Inhibitory Effect on the Mechanical Responses of the Cheliped Muscle of the Crayfish*,1),2)

By

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(With 7 Text-figures)

The neuromuscular system of Crustacea is very different in many respects from that of Vertebrata and is physiologically interesting material. In addition to the excitatory nerve fibres, the crustacean muscle is supplied by the inhibitory nerve fibre controlling its contraction. Therefore, mechanisms of the peripheral inhibition of the crustacean muscle have been studied by many investigators (van Harreveld and Wiersma 1937, Marmont and Wiersma 1938, Fatt and Katz 1953a, b, Hoyle and Wiersma 1958a,b,c, Dudel and Kuffler 1961).

Particularly, the crustacean muscle itself does not show the propagated contraction of all-or-none type even by a direct stimulus to the muscle fibre, and repetitive stimuli applied to the excitatory nerve evoke a local contraction of the muscle, producing small excitatory neuromuscular junction potentials. Also, the development of those excitatory junction potentials and of the muscular contraction are determined by the frequency of excitatory nerve impulses. Thus, the crustacean neuromuscular system can be highly facilitated (Fatt and Katz 1953 c, Murayama and Yamashita 1962).

For that reason, the frequency of repetitive stimuli to the inhibitory nerve fibre as well as to the excitatory one is regarded as a very important factor in the inhibitory phenomena of this neuromuscular system. Few investigations, however, have been made on inhibitory effects of altering the frequency over a wide range of repetitive stimulation of the excitatory and inhibitory nerve fibre on the contraction.

The object of the present experiment is to show how the muscular contraction can be inhibited mechanically when stimuli at various frequencies in various

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combinations are applied to the exciatory and inhibitory nerve simultaneously in the crayfish neuromuscular preparation, and to clarify the inhibitory mechanism of the crayfish cheliped muscle.

Material and Methods

Preparation: The nerve (an inhibitory nerve and an excitatory one)-muscle (the abductor muscle) preparation of the cheliped of the crayfish, *Procambarus clarkii*, was used for most of the experiments. The cheliped was removed from the animal at the point of natural autotomy. The shell of inside halves of the meropodite and of the carpopodite was entirely cut off with the underlying muscles, and the main nerve trunk was exposed. The proximal part of each nerve bundle was ligatured with thread to operate it freely. As is shown in Fig. 1, a slow nerve fibre and an inhibitory one supplying the abductor muscle were contained in the nerve bundle SB and LB–1, respectively. Thus, the nerve fibres could be used without isolation into a single axon, if only the tendon of the adductor muscle was completely cut off at the proximal part of the dactylopodite to avoid the effect of contraction of the adductor muscle evoked by stimulation of the LB–1 (for stimulation of the inhibitory nerve). This preparation was mounted on a slide glass fixed with rubber bands, and immersed in van Harreveld's solution (van Harrveld 1936) in a chamber.

Stimulation and recording: As a stimulator, a two channel tape-recorder (Akai 33D and R220) was used which was convenient to stimulate the excitatory nerve and the inhibitory one simultaneously in a combination of various frequencies of repetitive stimuli. Repetitive pulses (each pulse of the train was 0.2 msec. in duration) of various frequencies, which had been previously recorded on each channel of the electromagnetic tape using a square pulse generator, were reproduced and fed to two pairs of stimulating electrodes (one for the excitatory nerve and another for the inhibitory one) of Ag-AgCl type after amplification of the out-put pulses through a two channel main amplifier. These stimulating pulses reproduced were somewhat different from the original pulses in shape and were not complete square pulses, but it did not exert any improper influence on the responses of the muscle in this experiment. The frequency of stimulation is important in the present experiment.

On the other hand, a tip of the dactylopodite was connected with an isotonic lever, by means of which the mechanical responses of the abductor muscle were registrated on the smoke paper of a kymograph as usual.

Stimulation signal was recorded by means of a small lever connected with a vibrator of a moving coil type which was remodeled from a dynamic speaker. This vibrator was driven by another amplifier which was connected with the amplifier for stimulation as described above.

For extracellular recording of action potentials from an isolated single motor fibre, two pairs of glass capillary electrodes filled with van Harreveld's solution were used, and these electrodes were set in a moist chamber. One pair of electrodes was connected with a square pulse generator for stimulation and another pair with a D.C. amplifier through Ag-AgCl type electrodes, respectively. Potential changes were fed to the dual beam cathode ray oscilloscope after amplification.

A method for recording junction potentials from the muscle was given in detail by Murayama and Yamashita (1962).

All experiments were carried out at room temperature (21.5°–27.5°C).

Results

Efferent nerve fibres supplying the muscles and their electrical properties: By means of vital staining with a dilute solution of methylen blue and observation of the mechanical responses of the muscle by stimulation of the nerve, the distribution of the efferent axons for the distal muscles of the cheliped was investigated. The innervation pattern of the cheliped muscles is shown in Fig. 1. Especially, the

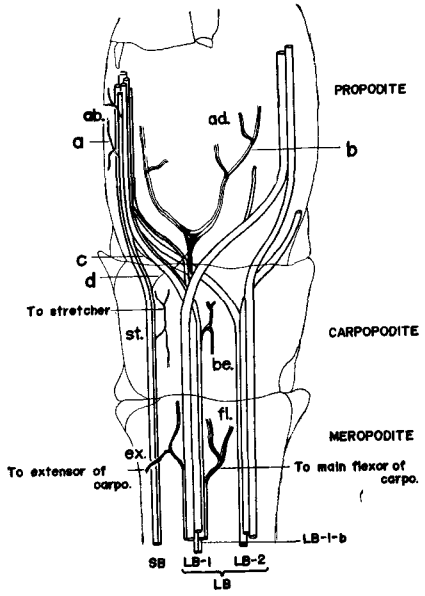

Fig. 1. Innervation of the cheliped muscle of the crayfish. a.: a slow motor axon and an inhibitory one (c.) supplying the abductor muscle, b.: a fast motor axon, a slow motor and an inhibitory (d.) supplying the adductor muscle. A fast and a slow motor axon supplying the adductor muscle (ad.) and an inhibitory axon supplying the abductor muscle (ab.) are contained in the bundle LB–1. A slow motor axon supplying the abductor muscle and an inhibitory axon supplying the adductor muscle are contained in the bundle SB.

adductor muscle was supplied by a fast motor nerve fibre, a slow one as a excitatory nerve and an inhibitory one. The abductor muscle received a slow motor nerve fibre and an inhibitory one. The results obtained in this experiment were essentially similar to those described by van Harreveld and Wiersma (1937) and Nagahama (1950). Accordingly, it is conceivable that the cheliped and the walking leg of Crustacea show, in general, the same innervation pattern as those described above, although there is a little difference in detail.

Excitability of the efferent nerve fibres supplying the adductor muscle and

the abductor one was examined by recording action potentials from each nerve fibre, which was completely isolated into a single nerve fibre. The results obtained are given in Table 1.

All the efferent nerve fibres responded in all-or-none fashion to produce

Table 1. Diameter and excitability of three kinds of the efferent nerve fibres supplying the adductor and the abductor muscle of the cheliped.

Nerve fibre	Diameter μ	Conduction velocity m/sec.	Refractory period msec.
Fast axon	47.5 (45.0—52.5)	7.5 (6.8—8.1)	2.5 (2.5—2.6)
Slow axon	31.9 (25.0—37.5)	2.8 (1.7—3.9)	3.6 (2.4—4.8)
Inhibitory axon	22.1 (7.3—30.0)	4.9 (3.9—5.8)	4.3 (3.2—5.4)

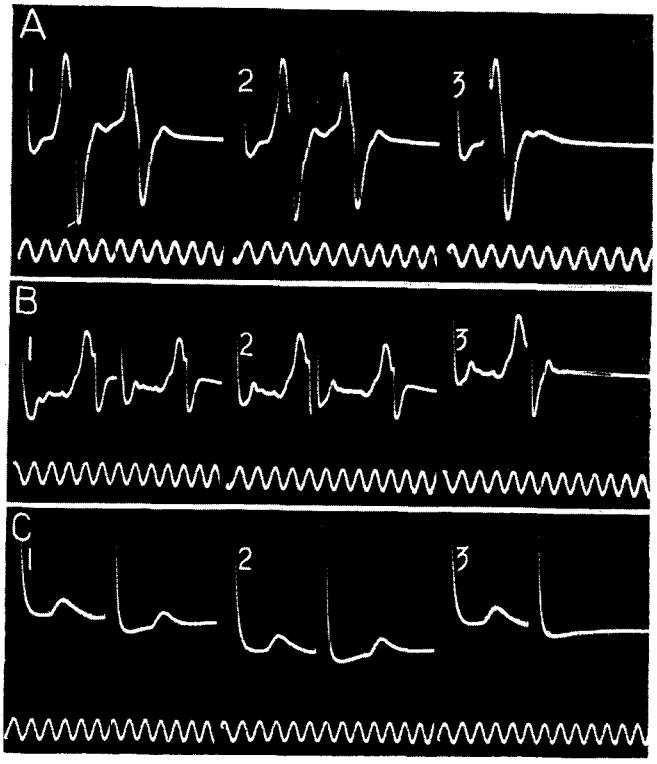

Fig. 2. Action potentials extracellularly recorded from a fast motor nerve (A), a slow motor one (B) and an inhibitory one (C). Successive two stimuli were given at various intervals to study refractory periods. Refractoriness of each nerve fibres is seen in the last records of each series. Time signals: 1000 cycles per second.

propagated action potentials to a single shock stimulus as is shown in Fig. 2. Although there was some difference in the excitability, the inhibitory nerve fibre was essentially the same as the excitatory one. The function of the inhibitory nerve fibre was only that it conducted impulses. Therefore, it appears that the peripheral inhibitory action takes place at the neuromuscular junctions.

Excitatory junction potentials (e.j.p.'s) and inhibitory junction potentials (i.j.p.'s): As the abductor muscle was supplied by only a single slow nerve fibre, it was convenient material to use for investigation of a pure slow contraction without isolation to a single slow nerve fibre for stimulation. In the following experiments, only the abductor muscle preparation was used.

The slow nerve-abductor muscle system was highly facilitated, and both the amplitude of the slow junction potentials (s.j.p.'s) and the height of a slow contraction of the muscle gradually increased (Murayama and Yamashita 1962), accor ding to increase in the frequency of repetitive stimuli. On the other hand, if only the inhibitory nerve was stimulated by a single shock to the inhibitory nerve, no detectable electrical change was obtained from the muscle; but by ca. 20 stimuli/ sec. the electrical changes of only a few mV (pure i.j.p.'s) was recorded. I.j.p.'s were the hyperpolarizing changes in the muscle of the low resting potential (e.g., 0.6 mV hyperpolarization at 70 mV in resting potential); the depolarizing one, on the contrary, in the muscle of the high resting potential (e.g., 0.3 mV depolarization at 80.6 mV in resting potential). And then various inhibitory effects on the e.j.p.'s were seen when the inhibitory nerve and the slow one were stimulated at various intervals. The most typical effect was the reduction of e.j.p.'s; in particular, when the inhibitory impulses arrived at the neuromuscular junctions from 2 to 3 msec. before the excitatory impulses, the e.j.p.'s were reduced exceedingly (Fig. 3B). This is in agreement with the results obtained by Marmont and Wiersma (1938) and Fatt and Katz (1953b). But other cases were observed: 1) no effect of inhibitory impulses on the e.j.p.'s and 2) algebracial summation of the i.j.p.'s on the e.j.p.'s (Fig. 3A). In any case, the mechanical inhibition of the contraction was similarly evoked, having no connection with those changes of the junction potentials.

These results may be taken to indicate that the inhibitory mechanism of the crustacean cheliped muscle cannot be clearly interpreted with the view of post-synatpic membrane conductance increase by the inhibitory impulses (Fatt and Katz 1953).

Mechanical inhibition of the contraction of the abductor muscle: The neuromuscular system of Crustacea, as mentioned above, showed a high degree of facilitation. Accordingly, it is conceivable that not only the contraction of the muscle but also the inhibitory effect on it is dependent on the frequency of repetitive stimulation to the excitatory and inhibitory nerve. Therefore, the relation of the frequency of stimulation of the slow nerve and the inhibitory one with the mechanical inhibitory effect on the contraction of the abductor muscle was

studied at first, apart from the electrical phenomena of the neuromuscular junctions. In the present experiment, it was unnecessary to check the intensity factor of stimulation to the nerve, for the abductor muscle was supplied by only a single slow motor nerve fibre and a single inhibitory one and responses of these nerve fibres were of all-or-none fashion.

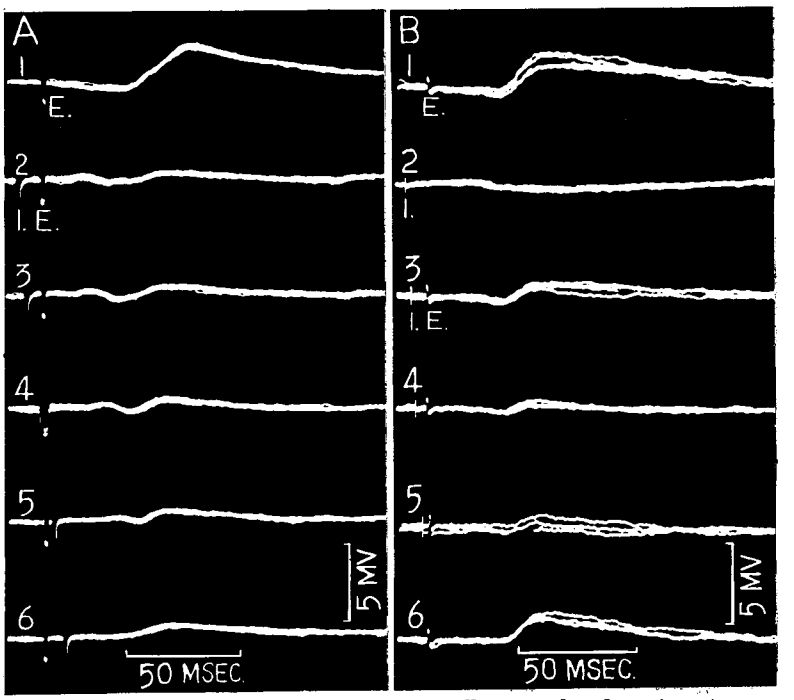

Fig. 3. Two typical examples of inhibitory effect on the slow junction potentials intracellularly recorded from the abductor muscle fibre in response to stimulation of the slow motor nerve and of the inhibitory one at various intervals. Each record resulted from manys uccessive sweeps synchronized with the frequency of stimulation. Reduction of the e.j.p.'s by inhibitory impulses is observed in B5, and the record B2 is pure inhibitory junction potentials. In each record, E. and I. are artifacts of the excitatory and inhibitory stimuli respectively.

If only the slow nerve was stimulated at a relatively low frequency (less than 10 stimuli/sec.), a visible contraction of the muscle fibre could be evoked, but this contraction could not move the dactylopodite. At more than 20 stimuli/sec., a powerful contraction of the muscle was elicited to move the dactylopodite. According to the increase in the frequency of repetitive stimuli, the neuromuscular preparation showed a remarkable facilitation and the rising speed of the contraction of the muscle increased; and the abductor muscle contracted in approximately the same degree as that of the contraction in response to stimulation of

the slow nerve fibre at the frequency of more than 60 stimuli/sec.

In the next place, the mechanical inhibitory effects on contractions evoked by stimulation of the slow nerve at the frequencies of 30, 60, 100 and 300/sec. were studied by stimulation of the inhibitory nerve at various frequencies from 10 to

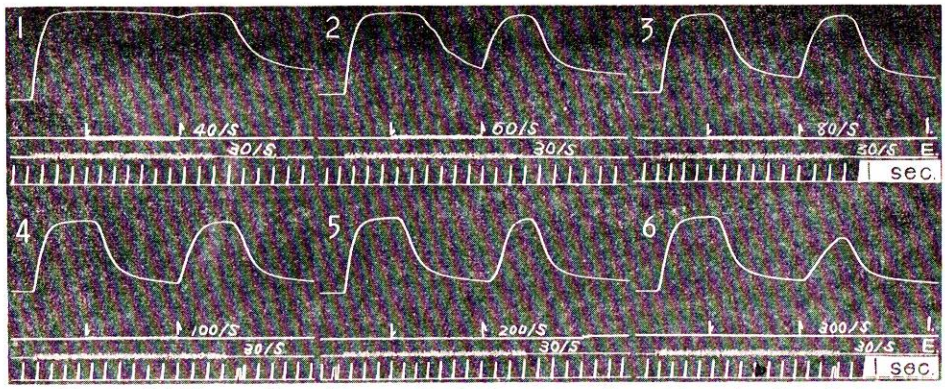

Fig. 4. Inhibitory effect of increase in the frequency of repetitive stimuli applied to the inhibitory nerve on the mechanical responses of the abductor muscle. An inhibitory nerve was stimulated at a frequency indicated in each record, while a slow motor nerve was stimulated at a frequency of 30/sec. In each record, from the top, the first signals: mechanical responses (the upward deflection: contraction, the downward one: inhibition of it), the second and the third signals: stimulation signals of the inhibitory nerve ↓ and ↑: marks of the beginning and the cessation of repetitive stimuli, respectively) and of the slow motor nerve respectively, the fourth signals: time scale indicating 1 second intervals.

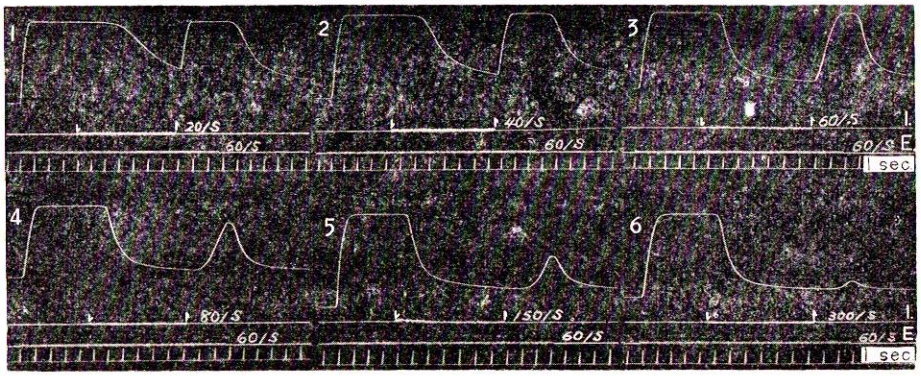

Fig. 5. Inhibitory effect on the mechanical responses of the abductor muscle. A slow motor nerve was stimulated at a frequency of 60/sec. For details, see Fig. 4.

300 stimuli/sec. for a certain period during the excitatory nerve stimulation. As the rate of the mechanical inhibition was gradually increased by the stimulation even of a relatively low frequency (20–30 stimuli/sec.) according to elongation of

its duration. In the present experiment, the mechanical inhibitory effect was investigated with a certain duration (3.8–7.5 seconds) of the inhibitory stimulation through one series of the experiment.

As is shown in Fig. 4, 5 and 6, the inhibitory effects appeared in three points of the mechanical response according to the frequency of repetitive stimulation of the inhibitory nerve. When the frequency of stimulation of the inhibitory

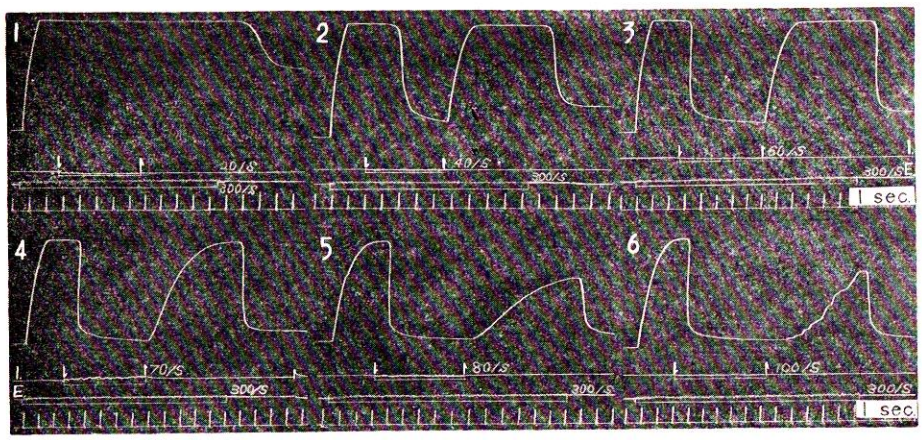

Fig. 6. Inhibitory effect on the mechanical responses of the abductor muscle. A slow motor nerve was stimulated at a frequency of 300/sec. For details, see Fig. 4.

nerve was increased: 1) the rate of relaxation of the muscular contraction gradually increased and the stimulation at a frequency of more than about 70/sec. completely inhibited the contraction of the muscle, 2) the time required for the mechanical inhibition to appear after the beginning of the inhibitory nerve stimulus (an inhibitory latent period) was gradually reduced, and 3) even after the cessation of the inhibitory stimulus at a frequency of more than about 70/sec., an inhibitory after-effect appeared on the re-contraction of the muscle, the rising speed of which was slowed down as a result the height of re-contraction of the muscle gradually decreased; and these three effects gradually increased. It was most interesting that these three effects of the inhibitory nerve stimulation did not depend on the frequency of repetitive stimulation of the excitatory nerve, but only on that of the inhibitory nerve stimulation and showed a certain tendency to increase with the latter frequency. Some typical examples of the results described above are shown graphically in Fig. 7. When the inhibitory nerve was stimulated in the rising phase of the contraction elicited by the slow nerve stimulation of 30/sec., the same tendency of the inhibitiory effects as described above was also observed.

On the other hand, when the inhibitory nerve was stimulated at a relatively higher frequency, a decrease in the height of re-contraction of the muscle by the

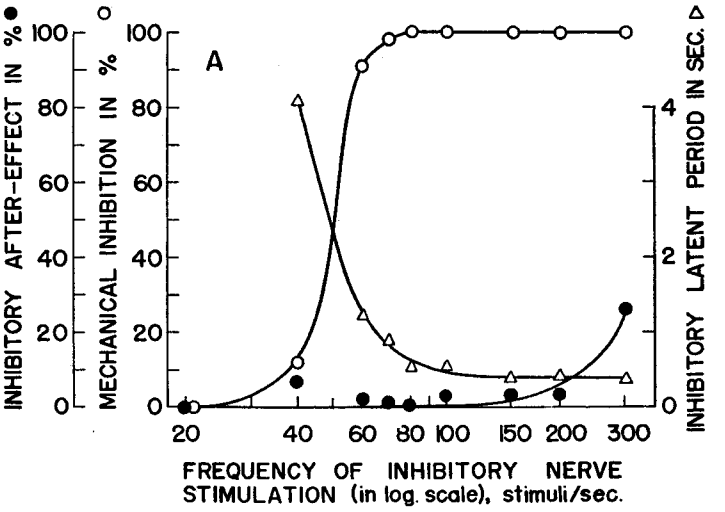

Fig. 7 (A)

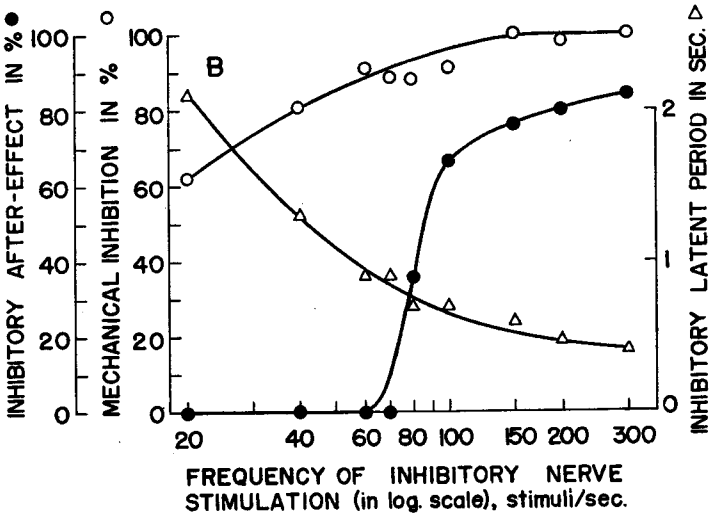

Fig. 7 (B)

Fig. 7. Graphs representing typical examples of the relation between the stimulus frequencies to an inhibitory nerve and the inhibitory effects. Hollow circles: the amount of reduction per cent of the amount of the normal contraction, triangles: the inhibitory latent period (time required for the mechanical inhibition to appear after the beginning of the stimulation of the inhibitory nerve), solid circles: inhibitory after-effect on the re-contraction of the muscle after the cessation of the inhibitory stimulus (the reduced height per cent of the height of the normal contraction). Frequency of stimulation of a slow motor nerve: 30 stimuli/sec. in A, 60 stimuli/sec. in B, 100 stimuli/sec. in C and 300 stimuli/sec. in D.

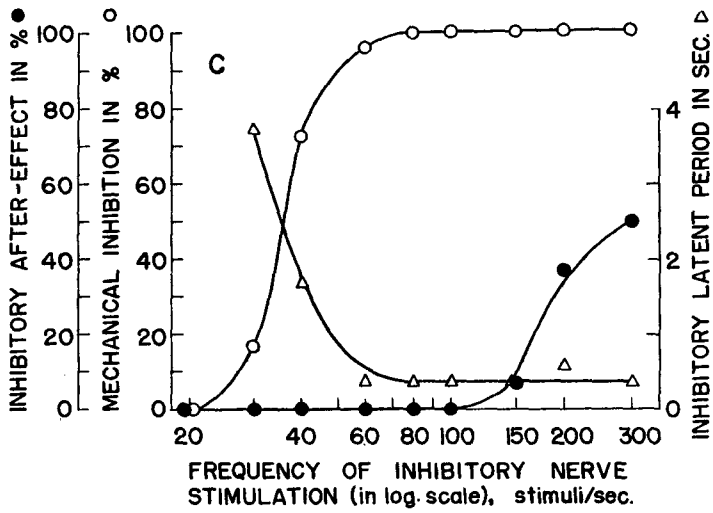

Fig. 7 (C)

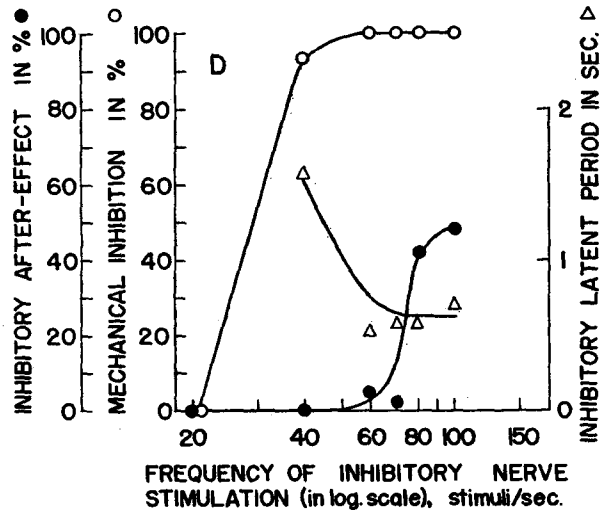

Fig. 7 (D)

successive excitatory stimulation was observed after the cessation of the inhibitory nerve stimulation. The inhibitory after-effect generally increased according to the increase in frequency of stimulation of the inhibitiory nerve, but a typical inhibitory after-effect was not shown in some preparations. However, the rising speed of the re-contraction was somewhat slower than that of the normal contraction and the

latency of the re-contraction after the cessation of the inhibitory nerve stimulation became longer in the same way.

Discussion

The results obtained from the efferent nerve fibres by means of electrophysiological method definitely indicate that the inhibitory nerve fibre conducts merely nerve impulses and its function is essentially almost the same as the other excitatory nerve fibres. This fact is very important in the physiological study, and then it seems possible that the peripheral inhibitory action takes place at the neuromuscular junctions.

The result which the inhibitory effect on the mechanical responses is not dependent on the change in the stimulus frequency applied to the excitatory nerve but only on that to the inhibitory nerve is different from the report by Marmont and Wiersma (1938) in which a certain ratio, the frequency of inhibitory stimulation/ that of excitatory stimulation, to set up a just complete inhibition was found. On the other hand, Dudel and Kuffler (1961) reported that the reduction of the e.j.p. in response to the inhibitory nerve stimulation was independent of the frequency of the excitatory stimulation, which is in agreement with the result obtained in the present study. In the walking leg of the crayfish, the muscular contraction evoked by the repetitive stimulation of the excitatory nerve at a relatively low frequency can be completely inhibited by the inhibitory stimulation at the frequency from 50 to 60/sec, but repetitive stimulation of the inhibitory nerve at higher frequencies is necessary for the complete inhibition of the contraction elicited by the excitatory stimulation at a relatively higher frequency of more than 50/sec. (Aoki 1963). This also seems to be fundamentally the same as the present results; the difference is only due to the duration of the train of repetitive stimuli applied to the inhibitory nerve (in Aoki's case the duration used was shorter than that in the present case). It may be important that the inhibitory impulses at a lower frequency can always completely inhibit even the strong contraction elicited by excitatory impulses at a higher frequency. It makes a quicker relaxation necessary in the rapid closure of the claw.

It is conceivable that the inhibitory transmission is due to a chemical transmitter substance from the fact that the repetitive stimulation of the inhibitory nerve at a high frequency elicites the inhibitory after-effect and the inhibitory impulses accelerate the relaxation after the cessation of the excitatory stimulation (Aoki 1963 and Ito 1956).

By the way, it is clear that the inhibitory effect on the mechanical response was determined only by the frequency of stimulation of the inhibitory nerve, independently of frequency of stimulation of the excitatory nerve. This finding, therefore, cannot easily support the view that the inhibitory transmitter substance neutralizes the excitatory one to inhibit the contraction of the muscle (Marmon and

Wiersma 1938). If the inhibitory action is due to such a competitive action between these two kinds of the transmitter substance, there should always exist a certain ratio, the stimulus frequency of the inhibitory nerve to one of the excitatory nerve just for the complete inhibition. The possibility is that the inhibitory transmitter substance changes primarily the permeability of the excitatory synaptic membrane to a certain kind of ions which is related to the excitation-contraction coupling, otherwise the inhibitory transmitter substance acts presynaptically on the excitatory nerve terminal to determine primarily the release of the excitatory transmitter substance. And in either case the facilitation by the excitatory transmitter substance at neuromuscular synapse may decrease owing to enhanced inhibition by the inhibitory transmitter substance. However, there is no direct evidence concerning whether the principal inhibitory mechanism is presynaptic or post-synaptic, for even the structural relation between the excitatory and the inhibitory terminals has not been clarified.

It is suggested that the inhibitory after-effect may be due, not to the fatigue of the neuromuscular system, but to the accumulation of the inhibitory transmitter substance released by the repetitive stimulation of the inhibitory nerve at a high frequency. It may be explained from the following facts that 1) the after-effect is always observed more or less in each preparation, 2) this increases according to increase in frequency of stimulation of the inhibitory nerve and 3) the repetitive stimulation of only the excitatory nerve cannot evoke a normal contraction of the muscle within a few minutes after the cessation of inhibitory nerve stimulation.

Summary

1. The inhibitory effects on the electrical and mechanical responses of the cheliped muscle of the crayfish, especially the relation between the mechanical inhibition and the frequency of repetitive simultaneous stimulation of the excitatory and inhibitory nerves were investigated.

2. The inhibitory nerve fibre itself produced an action potential of all-or-none type to a single shock. Although there was some difference in the excitability, it was essentially the same as the excitatory nerve fibre. The function of the inhibitory nerve fibre was only that it conducted impulses.

3. When the inhibitory impulse arrived at the neuromuscular junction from 2 to 3 msec. before the arrival of the excitatory impulse, the e.j.p. was clearly reduced. But cases were also observed in which no effect on the e.j.p.'s by the inhibitory impulses appeared or an algebracial summation of the i.j.p.'s on the e.j.p.'s occured, independently of the timing of the arrival of the inhibitory impulses. In any case, the mechanical inhibition of the muscular contraction was similarly evoked.

4. Inhibitory effects on the mechanical responses, independent of frequencies of repetitive stimuli (30–300 stimuli/sec.) applied to the excitatory nerve, increased according to the increase in the frequency of repetitive stimuli applied to

the inhibitory nerve. About 70 stimuli/sec. to the inhibitory nerve completely inhibited the muscular contraction. In addition, the latent period of inhibition was gradually reduced with the increase in the frequency of repetitive stimulation of the inhibitory nerve.

5. The inhibitory after-effect on the re-contraction of the muscle was observed after the cessation of stimulation of the inhibitory nerve, and it also increased gradually as the frequency of stimulation of the inhibitory nerve was increased. It is considered that the inhibitory after-effect may be due to the accumulation of the inhibitory transmitter substance released by stimulation of the inhibitory nerve at a higher frequency.

6. The relation between the frequency of the inhibitory stimulation and the inhibitory effects is discussed, and a possible explanation of the inhibitory mechanism is also presented.

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