Effects of External Ca-,Ba- and TEA-ions on the Electrical and Birefringent Properties of Activated Single Crayfish Muscle Fibres[1]

By

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(*With 8 Text-figures and 7 Tables*)

It was shown in the previous paper on the polarized light microscopy (Kakiuchi, 1964) that in the isolated single muscle fibres from dorsal extensor muscles of the crayfish a propagative contraction over their entire length was evoked by repetitive stimulation of just threshold intensity, while no electrical property of the activated materials had yet been given.

A question arises as to how the contractile element in a living muscle fibre operates during the fibre's activity.

The various effects of some external cations on the electrical activity of a single muscle fibre have been reported by Hodgkin and Katz, 1949, Fatt and Katz, 1953, and so on.

In the present investigation, it was undertaken to record simultaneously the microphotographic mechanograms by means of the polarizing microscope and the electrograms of the activated single crayfish muscle fibres under various external ionic (Ca-, Ba- and TEA-ions) conditions. The mechanism of crustacean muscle contraction was analysed specially concerning the linkage of electrical excitation to mechanical change in the ultra-structure.

Material and Methods: The research was performed on a fresh single muscle fibre isolated free of injury from dorsal extensor muscles of the crayfish, *Procambarus clarkii*. The small pieces of exoskeleton remaining at the both ends of the muscle fibre served as a convenient support for the preparation. Dimensions of single muscle fibre preparations used

were 150–380μ in diameter and 5.0–7.5 mm in length.

The control bathing solution used was van Harreveld's solution, but the test solutions of higher concentration in Ca-, Ba- and TEA-ions were made by substituting those ions for an equivalent amount of Na-ions in the van Harreveld's solution. In most of the experiments, the solutions of four different concentrations (control, 33%, 50% and 100%) were prepared by dilution of those hypertonic fluids with the control bathing solution.

single muscle fibre

Pool A   Pool E   Pool B

glass plate →   colophony wax bank ←

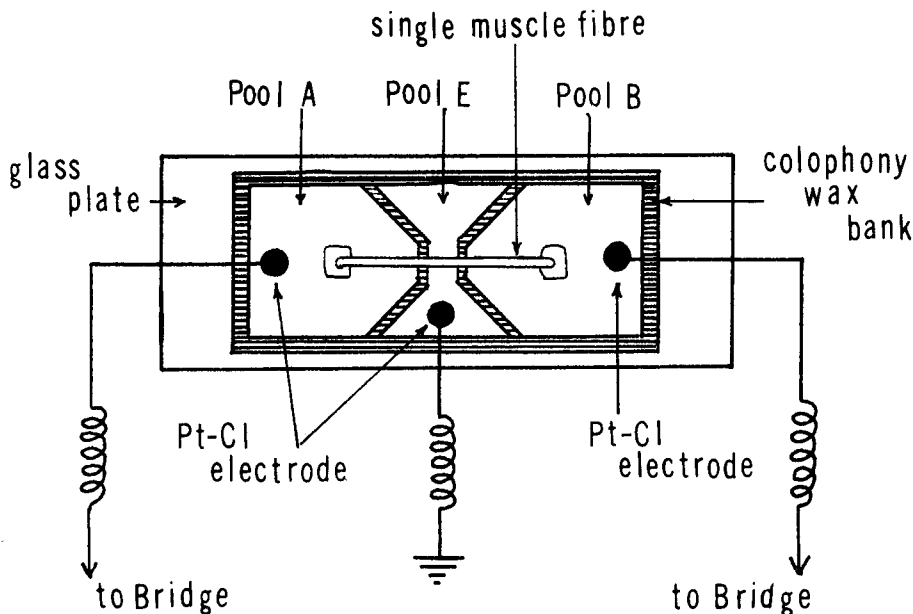Pt-Cl electrode   Pt-Cl electrode

to Bridge   to Bridge

Fig. 1. Schematic representation of the electrode assembly. Three pools A, B and E are provided on a glass plate. They are partitioned off by the colophony wax banks. The interval between pools A and B, namely the narrow part of the central (E), is 3 mm in width. Each pool possesses disc-shaped platinous chloride electrodes whose diameters are approximately 3.5 mm.

For the purpose of simultaneous recording of both electrical and optical phenomena after activation, the single muscle fibre preparations were placed across three small pools on a glass plate partitioned off by a colophony wax bank (Fig. 1). Only small amounts of experimental solution (less than 0.5 cc for each pool) were required to effect the muscle fibre. Each of the pools had a low impedance platinous chloride electrode, through which stimulating current was applied to the preparation and the bio-electric potential change was led out extracellularly. In order to prevent electrical leakage due to the presence of saline covering the muscle preparation between the pools, small amounts of petroleum jelly were coated on the muscle fibres at the partition banks for insulation. The insulating petroleum jelly banks brought the threshold for electrical stimulation of the single muscle fibre to a low level.

Before stimulation and recording, the preparation was adapted to the new solution for more than 15 minutes after solution change.

The stimulating and recording systems are illustrated in Fig. 2.

The electrical recording equipment consisted of a bridge circuit and a differential input emitter follower electrometer. The bridge circuit was set to abolish the stimulus
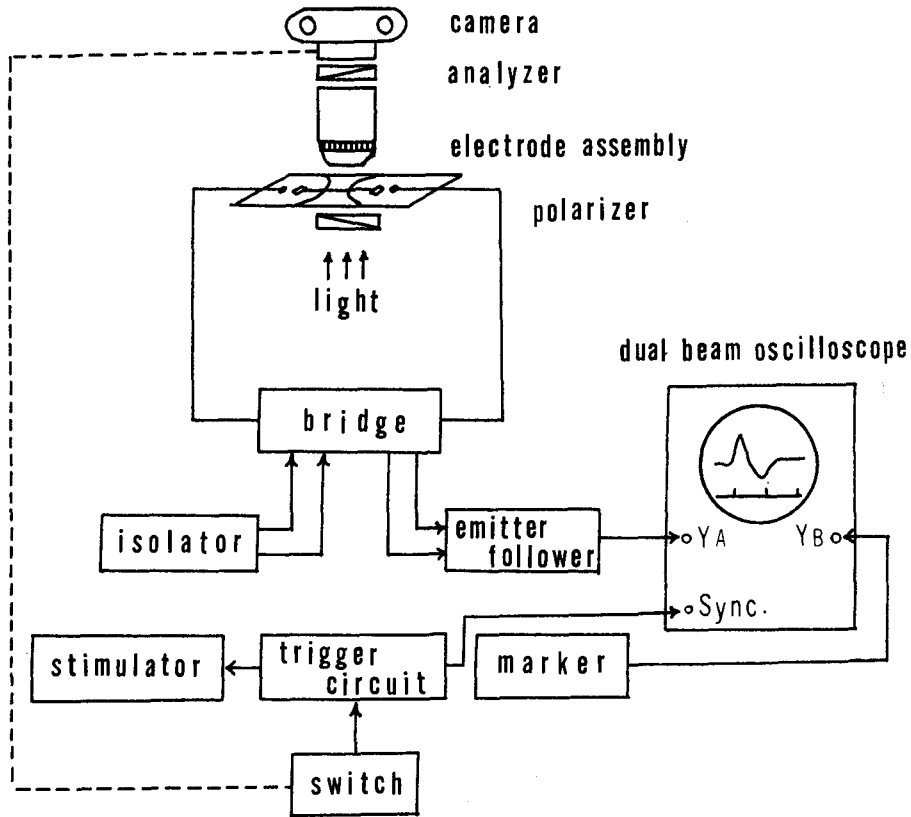

Fig. 2. Schematic diagram of the experimental arrangement. It consists mainly of a stimulating system, an electrical recording system and a polarizing microscope. These three are coupled with each other electrically or mechanically in the trigger circuit for synchronization. The borken line shows a mechanical coupling between the shutter of the camera and the switch of the trigger circuit.

artefact which appeared in the input stage because of the system's employing the common electrodes for stimulation and recording. Due to a lack of balancing capacitance component, however, the transient artefact was not completely eliminated by this system. The input transistors were of the silicon-planar type and operated in a low base current

(less than 0.005μA), so that the input impedance was estimated as more than 10MΩ. The out-put was connected to the first channel of a dual-beam oscilloscope (Nihon Kohden, VC–5). The voltage and time calibration was carried out by a train of square pulses from a pulse generator; the amplitude and the interval corresponded to the voltage and the time. Calibration signals were fed into the second channel of the oscilloscope. The rise time of the recording system was frequently checked with a square wave derived from the stimulator and inserted in series with the electrodes. The over-all rise time was between 80 and 150μ sec. The beams of the oscilloscope were externally swept by pulses from the triggering circuit.
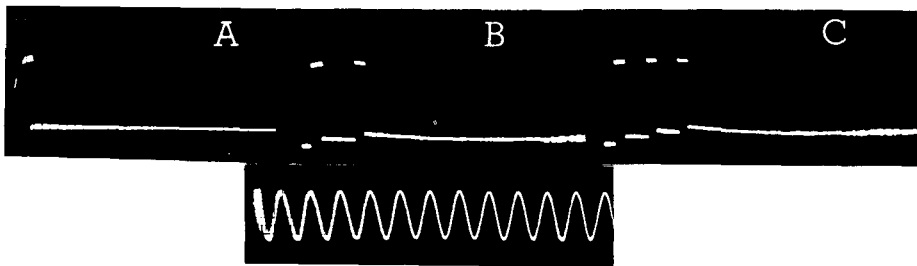

Fig. 3. Oscilloscope records of stimulating pulses used for activation. A: single pulse, B: double pulses and C: triple pulses. The double pulses were generally employed in the present research. Their amplitude ranged between 1.74–2.0 volts. The lower time signal is 1000 cycle per second.
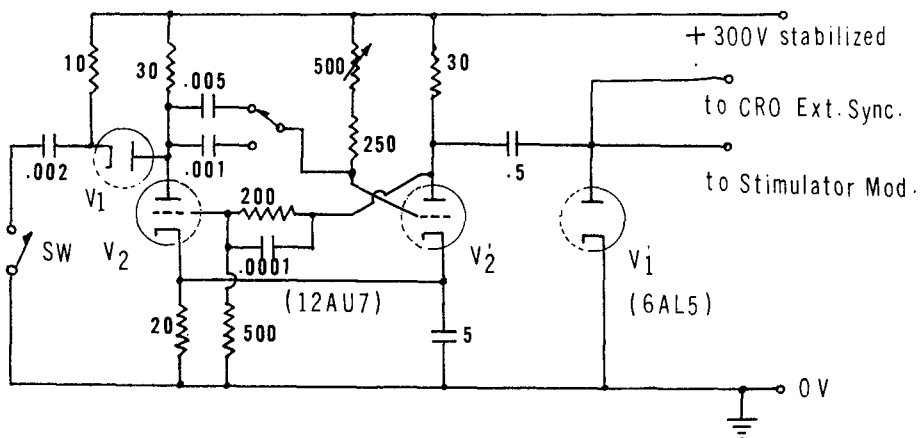

Fig. 4. Circuit diagram of the trigger circuit. By manipulation of the switch, the monostable multivibrator (V_2 and V_2') generates square pulses, width of which is 1–5 msec and variable. The output square pulse is fed into the oscilloscope and to the stimulator for both triggering and modulation. The DC regenerator (V_1') delivers DC component to the output square pulse the bottom of which was arranged at the zero volt level. The values of capacitors and resistors are shown in μF and KΩ respectively.

A square wave voltage generator (Nihon Kohden, MSE–2) was used for stimulation. This generator also provided, together with the triggering circuit, single, double and triple square pulses (see Fig. 3) whose width and interval were approximately 0.5 msec, and whose amplitude varied from 1.74 to 2.0 volts.

Photographing the change in birefringence during activity was performed through a polarizing microscope (Nikon, POH). The shutter of the camera was mechanically connected with the switch of the triggering circuit for synchronization with the muscular contraction. The exposure time of camera was 1 ± 0.1 msec.

The synchronization which made the simultaneous recording of the electrical and optical change in the materials possible, was achieved by the triggering circuit (Fig. 4), where the trigger pulses to operate both recording and stimulating systems and the gate pulses for the production of stimulating double and triple pulses, were generated.

All experiments were carried out at room temperature ($18°$–$22°C$).

Results

Activation of the muscle fibres: Only the propagating action potentials were treated in this investigation. Fig. 3 shows the wave form of three kinds of the square pulse stimuli employed. The pulse width and pulse interval were kept unvaried throughout the study, the amplitude ranged between 1.75–2.0 V and this was suitable for the preparations. A single pulse stimulus did not evoke action potential of the muscle fibre in the control bathing solution, but it evoked the burst of action potentials in the test solutions of higher concentration (of $CaCl_2$, $BaCl_2$, and TEA-Cl) than 50%, 80%, and 90%, respectively. Adding to this fact, no propagating action potential was initiated in the control bathing solution by any single pulse stimulus in spite of an increase in the duration and the amplitude up to 3 msec and 5 V, respectively. The excessively strong stimulus injured the muscle preparations. The stimulation with double pulses could just evoke action potentials in most preparations. The stimulation with triple pulses also had the same effect as that of the stimulation with double pulse. There was no significant difference in the size and the duration between action potentials elicited by both kinds of stimuli (A and B in Fig. 5).

Recording of propagated contraction wave: The contraction wave propagating along the muscle fibre was photographed through the polarizing microscope and it was clearly found as a dark portion of the fibre on the microphotograph. The muscle fibre preparation placed on the glass plate having an electrode assembly was accurately set at voluntary position by controlling the mechanical stage of the microscope. This was necessary for catching the contraction wave in the microscope field. The timing between stimulation and operation of the camera shutter was also important. The microphotography followed the application of stimuli by approximately 5 msec because of the mechanically delayed switching of the triggering circuit.

The correlation between the size of action potentials and the degree of change in the birefringence is shown in Fig. 5, in which a muscle fibre was activated (A)
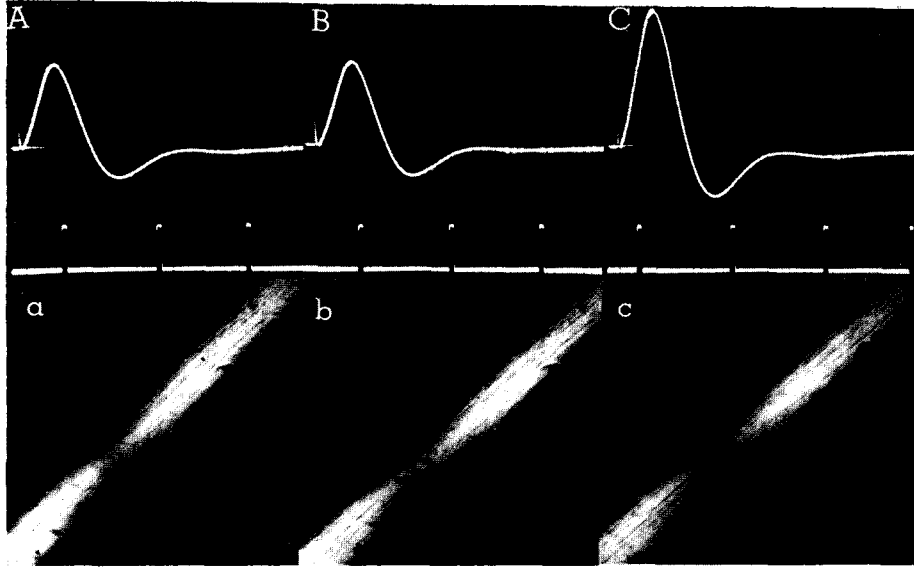
Fig. 5. Action potentials (A-C) and contraction waves (a-c) recorded simultaneously from a muscle fibre treated with Ca-solution at the following concentrations: 33% Ca-ions substituted for Na-ions in A, B, a, b, and 50% Ca-ions substituted for Na-ions in C and c. Activation was performed by double-pulse stimuli in A, C and c. In B and b, the muscle fibre was activated by triple-pulse stimuli. The diameter of the fibre was 150μ. The exposure time of the camera was 1 msec. The amplitude of the calibration mark indicates 20 mV and the interval 20 msec.

by double pulses in 33% CaCl$_2$ solution, (B) by triple pulses in 33% CaCl$_2$ solution, and finally (C) by double pulses in 50% CaCl$_2$ solution. (a)-(c) in Fig. 5 are the polarized light microphotographs of activated muscle fibre simultaneously taken with recording of the action potential. In the figure, the microphotographs (a)-(c) correspond to the records (A)-(C) of action potential respectively. It is evident that the degree of darkness which locally appeared in the muscle fibre is proportional to the amplitude of the action potential shown in Fig. 5.

Effect of Ca-ions: Previous to recording, some inspections were performed. No striking shrinkage of the muscle fibre was observable under the microscope even in 100% CaCl- solution. However, the stimuli were applied to the muscle fibres repeatedly up to eight times under the external condition, because the fibre would become completely unexcitable and was injured by excessive application of stimuli.

The effect of Ca-ions on muscle fibres was investigated by means of double-pulse stimulation. The oscilloscope records obtained are shown in Fig. 6, the amplitudes and the durations of the action potentials were measured on the
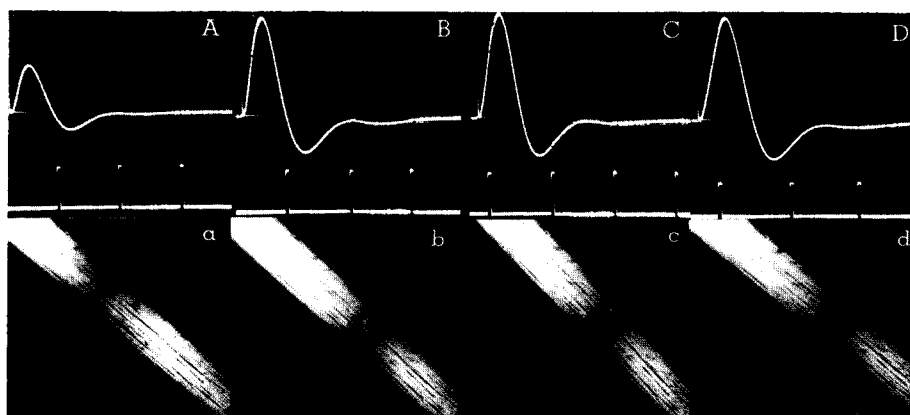
Fig. 6. Action potentials (A-D) and contraction waves (a-d) recorded simultaneously from a muscle fibre treated with Ca-ions solution at various concentrations: 33% Ca-ions substituted for Na-ions in B and b, 50% Ca-ions for Na-ions in C and c, 100% Ca-ions for Na-ions in D and d, and the control solution in A and a. The muscle fibre was activated by double pulse stimuli. Each record was taken 15 minutes after the treatment. The diameter of the muscle fibre was 220μ, and the exposure time was 1 msec. The amplitude of the calibration marks indicates 20 mV and the interval 20 msec.

Table 1. Effects of Ca-, Ba-and TEA-ions on the action potential

Test solution		Action potential	
		Amplitude in mV	Duration in msec
control		23—34	29—36
CaCl$_2$*	33%	45—58	35—38
	50%	49—62	36—40
	100%	64—78	36—39
BaCl$_2$*	33%	50—68	33—37
	50%	59—71	38—42
	100%	65—78	38—48
TEA-Cl*	33%	25—33	40—52
	50%	52—64	46—59
	100%	60—68	54—72

*CaCl$_2$, BaCl$_2$ and TEA-Cl are substituted for NaCl in van Harreveld's solution at proportions as are shown in the table.

records, and the values are summarized in Table 1. The variation of measured values of action potentials is refered to in the discussion (p. 172). As is shown in Table 1, the amplitude of action potentials is increased with the Ca-ion's concentra-

tion, although no linear correlationship is perceived between the amplitude and the concentration of Ca-ions. No remarkable increase in duration was obtained with Ca-ions. Washio, et al. (1961) reported that the birefringence decreases with the height of contraction of the fibre and with the degree of disorder of the submicroscopical arrangement of the contractile elements. In this point of view, the patterns of change in birefringence of a muscle fibre in Ca-test solutions (a-d in Fig. 6) reveal the degree of muscle contraction simultaneously recorded with the amplitude of action potential. Consequently, it is accepted from the figure that the darkness of the pattern increases as the amplitude of the action potentail increases.

   Effect of Ba-ions: The effects of Ba-ions was carried out in the same way as that of Ca-ions. The records are shown in Fig. 7, and the resultant values of action potentials are in Table 1. The results obtained were similar to those obtained from the experiments on Ca-ions, and also to the result of Murayama and Yamashita (1962) on the adductor and the abductor muscles of crayfish cheliped.
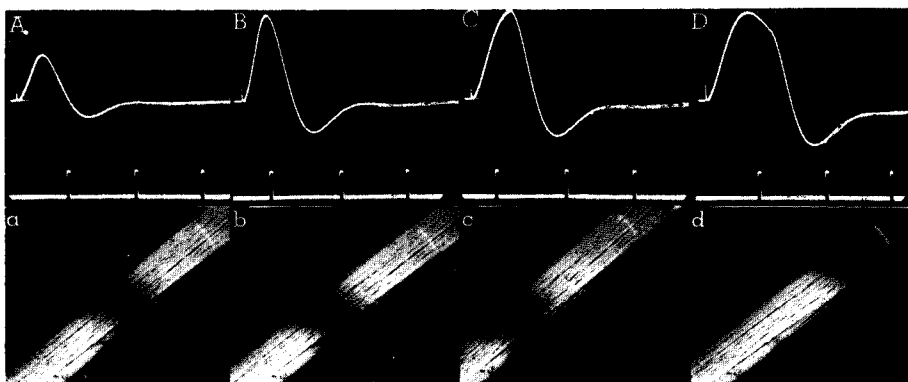

Fig. 7. Simultaneously recorded action potentials (A-D) and contraction waves (a-d) of a muscle fibre treated with Ba-ions solutions: 33% Ba-ions substituted for Na-ions in B and b, 50% Ba-ions for Na-ions in C and c, 100% Ba-ions for Na-ions in D and d, and the control solution in A and a. Double-pulse stimulation was used, the diameter of the fibre was 280μ and the exposure time was 1 msec. The amplitude and interval of the calibration marks indicate 20 mV and 20 msec, respectively.

   The length of contraction wave was measured on the microphotograph as the length of darkened part of the fibre where the birefringence was decreased. The darkened part was more dark and wide in range in the Ba-ions solutions than in the Ca-ions solutions, and the contraction wave stronger in the Ba-ions solutions than in the Ca-ions solutions. As is shown in Fig. 7, the length of contraction wave in the isotonic $BaCl_2$ solution is increased to about 130% of that in the control bathing solution, and the duration of action potential is increased to 140%

of that in the control. It may be considered that there is a parallel between the length of contraction wave and the duration of action potential.

Effect of TEA-ions: TEA-ions prolonged the duration of action potential according to their concentration with an accompanying increase in the amplitude. No striking prolongation such as that in TEA-Cl solutions was seen in any solutions of $CaCl_2$ or $BaCl_2$. The records are shown in Fig. 8 and the values of action potentials in Table 1. The wave forms of action potentials in the test solutions of higher concentration of TEA-ions are different from those in the cases of Ca-ions or Ba-ions, and they have plateaus.

Propagation velocity: The propagation velocity of action potential was estimated. The action potential traveled the distance (3 mm) between A- and B pools in Fig. 1. From the records in Figs. 6–8, the propagation velocity (0.15–0.25 m/sec) was obtained.

On the other hand, concerning the patterns of change in birefringence, the propagation velocity of contraction wave was measured by analyzing the micro-photograph. From the exposure time of approx. 1 msec and from Figs. 6–8, the propagation velocity (0.3–0.4 m/sec) was estimated.
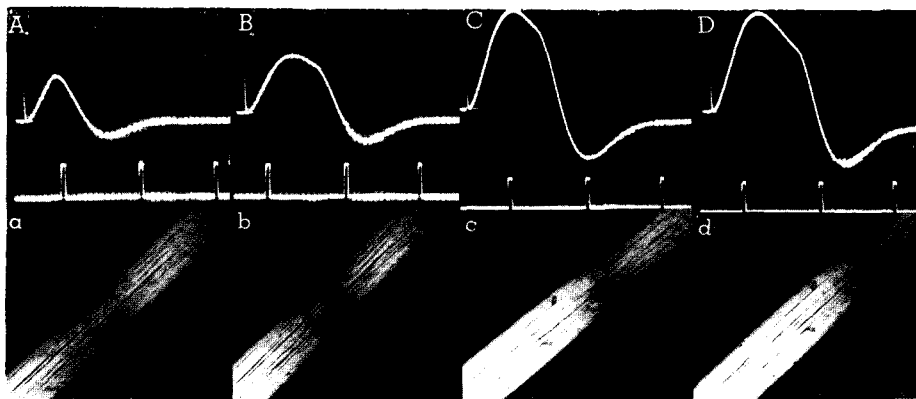

Fig. 8. Action potentials (A-D) and contraction waves (a-d) recorded simultaneously from a muscle fibre treated with TEA solutions: 33% TEA-ions substituted for Na-ions in B and b, 50% TEA-ions for Na-ions in C and c, 100% TEA-ions for Na-ions in D and d, and the control solution in A and a. Double-pulse stimulation was used, the diameter of the preparation was 350μ, and the exposure time 1 msec. Calibration mark indicates 20 mV in height and 20 msec in width.

Discussion

The effects of external Ca-, Ba- and TEA-ions on the electrical properties of muscle fibres have been reported by many authors, namely, Hodgkin and Katz (1949), Tamasige (1956), Fatt and Ginsborg (1958), Werman, MacCann and Grund-

fest (1961), Murayama and Yamashita (1962), Abbott and Parnas (1965). The results obtained from the present study agree generally with those obtained by them.

The remarkable variation in values of action potentials in the same solution in Table 1, may be ascribed rather to the irregular order of the change of the test solutions, than to the individual variation; the change of test solutions was carried out usually from the lower to the higher concentration, but in some cases order was reversed to check the excitability of the fibres.

The values of propagation velocity of action potentials, 0.15–0.25 m/sec, coincide well with that of the 0.2 m/sec reported by Katz and Kuffler (1946) and Fatt and Katz (1953) on crustacean muscle fibres.

In the previous paper (Kakiuchi, 1964), the value of 0.8 m/sec for the propagation velocity of the contraction wave was obtained on the same muscles, but the value obtained from the present study using double-pulse stimulation was 0.3–4.0 m/sec. There is a remarkable difference between the values. The former value of 0.8 m/sec was estimated from the fact that only one contraction wave is elicited in response to a single electrical shock. In the muscle fibre soaked in van Harreveld's solution, at least two successive stimuli were necessary for a contraction wave to be elicited and propagated. The calculation of conduction velocity in the former case must be carried out considering that a contraction wave is elicited by two peaks of the sine-wave current stimuli. The time factor T_1-T_2 in the formula should be multiplied by a factor of two, and from this the propagation velocity is 0.4 m/sec. The value is well in accordance with the value of 0.3–0.4 m/sec in the present case.

An accurate quantitative measurement could not be made on the microphotographs shown in Figs. 6–8. However it may be concluded that the amplitude of action potential is proportional to the degree of disorder of the submicroscopical arrangement of the contractile elements, that is, to the height of the contraction wave, and that the duration of action potential is also proportional to the disordered area of the contractile elements, namely, to the wave length of the contraction wave.

Finally, it is concluded that an action potential and contraction wave are derived from an activation mechanism in the stimulated muscle fibre, and that the mechanism is activated by a disturbance of electro-chemical equiblirium in the fibre by entrance of calcium or other ions into the cell interior, and then the submicroscopical arrangement of contractile elements which consist of long protein chains is put into disorder by a resultant change in electrical field.

Summary

1. The effect of Ca-, Ba- and TEA-ions on the action potentials and on the birefringence changes of isolated single muscle fibres of crayfish dorsal extensor muscles during activity was investigated.

2. These ions increased the size and the duration of action potentials of the

muscle fibre, as their concentration was increased. TEA-ions had a striking effect on the prolongation of the duration.

　　3.　Propagating action potentials and contraction waves of the crayfish muscles were recorded simultaneously. The propagation velocity of action potentials 0.15–0.25 m/sec, was measured. That of the contraction waves was of the same order, although the accuracy of measurement of the latter was certainly low.

　　4.　It was concluded that the amplitude and duration of action potentials are proportional to the height of the contraction wave and to the wave length, respectively.

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