing following axotomy (Muramoto, 1993), axotomy-induced firing (AIF) and the present SF activity differ greatly. The distinction is as follows. (1) The AIFs response is to axotomy, while the SFs occur without any stimulation. (2) Characteristics in SF firing patterns (Fig. 3) were different from those of the AIFs observed previously (Fig. 3 in Muramoto, 1993). (3) The duration of the SFs (3.63 ± 1.01 hr, $n=19$) was much longer than that of the AIFs following the A6-cut observed in this study (0.11 ± 0.032 hr, $n=19$) or that of the AIFs following axotomy of various levels of the AML axon observed in the previous study (≤ 67 min; Muramoto, 1993). (4) There was a long latency (1.91 ± 0.35 hr, $n=19$) until SF occurred after the axotomy (A6-cut), and no significant correlation was found statistically between latency and duration of SFs ($P>0.05$). These observations offer further corroborating evidence that

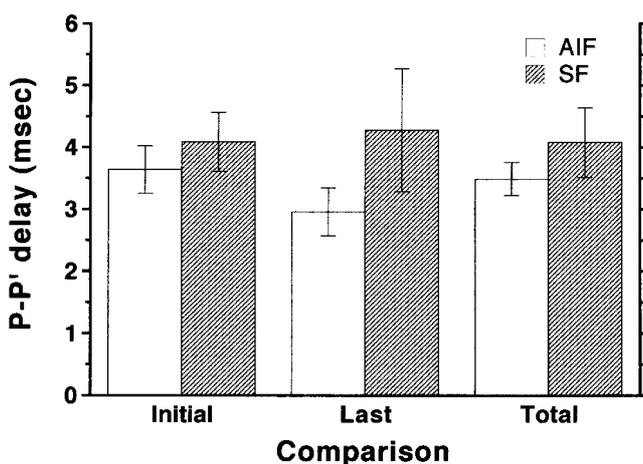


Fig. 8. Comparison of P-P' delays between AIFs and SFs. The mean P-P' delays with SE for AIFs following the A6-cut and the SFs occurring after the A6-cut on the same preparations ($n=4$) are depicted. Initial, the P-P' delay data obtained from the mean of the initial 50 impulses during AIF or SF. Last, the P-P' delay data obtained from the last 50 impulses. Total, the total P-P' delay data obtained from every successive 50 impulses during AIF or SF.

the anucleate axonal segment of the AML axon itself can exhibit a repetitive firing without stimulus.

An earlier study showed that AIFs are responsible for depolarization occurring at the axotomized end of the AML axon, which decreases exponentially during an AIF (Muramoto, 1998). On the contrary, SFs seemed to be resulting from depolarization at its proximal end, which increases gradually during a SF. A gradual increase in firing frequency and a gradual decrease in amplitude (Fig. 4) support this idea. Further evidence for this idea is that the application of high K^+ saline (54 mM; 10-fold concentration of normal saline), which is capable of producing membrane depolarization, to the bath of an isolated axonal segment could induce prolonged spontaneous firing with characteristics similar to those of SF activity (Muramoto, unpublished data). It seems to require a long time period for the proximal end of the AML axonal segment to return to the normal resting potential and to depolarize again to the spike discharge threshold, because it took 4.48 ± 1.07 hr ($n=3$) for the second SF activity to set up after cessation of the first SF activity. The mechanisms underlying SF need to be investigated further.

Effect of trophic factors

It is generally presumed that axonal proteins essential for trophic support are synthesized in the soma and supplied to the axon via axonal transport (Koenig and Giuditta, 1999). In addition, in crayfish or squid giant axons, endogenous synthesis of axoplasmic proteins appears to be present in the periaxonal glia (Gainer *et al.*, 1977; Lasek *et al.*, 1977; Sheller and Bittner, 1992; Tanner *et al.*, 1995). Koenig and Giuditta (1999) pointed out that the total axonal protein or RNA content of an axon can be much larger than that of its cell body, depending on the diameter and length of the axon. Anucleate axons in crustaceans can survive for months (Hoy *et al.*, 1967; Wine, 1973; Bittner and Johnson, 1974; Atwood *et al.*, 1989) and the survival time appears to be dependent on the presence of axoplasmic proteins (Bittner and Mann, 1976; Sheller and Bittner, 1992; Tanner *et al.*, 1995).

For crayfish giant axons, axonal length seems to be a non-critical factor in determining survival times of isolated axons, since anucleate axonal segments of different length showed the same range of survival times (Bittner, 1988). Similarly, length of the AML axonal segment seems to be independent of prolonged enhancement of SFs, because the data obtained showed no significant correlation ($P>0.05$) between length of the AML axonal segment and latency or duration of SFs. Moreover, no significant correlation ($P>0.05$) was found between size of the axonal preparation and latency or duration of SFs. These data suggest that trophic support is not an important factor for the prolonged enhancement of SFs. Furthermore, these data indicate that SFs are not dependent on other axons and some other structures surrounding an isolated AML axonal segment, supporting the idea that only the AML axon can exhibit SF activity.

The mechanism where by prolonged firing occurs spontaneously is mysterious, and will be the subject of further study.

The site of SF initiation

In ordinary motoneurons, the site of initiation of the action potentials is found at the initial segment (Eccles, 1964; Stuart and Sakmann, 1994), and in some invertebrate neurons, it is found at separate regions of their neuronal arborization (Gu *et al.*, 1991; Zec̆ević, 1996). However, the initiation site of SF activity does not localize in such a restricted region. The following observations support this idea: (1) The present axonal segment preparation possessed no soma and no initial segment (see Materials and Methods). (2) SF was found despite the length of the axonal segments, irrespective of whether bifurcation (B; Fig. 1, inset) was present or not. (3) There was no significant correlation ($P>0.05$) between length of the axonal segments and duration of SFs. These observations indicate that any length of the AML axonal segment can exhibit SF activity, suggesting that SF activity occurs at any region of this segment.

The simultaneous recordings of SF activity in this study demonstrate that AML impulses are initiated proximally and then actively propagated in a proximal to distal direction along the AML axonal segment. The evidence for this idea is as follows. First, during the long period of a SF, the P–P' delay always showed almost constant positive values in most cases (9/10=90%), indicating that SF is initiated at or near the electrode located proximally. Second, the AML impulses recorded proximally showed some fluctuation or diminution in their amplitude, whereas such change was not observed in those recorded distally, indicating that the AML impulses actively propagate in a proximal to distal direction. These observations suggest that impulse initiation site was at a proximal region of the AML axonal segment.

The exact region of the initiation can be assessed by comparing the P–P' delay for AIFs and SFs, since it was shown earlier that AIFs are initiated and maintained locally at the axotomized end of the AML axon (Muramoto, 1993). This comparison showed that no significant difference ($P>0.05$) in P–P' delay was found between the AIFs following the A6-cut and the SFs observed 1.91 ± 0.35 hr after the A6-cut. It was thus concluded that the initiation site of the SF activity was at the proximal end of the AML axonal segment.

Electrophysiological and ultrastructural analyses on cultured *Aplysia* neurons have shown that the dynamics of transection and recovery of their proximal and distal transected axonal segments is essentially identical (Spira *et al.*, 1993). However, for the AML axonal segment, the present study revealed that spontaneous firing is initiated proximally and propagates orthodromically in a proximal to distal direction, but not vice versa. The mechanism underlying this process is unknown and will be the subject of further studies.

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