Effects of Reduction of Calcium Ions on the Electrical Properties of an Isolated Single Muscle Fibre from the Crayfish[1),2)]

By

Yoshimi Miyamoto[3)]

Zoological Institute, Hokkaido University
(*With 6 Text-figures*)

The effects of changes in extracellular calcium ions upon properties of the frog muscle membranes have been investigated by many workers. An increase in the concentration of calcium ions in a bath solution, increased the threshold of stimulation and membrane resistance (Tamasige, 1951 and Jenerick and Gerard, 1953). An increase in the amplitude of the membrane potential, or the action potential, has also been reported following soaking of the muscle in fluid containing high concentrations of calcium (Ishiko and Sato, 1957).

On the other hand, when the amount of external calcium is reduced, the threshold decreases slightly and a transient increase in the excitability of the cell membrane has been observed (Hisada and Miyamoto, 1961). In addition, recent investigations have shown that perfect removal of external calcium generates spontaneous discharge of the action potential, because the cell membrane becomes unstable because of the remarkable decrease in the membrane resistance (Bülbring et al., 1956 and Curtis, 1961). These experiments clearly indicate that the excitability of the muscle membrane is controlled by the amount of external calcium.

Crustacean muscle has somewhat different electrical properties from frog muscle. It has high membrane capacitance ($40\mu F/cm^2$), and low membrane resistance ($100\Omega.cm^2$). The action potential obtained from the muscle seldom propagates and its amplitude varies with stimulus intensity, which has been referred to as a "graded response" in contrast to the "all-or-none response" of the frog muscle, or of the other excitable tissues (Fatt and Katz, 1953). This graded response can be converted to an all-or-none response by adding alkali-earth ions such

1) Contribution No. 658 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

2) This work has been supported in part from governmental Grant No. 93002, (to Prof. M. Tamasige) in Aid for Fundamental Scientific Research.

3) The present address of the author is the Department of Physiology, Research Institute of Applied Electricity, Hokkaido University, Sapporo, Japan.

as Ba^{++} or Sr^{++} (Fatt and Katz, 1953, Werman et al., 1961 and Murayama and Yamashita, 1962).

The present author has previously shown that a transient increase in membrane excitability can be produced by low calcium treatment (Hisada and Miyamoto, 1961 and Miyamoto, 1962, 1963). Therefore, if the amount of calcium ions in the physiological saline is greatly reduced, a transient increase in membrane excitability may also be expected without changing the other ionic components of the fluid. For the purpose of confirming this assumption, an isolated single muscle fibre of a cryafish was used in calcium fluids of various concentrations. In the present study, the membrane resistance and potential were measured in order to determine the static property of the muscle membrane. In the next stage, action potentials in response to various types of stimulation were measured to analyze the nature of these potentials and to determine the threshold, chronaxie, and conduction velocity etc. From the results of these experiments, the difference between the graded and all-or-none responses and the role of calcium in the excitation of the cell membrane are discussed.

Material and Method

Single muscle fibres which were entirely free of any injury were isolated from the abdominal extensor muscle of the crayfish, *Procambarus clarkii*, leaving small portions of shell at both ends. These fibres were soaked for 5–30 minutes in normal saline or in test solutions containing various concentration of calcium. The physiological saline had the following ionic composition, in m. mol/l: Na, 79.3; K, 2.9; Ca, 4.7; Mg, 0.6; Cl, 131.7. Test solutions were made by reducing the amount of calcium in the normal saline. The slight change in the osmotic pressure of the solutions which resulted from the reduction of calcium, produced no obvious effects on the muscle membrane (Murayama and Yamashita, 1962). All of the solutions were buffered to a pH of 7.2 by adding $NaHCO_3$.

Stimulation and recording: The resting potential and the membrane resistance were measured by microelectrode technique. The glass microelectrodes used had tip diameters of 0.5μ, and a current resistance of 10MΩ. Under the microscope, the microelectrode was inserted into the muscle cell, which was mounted in a plastic chamber filled with bath solution. In order to measure the effective membrane resistance of the muscle fibres soaked in normal saline or in the test solutions, a single low resistance intracellular microelectrode was used, both for recording potential and for applying current. The potential drop across the electrode tip was balanced by a Wheatstone bridge device. Through the Wheatstone bridge, an outward current of 70 msec and constant intensity was applied to the muscle fibre. The membrane potential and the tonic potential through the microelectrode, which reflect changes in the membrane resistance, were amplified and displayed on a cathode ray oscilloscope. The action potentials produced by the muscle fibre in response to the electrical stimulation with various intensities and durations were recorded extracellularly through a pair of Ag-AgCl electrodes. All experiments were carried out at room temperature.

Results

Effects of reduction of calcium upon the membrane potential and resistance of the muscle fibre from a crayfish. Changes in the membrane potential and the

membrane resistance produced by immersion of the muscle for 5 to 30 minutes in normal and in various low calcium salines are summarized in Table 1. The values are given as percentages of the initial response.

Table 1.

Concentration of calcium in the bath solution		Duration of immersion				Number
		5 min		30 min		
			Mean		Mean	
Normal	m.p	76.2-123.8	100%	71.4-133.3	91.4%	18
	m.r	78.4-147.1	100	49.0- 98.0	60.8	18
1/4 calcium	m.p	61.9-142.9	109.5	66.7- 95.2	79.0	17
	m.r	49.0-117.6	76.5	29.4- 49.0	39.2	17
1/16 calcium	m.p	81.0-128.6	102.9	19.0- 76.2	47.6	20
	m.r	39.2- 78.4	64.7	29.4- 58.8	49.0	20
Calcium-free	m.p	47.6-128.6	92.4	28.6- 66.7	51.4	19
	m.r	39.2- 58.8	49.0	19.6- 27.4	21.6	19

Effect of reduction of calcium ions on the membrane potential and membrane resistance of an isolated single muscle fibre from a crayfish.
Symbols. m.p: membrane potential.
m.r: membrane resistance.

Membrane potentials measured 5 min after immersion showed no obvious decrease, even if a calcium-free fluid was used as the test solution. With this solutions, the membrane potential decreased to as much as only 8% of the initial response. On the other hand, membrane resistance decreased remarkably in the low calcium fluid. A decrease of 23.5% was measured in a fibre soaked in 1/4 calcium fluid, and in calcium-free fluid membrane resistance decreased to half of the initial response. Membrane potential and resistance decreased remarkably in muscle fibre soaked for a long time in low calcium fluids. In general, the membrane potential decreased with time. The decrease reached 10% after 30 minutes, even in normal saline. The rate of decrease with time was much greater in the low calcium fluid than in the normal saline. When calcium-free solution was used, the membrane potential decreased to 51.4% of the initial response after 30 minutes. The rate of decrease in the membrane resistance was more obvious than in the membrane potential when the muscle was immersed in the low calcium fluid for a long time. After immersion in calcium-free fluid, the membrane resistance was reduced to 20% of the initial response.

Effects of reduction of calcium upon the action potential of crayfish muscle. Latency, chronaxie and conduction velocity of the action potential. The electrical properties of the crayfish muscle membrane are somewhat different from those of the other excitable tissues such as frog muscle or the squid axon. In contrast to its

higher capacitance ($40\mu F/cm^2$), a much lower resistance has been reported. ($100\Omega.cm^2$, which corresponds to 1/40 of that of the frog muscle). The action potential of the muscle is also different from that of other excitable membranes. In the crayfish muscle, a single pulse usually evokes only a local response, which never propagates to other areas. This type of action potential is called a "graded response" (Fatt and Katz, 1953). In Fig. 1, A, action potentials generated by a

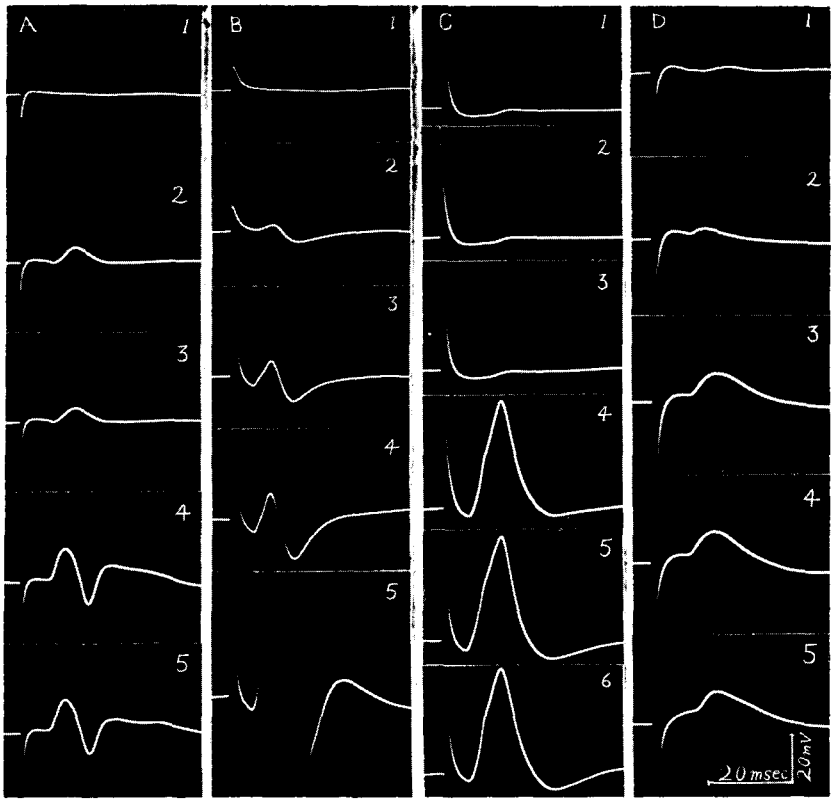

Fig. 1. Action potentials recorded extracellularly from crayfish isolated single muscle fibres soaked in normal or low calcium solutions. Stimulation: Single pulse duration, 0.2 msec. Intensity increased from top to bottom. Solution: A, Normal (van Harreveld's) solution, B, 1/2 calcium, C+D, 1/4 calcium. Time: A, B, C, 5 min; D, 30 min.

stimulus of 0.2 msec duration and various suprathreshold intensities are shown. The stimulating and recording electrodes consisted of four Ag-AgCl rods, with tip diameters of 0.5 mm. They were arranged parallel to each other at intervals of 1 mm. In response to a stimulation of the suprathreshold, the muscle membrane produced an action potential of a few mV. The amplitude of the action potential increased with the stimulus intensity. As long as the intensity was at a relatively

low level, the action potential was monophasic, which was considered to be a local response. When the stimulus intensity was increased slightly, the action potential changed from monophasic to biphasic, indicating that the conducting ability of the action potential increased with stronger stimulation. But even though the stimulus intensity was increased approximately 90V, no action potential was observed which propagated over the entire length of the fibre. Monitoring the mechanical response of the muscle fibre with the microscope, confirmed that there was only a local contraction near the stimulating electrodes. Next, muscle fibres soaked for 5 min in 1/2 calcium were used (Fig. 1, B). In most cases, the action potentials recorded were almost the same as those observed in normal saline, and microscopic observation also showed no significant changes. However, in a few cases, there was a completely propagated action potential with a higher amplitude ($>$ 40 mV) and a powerful contraction. The frequency of appearanace of such propagated action potentials increased with a decrease in the concentration of calcium in the bath solution (Fig. 1, C). The amplitude of the propagated action potentials was not dependent upon the suprathreshold stimulus intensity, and the general characteristics of the action potential appeared to be the same as those of the "all-or-none response" observed in the frog muscle. However, the transient increase in the membrane excitability resulting from immersion in low calcium fluid ceased within a relatively short period. A propagaged action potential was never observed in muscles soaked in 1/4 calcium fluid for 30 minutes (Fig. 1, D). With long immersion in the low calcium solution, the excitability of the muscle membrane decreased remarkably, and only local response was obtained. When the muscle fibre was immersed in calcium fluids of less than 1/4 normal concentration, the muscle fibre lost its excitability within a few minutes. In such low, or calcium free fluids, spontaneous discharges of the repetitive action potentials were often observed for periods of two to five minutes. These repetitive action potentials and contractions have also been seen even more frequently in frog muscle soaked in low calcium fluid (Miyamoto, 1962 and 1963).

In Fig. 2, the latent period between the onset of stimulation and the action potential was plotted against the stimulus intensity. This was measured in fibres soaked in normal and in various low calcium fluids. The curves obtained were hyperbolic as have also been obtained from other excitable tissues. The relationship between the stimulus intensity and the latent period of the action potential in muscle soaked in 1/2 calcium fluid was almost the same as that observed in normal saline. However, a slight increase in the threshold and chronaxie were measured in the muscle fibres soaked in 1/4 calcium fluid. These results indicate a decrease in the membrane excitability. A discontinuous curve was obtained in muscle fibre soaked for 5 min in 1/4 calcium fluid. The first part of the curve was plotted from propagated action potentials and the last part from local ones. The shape of the curve indicates the transition in the nature of the action potential, from local to propagative.

In Table 2, the conduction velocity and the chronaxie of the action potential, as determined from fibres soaked in low calcium fluids of various concentrations, are summarized.

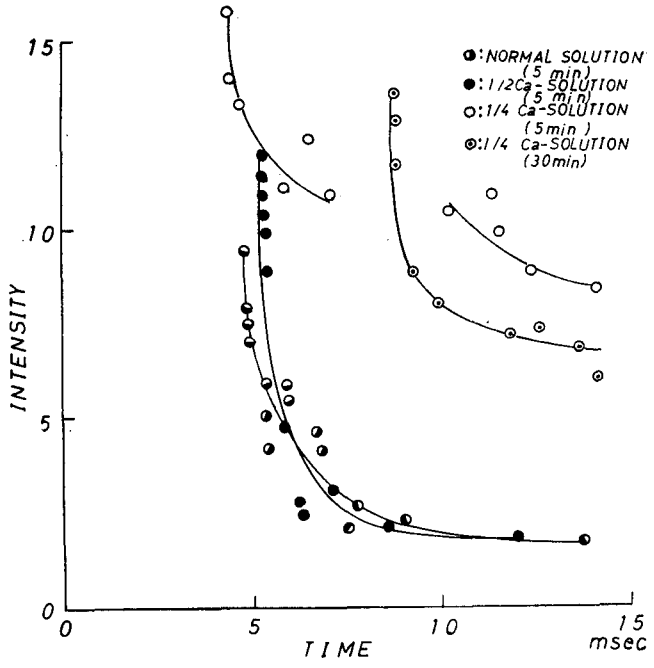

Fig. 2. Intensity-time relation of action potentials in normal and low calcium solutions. Stimulus intensity is shown as relative value.

Table 2.

Calcium concentration	Conduction velocity of action potential		Chronaxie	Number
		Mean	Mean	
Normal saline (5min)	16.4–19.2	18.2 cm/sec	5.8 msec	9
1/2 calcium (5min)	12.8–23.3	17.6	6.2	13
1/4 calcium (5min)	16.4–19.9	16.9	4.5*	11
1/4 calcium (30min)	None		8.6	9

Chronaxie and conduction velocity of action potentials in crayfish muscle fibres, measured in normal or low calcium solutions.

* In this case, the chronaxie was measured in the fibre which produced the propagated action potentials.

The conduction velocity of the action potential was nearly constant, but still slightly dependent upon the external calcium concentration. On the other hand, the chronaxie clearly increased with a reduction in the external calcium. This increase in the chronaxie indicated a decrease in membrane excitability. However, the chronaxie as calculated from the latency-intensity relation plotted from action potentials propagating along the entire length of the muscle fibre, was smaller than normal. This phenomenon indicated a transient increase in the membrane excitability of muscles in the lower calcium fluids. The minimum chronaxie was 4.5 msec, which is very close to that observed in frog muscle fibre (2.5–3.0 msec, Hisada and Miyamoto, 1961).

The action potentials evoked by prolonged current stimulation. In Fig. 3, the

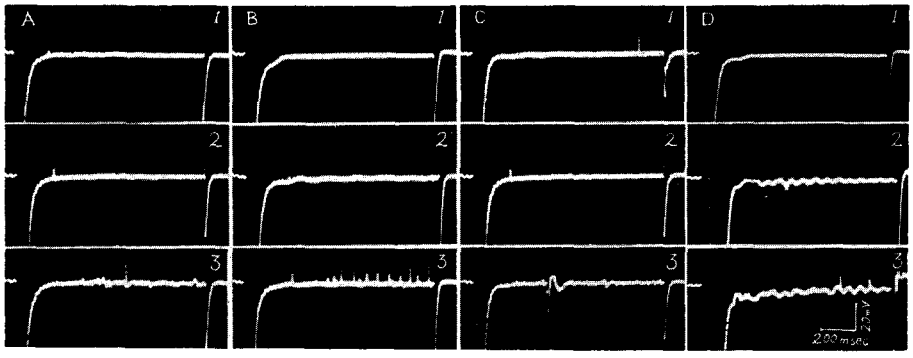

Fig. 3. Action potentials produced by prolonged current stimulation. Solution: A, Normal (van Harreveld's) solution; B, 1/2 calcium; and C+D, 1/4 calcium. Stimulus intensity increases from top to bottom. Time: A, B, C. 5 min; D, 30 min.

action potentials produced by muscle fibres in response to current stimulation of 1 sec duration and various intensities, are shown. In frog muscles, repetitive discharges of the action potential were usually observed (Miyamoto, 1962), but crayfish muscle immersed in normal saline responded to prolonged stimulation with only a single discharge of a local response (Fig. 3, A). With strong stimulation, the muscle fibre produced a subthreshold oscillation (often observed in frog muscle; Hisada and Miyamoto, 1961). In lower calcium solutions, the subthreshold oscillation was usually followed by a train of repetitive action potentials (Fig. 3, B). But, if the amount of calcium ions in the bath were decreased to less than 1/4 of normal concentration, the excitability of the muscle membrane was reduced and the repetitive action potentials again disappeared (Fig. 3, C, D). In Fig. 3, C, the propagated action potential previously mentioned is also shown. Microscopic observation showed that the muscle fibre was greatly shortened by prolonged current stimulation, and that its final length was approximately half of its initial length. Repeated application of the prolonged current to the muscle fibre produced an irreversible contrac-

tion and destruction of the muscle membrane.

Action potentials evoked by repeated stimulation.

In Fig. 4, the action potentials of muscle fibres in response to repeated stimuli of 0.2 msec duration and various frequencies, are shown. In this experiment, the stimulus intensity was kept constant. With repeated application, the amplitude of the action potentials gradually increased untill they reached a plateau. Superimposed recordings of action potentials had a monophasic shape indicating lower propagation capacity. With an increase in stimulus frequency, the amplitude of the action potentials reached to a plateau within a relatively short period, and the monophasic shape of the action potential became biphasic.

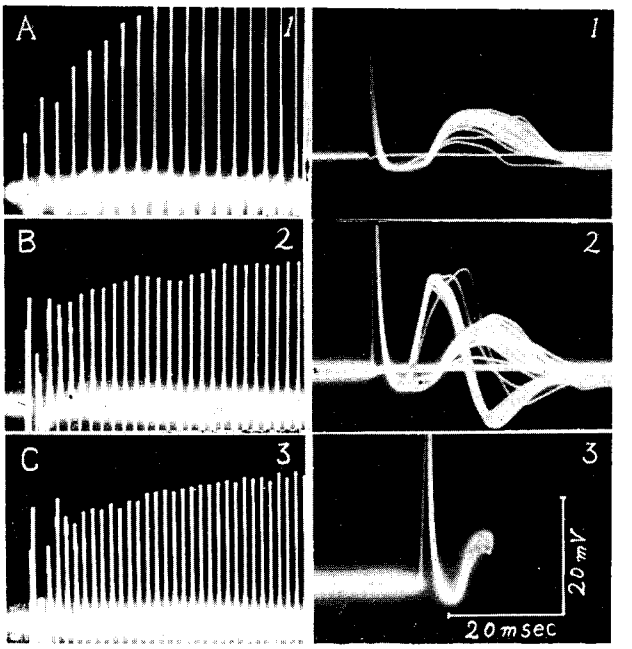

Fig. 4. Action potentials produced by repeated stimuli (normal saline). Stimulus frequency: A, 10c/s, B, 20 c/s and C, 30 c/s.
Superimposed records of the action potentials are given at right.

In Fig. 5, the results obtained from the same experiment, using lower calcium fluids are shown. When these lower calcium solutions were used, no action potential was generated by successive stimuli after the single, initial discharge produced by the first stimulus. In the other cases, there was a pause of constant duration between action potentials even though stimuli were repeated constantly during the period of the pasue. This pause, or refractory period, in the action potential, was not observed in the controls in normal saline. The

duration of the pause seemed to depend upon the concentration of external calcium; the lower the calcium, the longer the refractory period. In order to determine the nature of this refractory period, two successive stimuli, of 0.2 m sec duration, were applied to muscle soaked in lower calcium fluids, and the interval between these successive stimuli was varied (Fig. 6). When the interval between these stimuli was less than 50msec, the second action potential was inhibited by the refractory

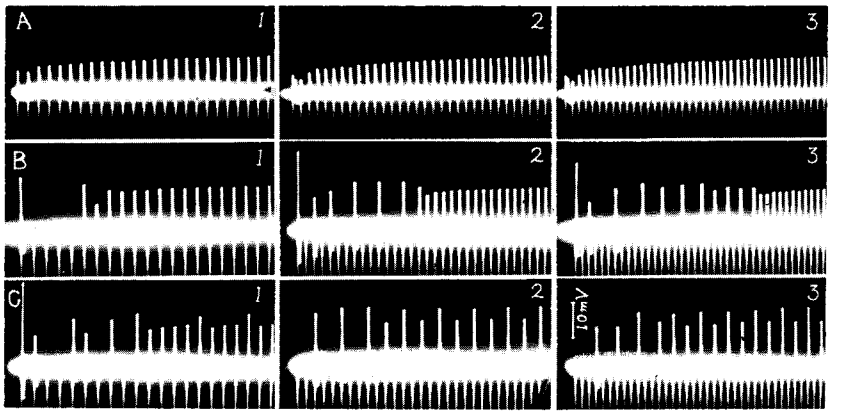

Fig. 5. Action potentials produced by repeated stimuli in low calcium fluids. Stimulus frequency: 1, 10 c/s; 2, 20 c/s; and 3, 30 c/s.
Solution: A, Control normal saline; B + C, 1/4 calcium. Time: A+B, 5 min; C, 30 min.

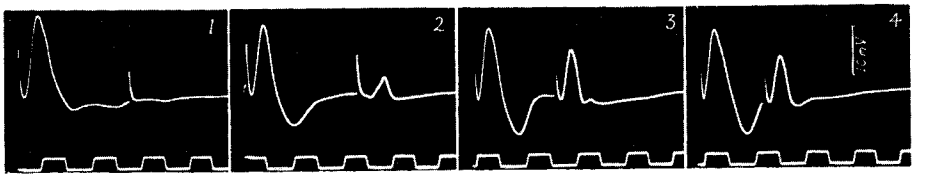

Fig. 6. Refractory period of action potentials in low calcium solution (1/4 normal). Explanation given in text. Time signal is 50 c/s.

period of the first one. Increasing the stimulus intensity, while keeping the interval constant, brought about the second response. If the intensity of the second pulse was strong enough, the refractory period decreased to 20 mseconds, so, the pause in the action potential observed in the lower calcium fluids should be referred to as relative refractory period. It has been reported that the increase in the refractory period which results from a deficiency in calcium ions bound to the cell membrane, is the most important factor in the mechanism of anaesthesia (Yamaguchi and Okumura, 1963).

Discussion

It has previously been reported that (1) the threshold response of muscle fibre to electric stimulation, decreases in low calcium fluids; (2) repeated discharge of action potentials are evoked by prolonged current stimulation, and the frequency of the repeated action potentials depends upon the concentration of external calcium, and (3) spontaneous discharges of action potentials are often observed in the muscles exposed to lower calcium solutions, without any electrical or mechanical stimulation (Hisada and Miyamoto, 1961; Miyamoto, 1962 and 1963). Calcium ions have also been reported to inhibit potassium depolarization (Tamasige, 1952 and Miyamoto, 1963). The role of calcium ions in the mechanism of initiation of the action potential has been investigated by experiment using the frog muscle fibre. The results indicate that release of calcium ions from their bonds to the membrane is the first process in membrane excitation (Miyamoto, 1963).

The results obtained from the present investigation of crayfish muscle fibres support this conclusion as to the role of calcium. When muscle fibre was soaked in lower calcium fluids, the following observations were made: (1) threshold and chronaxie of the action potential increase (2) the repetitive action potentials appear more frequently in response to prolonged current stimulation and (3) an action potential of the all-or-none type, usually rarely observed in crayfish muscle, may be easily obtained. One explanation of these observations may be that the excitability of the muscle membrane is controled by the amount of external calcium, which determine the rate of release of calcium ions bound to the cell membrane. The more calcium ions release, the more unstable the membrane becomes, therefore, slight stimulation may evoke a vigorous response in muscle membranes soaked in low calcium fluids.

The ability of the action potential to propagate has been explained by the theory of membrane resistance. Tamasige (1953) reported that higher membrane resistance is absolutely necessary for propagation of the action potential. That propagative action potentials were observed in the low calcium solutions used in the present investigations, appeares inconsistant with this theory, since membrane resistance decreases with the reduction of calcium, but when the immersion in lower calcium fluids was of short duration (less than 5 min), the membrane potential retained its initial level, and the decrease in the membrane resistance was negrigible. If membrane resistance was significantly decreased by long immersion periods, no propagated action potential was obtained, and excitability of the membrane was less than normal. Therefore, the propagated action potential observed must have been generated by dissociation of calcium from the cell membrane while the membrane retained normal potential and resistance levels.

Werman et al., have recently reported that surplus of external calcium changes the graded response of lobster or insect muscle into an all-or-none response. Moreover, Murayama and Yamashita (1962), reported that some alkali-earth ions such as Ba^{++} or Sr^{++}, penetrate the muscle cell and generate all-or-none responses

in crayfish muscle. The electrical charactelistics of the propagated responses observed in the present investigation one is somewhat different from those reported by Murayama and Yamashita or by Werman et al., since the duration of the action potential they reported is significantly longer than those obtained in the present experiment. Fatt and Katz (1953) have reported propagated action potentials in crab muscles which are the same as those obtained in the present study.

Yamaguchi and Okumura (1963) reported that the refractory period of the action potential is significantly prolonged by anaesthesia, and from that observation they assumed that anaesthesia may remove the calcium ions bound to the cell membrane. The present findings as to the relation between the extracellular calcium concentration and generation of the action potential will adequately support their assumption.

The successive experiments with frog muscles and the present experiments with crayfish muscles, clearly indicate that the excitability of the muscle membrane depends directly upon the concentration of external calcium. The present experiments prove that the graded response of the cell membrane of the crustacean muscle can be converted to a all-or-none response by using low calcium fluids. Previous experiments have also shown that the all-or-none, propagated response of the frog muscle membrane may be changed to a graded, local response, under suitable conditions. These resiprocal transitions indicate that the essential mechanism in the excitation of the cell membrane will be the same, and that the difference in the nature of the action potential is produced by a combination of the electrical properties of the cell membrane and the external condition surrounding the muscle fibre.

Summary

1. Membrane potential and resistance were measured in an isolated single muscle fibre from the crayfish, *Procambarus clarkii*, which was immersed in normal saline or low calcium fluids.

2. The lower the external calcium, the lower the membrane resistance or potential. The rate of decrease in the membrane potential or resistance was compared with the immersion time.

3. Non-propagated action potential in response to a single stimulus was recorded extracellularly from muscle fibres soaked in normal saline, and the propagated action potential of an all-or-none nature, from muscle soaked in low calcium fluids.

4. The conduction velocity of the action potential was inaltered by variation in the amount of external calcium, but the chronaxie was slightly dependent. The mean values calculated for muscles in normal saline were 18.2 cm/sec and 5.8 msec, respectively.

5. When muscle fibre was immersed in low calcium fluid for a short period, it produced a train of repetitive action potentials following a subthreshold oscilla-

tion, in response to prolonged current stimulation.

6. The refractory period of the action potential increased significantly in the low calcium fluid when the muscle fibre was repeatedly stimulated.

7. The role of calcium in the elicitation of the propagated action potential was discussed and it was concluded that calcium ions controlled the excitability of the muscle membrane.

The author wishes to express his sincere thanks to Professor Mituo Tamasige for his kind guidance through the course of this investigation and for revision of the manuscript.

References

Bülbring, E., M. Holman and H. Lüllmann 1956. Effects of calcium deficiency on striated muscle of the frog. J. Physiol. **133**: 101–117.

Curtis, B.A. 1963. Some effects of Ca-free choline-Ringer solution on frog skeletal muscle. J. Physiol. **166**: 75–86.

Fatt, P. and B. Katz 1953. The electrical properties of crustacean muscle fibre, J. Physiol. **120**: 171–204.

Hisada, M. and Y. Miyamoto 1961. Effects of reduction of calcium on electrical activities of muscle membrane. Jour. Fac. Sci. Hokkaido Univ. Zool. **14**: 536–543.

Ishiko, N. and M. Sato 1957. The effect of calcium ions on electrical properties of striated muscle fibres. Jap. J. Physiol. **7**: 51–63.

Jenerick, H.P. and R.W. Gerard 1953. Membrane potential and threshold of single muscle fibres. J. cell. comp. Physiol. **42**: 79–102.

Miyamoto, Y. 1962. Repetitive electrical and mechanical responses of an isolated single muscle fibre to prolonged electrical currents. Jour. Fac. Sci. Hokkado Univ. Zool. **15**: 111–122.

———— 1963. Effects of changes in extracellular calcium concentration on the electrical and mechanical responses of an isolated single muscle fibre. Jour. Fac. Sci. Hokkaido Univ. Zool. **15**: 235–247.

Murayama, K. and Y. Yamashita 1962. The effects of Barium, Strontium and TEA ions on the production of action potentials in the Cheliped muscle of the crayfish. Jour. Fac. Sci. Hokkaido Univ. Zool. **15**: 123–136.

Tamasige, M. 1951. Effects of potassium ions upon the electrical resistance of an isolated frog muscle fibre. Annot. Zool. Japon. **25**: 15–20.

———— 1952. Effects of potassium and calcium ions upon the contractile mechanism of single muscle fibre. Annot. Zool. Japon. **11**: 101–115.

———— 1953. Relation between mechanical response and membrane resistance of single muscle fibre. Jap. J. Zool. **11**: 105–115.

Werman, R., F.V. McCann and H. Grundfest 1961. Graded and all-or-none electrogenesis in arthopod muscle. 1. The effects of alkali-earth cations on the neuromuscular system of *Romalea microptera*. J. Gen. Physiol. **44**: 970–996.

Werman, R. and H. Grundfest 1961. Graded and all-or-none electrogenesis in arthopod muscle. II. The effects of alkali-earth and onium ions on lobster muscle. J. Gen Physiol. **44**: 997–1027.

Yamaguchi, T and H. Okumura 1963. Effects of anaesthetics on the electrical properties of the cell membrane of the frog muscle fibre. Annot Zool. Japon. **36**: 109–117.