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Effects of Changes in Extracellular Calcium Concentration on the Electrical and Mechanical Responses of an Isolated Single Muscle Fibre^{*,1),2)}

By

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(With 7 Text-figures)

The exact nature of the process which links the electrical and mechanical responses in muscle activity is still unknown. When a muscle is stimulated electrically, a transient change in the membrane potential is observed to precede the muscular contraction (Kuffler, 1946 and Miyamoto, 1962). A phasic contraction and a remarkable decrease in the membrane resistance of the muscle are also induced when the muscle is soaked in a potassium solution (Tamasige, 1951), but potassium ions injected into the muscle intracellularly cause neither contraction nor membrane change (Kamada and Kinoshita, 1943). On the contrary, recent investigation of the effects of alkaloid caffeine upon muscle has shown that (1) the contracture induced by caffeine is not mediated by changes in resting potential or in ionic permeability of the muscle membrane; (2) the caffeine-induced contracture can be produced while the muscle is depolarized by potassium; and (3) the contracture is not affected by altering the concentration of extracellular calcium (Axelsson and Thesleff, 1958). These findings lead one to suspect that there is no correlation between the change in the membrane permeability and activation of the contractile mechanism in the case of responses induced by caffeine.

On the other hand, Bianchi (1961) has reported that caffeine causes a decrease in the amount of calcium bounded to the cell membrane of the muscle. This report suggests that if the muscle is kept in a calcium-poor solution, caffeine may generate a change in the membrane potential of the muscle, because the release of calcium ions from their binding sites on the cell membrane has been

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considered as the first process in the elicitation of electrical response (Miyamoto, 1962).

In the present investigation, simultaneous recordings were made of the electrical and mechanical responses of an isolated single muscle fibre in order to determine the effects of caffeine and potassium upon the muscle. The use of a microelectrode technique makes it possible to observe the exact nature of the transient changes in membrane potential and in membrane permeability during the application of the test solutions. Especial attention was given to the role of calcium ions in the mechanism of excitation and contraction coupling of the muscle in the present investigation.

Material and Methods

Single muscle fibres entirely free from injury were isolated from the iliofibralis muscle of the frog, *Rana japonica*. The apparatus for simultaneous recording of electrical and mechanical responses was the same as that described in a previous paper (Miyamoto, 1962). This method is in principle identical with that first introduced by Tamasige (1953). Signals of the responses of the muscle fibre were amplified and displayed on a double beam oscilloscope, one for the electrical responses and the other for the mechanical (see Fig. 1). Isotonic 128 m.mol potassium chloride and Ringer's fluids containing various concentrations of calcium were prepared as test solutions. The calcium-Ringer's fluid was made by mixing isotonic calcium chloride with isotonic calcium-free Ringer's fluid at various volume ratios. Caffeine was dissolved in these test solutions or in the normal Ringer's fluid at concentration of 5 m.mol/l to 25 m.mol/l. In order to measure transient changes in the membrane potential and resistance during the excitation of the muscle in response to caffeine or potassium, a single intracellular microelectrode of a low resistance (10 Mohms) was used both for recording the potential and for applying currents. The potential drop due to the high input impedance of the electrode was balanced by a Wheatstone bridge. This method is the same as that used in the previous investigation (Hisada and Miyamoto, 1961). Hyperpolarizing rectangular pulses (100 msec duration, 10 c.p.s. period) were applied to the muscle through the electrode and the anelectrotonic potential induced by this current was estimated as an effective membrane resistance since cytoplasm resistance is negligibly small compared with membrane resistance in frog muscles. Caffeine dissolved in isotonic potassium phosphate was injected into the muscle cell with a glass micropipette (tip diameter, 0.5 μ) under a microscope. The Ringer's fluid used had the following ionic composition, expressed in m.mol/l: Na, 126.4; K, 2.0; Ca, 1.3; Cl, 127.6; H_2PO_4 , 2.0; H_2CO_3 , 1.4. All solutions were buffered with isotonic $NaHCO_3$ solution to pH=7.2. The experiments were carried out at room temperature, 25°-28°C.

Results

Responses induced by isotonic potassium chloride. It is known that reversible contracture and membrane depolarization of the muscle are elicited by soaking the muscle in a solution containing potassium ions of suprathreshold concentration (Tamasige, 1951). In the present investigation, isotonic contraction and electrical response of an isolated muscle fibre induced by the isotonic potassium solution were

recorded simultaneously. When the muscle fibre was completely fresh, the muscle contracture induced by potassium ceased within a short period. When the muscle was kept in Ringer's fluid for a longer period of time, the duration of the contracture increased and there was an increase in both the rates of contraction and

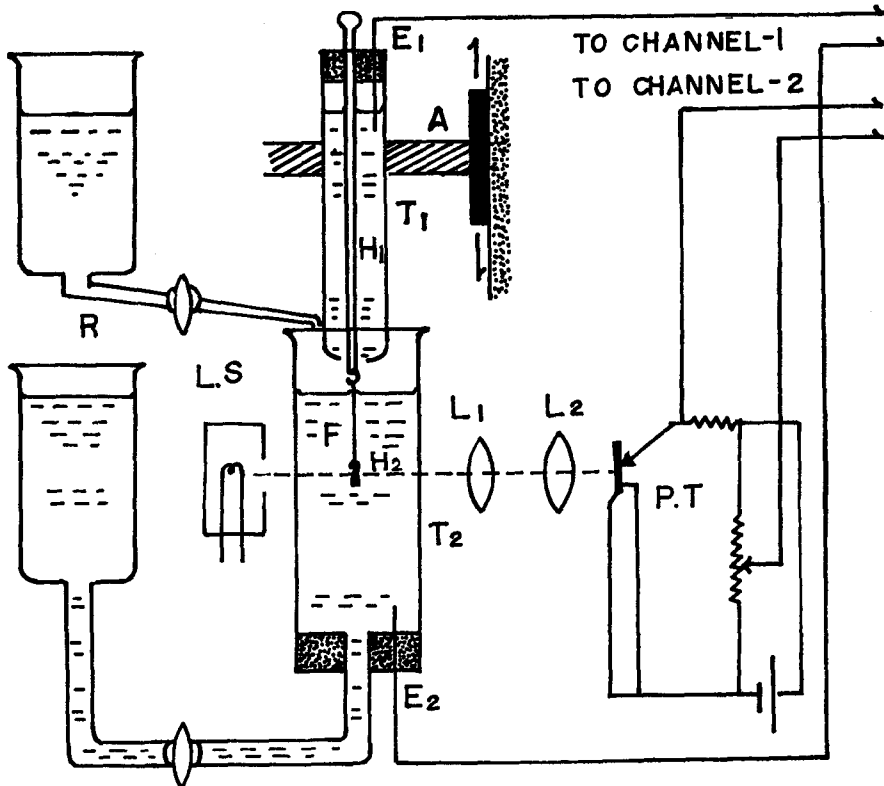


Fig. 1 Diagram of the experimental arrangement. A: Adjustable stand; E₁, E₂: Non-polarized electrodes; F: Single muscle fibre; H₁: Glass hook to suspend the muscle fibre; H₂: Small stained glass hook; L₁, L₂: Convex lenses; L.S.: Light source; P.T.: Photo-transistor; R: Reservoir for exchange of the bath solution or for application of the test solution; T₁, T₂: Upper and lower glass vessels. The upper part of the fibre is drawn up for a length of about 4-5 mm into the Ringer's fluid in which an Ag-AgCl electrode is set up. The other end is suspended in the Ringer's fluid in the lower vessel in which a second electrode is set up. Another explanation is contained in the text.

relaxation. Contracture of the muscle induced by extracellular potassium ions was completely inhibited 120 min after preparation. A train of repetitive action potentials and twitches activated by the action potentials appeared in the initial period of the electrical response of the fresh fibre exposed to the potassium solution. The reaction time required for the elicitation of the repetitive responses was

prolonged with time after the preparation, so in stale muscle these repetitive responses were preceded by the onset of contraction without electrical change. The repetitive responses were inhibited in the muscle fibre kept in Ringer's fluid for 40 min (see Fig. 2, A and B). A possible explanation of the phenomenon might be an irreversible enhancement in ionic permeability of the muscle membrane as has been previously considered (Tamasige, 1953).

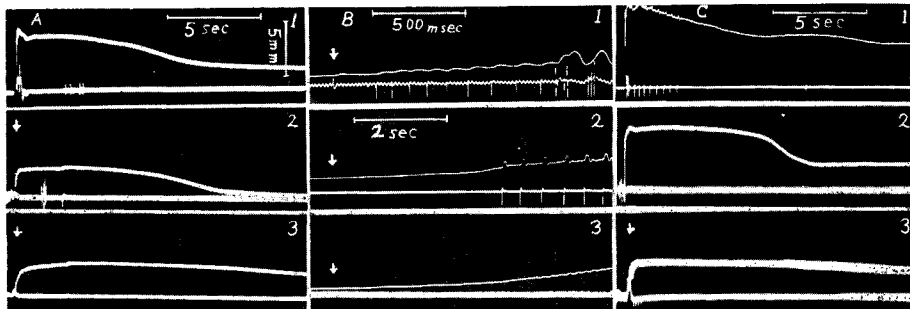


Fig. 2. Mechanical and electrical responses of an isolated single muscle fibre induced by isotonic (128 m.mol/l) potassium. Upper trace: Mechanical response. Lower trace: Electrical response. Ser. A. Typical change with time following preparation in the potassium-induced responses. (1) Record obtained in a fresh fibre, 5 min after preparation. (2, 3) Records in stale fibres, 20 and 40 min after preparation. Ser. B. Records showing the initial periods of potassium-induced responses of the muscle fibres, in which the time scales are prolonged. Time following the preparation of each fibre in the series is the same as that in series A. Arrows indicate the time at which the test solutions were applied. A train of action potentials and twitches appearing in the initial periods of the response appeared late in the stale fibres. Ser. C. Changes in potassium-induced responses of the fibres produced by previously soaking the muscle in calcium Ringer's fluids of various concentration for 5 min. (1) Record obtained from the fibre previously exposed to 0.1 m.mol calcium Ringer's fluid, (2) 1.3 m.mol, (3) 13 m.mol. Note the similarity between the responses in series A, B and C.

In the next stage, changes in the potassium-induced responses of the isolated muscle produced by short (5 min) preliminary exposure to Ringer's fluid containing calcium at various ratios were examined. The repetitive twitches and action potentials usually appearing in the initial period of the response were inhibited by an increase in the calcium concentration of the Ringer's fluid. The potassium-induced contracture of the muscle previously exposed to a high calcium fluid was of relatively long duration. The prolongation in the duration of the contracture resulted primarily from substantial increase in the duration of the contraction and relaxation phases. The muscle fibre previously exposed to a calcium-poor solution for a short period (less than 5 min) responded to the potassium solution with repetitive twitches of higher frequency. However, with a longer period of soaking

in the calcium-poor solution the contracture induced by the potassium solution was suppressed (see Fig. 2, C). It has already been reported that the excitability of muscle membrane decreases with increasing time or with an increase in the level of extracellular calcium when the muscle is stimulated by electrical current (Miyamoto, 1962). These facts indicate that release of the calcium ions bonded to the muscle membrane is the first process related to the elicitation of the membrane excitation of the muscle. The opposite effect of extracellular calcium from that of potassium upon the cell membrane has been reported by Tamasige (1950).

Contracture induced by caffeine. As a preliminary test, the sensitivity of an isolated muscle fibre to caffeine contained in the Ringer's fluid was examined. A graded contracture of the muscle could be induced by caffeine at a concentration of 5 m.mol/l within 10 sec, but no contracture was evoked by caffeine at concentrations of 4 m.mol or less after 5 min exposures. No change in the sensitivity of the muscle to caffeine with increased time following preparation was observed in the present experiment.

With an increase caffeine in concentration in the bath solution, the maximum rate of contraction became higher (see Fig. 3). Neither repetitive action potentials, which appeared in the initial period of the potassium induced-contraction, nor the repetitive twitches resulting from the electrical responses could be observed in muscle fibres exposed to caffeine. The contracture lasted as long as the drug was in the bath solution, but it was reversible when the caffeine was removed. An irreversible contracture was obtained from the muscle fibre soaked in Ringer's fluid containing caffeine of concentrations above 7.5 m.mol/l, in which the entire length of the muscle fibre was shortened to about 60% of the resting length.

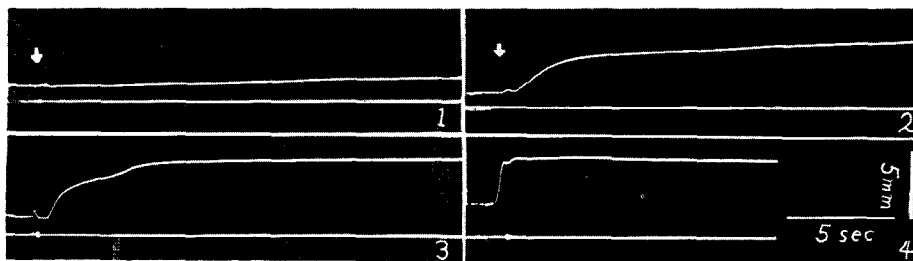


Fig. 3 Typical changes in contracture of the muscle fibres induced by caffeine of various concentrations dissolved in normal Ringer's fluid. Arrows indicate the time when the test solutions were applied. (1) Record obtained from fibre exposed to 5 m.mol caffeine Ringer's fluid, (2) 7.5 m.mol, (3) 12.5 m.mol, (4) 25 m.mol.

Effect of calcium on the contracture induced by caffeine. Next, effects of changes in the concentration of external calcium on the contracture induced by caffeine were studied. No remarkable change in the sensitivity of the muscle to caffeine nor any change in the maximum rate of the contraction were produced by

altering the concentration of calcium in the Ringer's fluid surrounding the muscle fibre. However, a slight decrease in the maximum rate of contraction was obtained from muscle soaked in isotonic calcium solution containing caffeine at supra-threshold concentration (see Fig. 4, A and Fig. 5, A).

A very interesting fact was obtained from the caffeine-induced response of the muscle with a decrease in the external calcium in the bath solution. The muscle membrane became very sensitive to caffeine in the calcium-poor solution, in which a train of repetitive action potentials and twitches were observed (see Fig. 6). In contrast to the initial twitches which appeared in the potassium-induced contracture, the repetitive responses in this case required a longer reaction time (5 to 10 sec), thus the repetitive responses were usually preceded by the caffeine-contracture without any membrane change. The discharges of the propagated

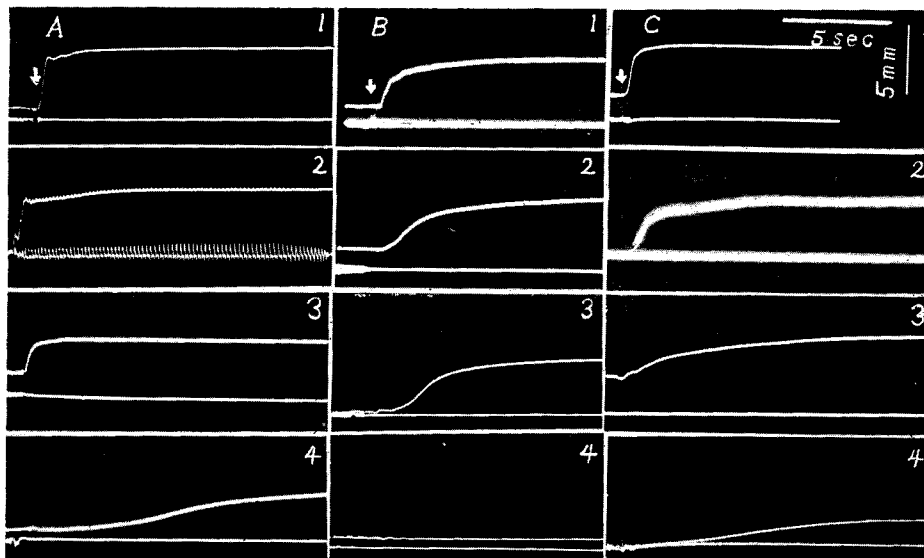


Fig. 4 Effects of calcium and potassium on the contracture induced by caffeine. Ser. A. Changes in the caffeine-induced contractures of the muscle fibres produced by soaking the fibres in various concentration of calcium Ringer's fluid. (1) Records obtained from the fibre exposed to normal 1.3 m.mol calcium Ringer's, (2) 0.1 m.mol, (3) 13 m.mol, (4) isotonic 83 m.mol calcium solution. 25 m.mol of caffeine was added to each solution. Note the train of repetitive action potentials of the fibre appearing in response to the 25 m.mol caffeine-Ringer's fluid containing 0.1 m.mol calcium. Ser. B. (1) Inhibition of caffeine-induced contracture of the muscle fibre produced by preliminary exposure to the isotonic 128 m.mol potassium solution for 5 min, (2) 20 min, (3) 40 min, (4) 24 hr. 12.5 m.mol of caffeine was added to each solution. Ser. C. Recovery of sensitivity of the potassium-depolarized fibre to caffeine obtained by returning the muscle fibre to normal 1.3 m.mol Ringer's fluid. Concentration of caffeine is the same as that in series B.

responses lasted for periods of 8 to 10 sec and they gradually diminished. Analysis of the result as to the frequency of the occurrence of the propagated responses indicated that some constant ratios may exist between concentrations of calcium and caffeine in the bath solutions in the elicitation of propagated responses. Repetitive responses were evoked in 80% of the fibres exposed to the Ringer's fluid containing 17.5 m.mol caffeine and 0.3 m.mol calcium. The high percentage of occurrence of these responses was reduced to 50% by doubling the external calcium level of the bath solution, in which the level of caffeine was not altered. The repetitive responses were completely inhibited when the fibre was exposed to the Ringer's fluid containing 17.5 m.mol of caffeine and 1.3 m.mol of calcium.

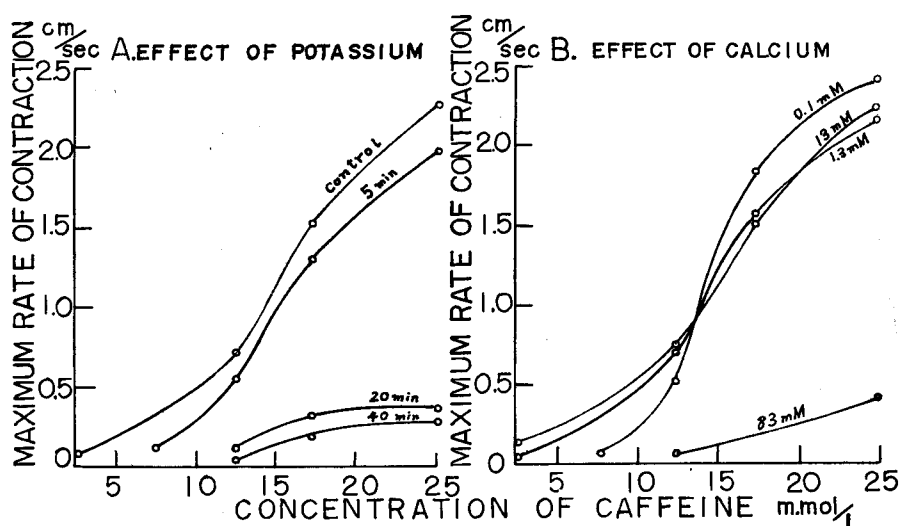


Fig. 5 Effect of external calcium and potassium upon the contracture induced by caffeine. A. Changes in the maximum rate of contraction of the isolated muscle produced by exposing the muscle to Ringer's fluids containing various amounts of calcium. Numerals along the curves indicate concentrations of calcium in each solution. Note that the external calcium ions produce no remarkable effects on the contracture induced by caffeine except for sudden decline in the response of the fibre kept in isotonic 83 m.mol calcium solution. B. Inhibition of the maximum rate of rise of the caffeine induced contracture of the muscle fibre produced by keeping the muscle in isotonic 128 m.mol potassium solution. Numerals along the curves indicate the periods (min) of potassium-treatment.

Effects of potassium on the contracture induced by caffeine. Axelsson and Thesleff (1958) have reported that caffeine contracture can be produced in a muscle completely depolarized by potassium. The results obtained in the present investigation have also shown that caffeine still effected muscles depolarized by isotonic potassium solution. But the effect of the caffeine on the muscle fibre was reduced

with an increase in the period during which the muscle was kept in the potassium solution (see Fig. 4, B and Fig. 5, B). Thus, an increase in the threshold to caffeine (two-fold increases in the threshold were obtained from muscles kept in the potassium solution for 180 min), a decrease in the maximum rate of contraction and a prolongation of the reaction time were produced by exposing the muscle fibres to the potassium solution for a long period. A remarkable decrease in the sensitivity of the muscle to caffeine was observed in fibres treated by potassium for 24 hr. A shortening of only 20% of the initial length was induced by caffeine of 50 m.mol. The muscle sensibility to caffeine was somewhat recovered by returning the muscle to the normal Ringer's fluid, but this treatment was ineffective for fibres completely depolarized by potassium (see Fig. 4, C). It has already been reported that the excitability of the muscle to an electrical stimulus is markedly reduced by application of extracellular potassium (Tamasige, 1953). The results obtained in the present study suggest that the ineffectiveness of caffeine upon the potassium-treated muscle is due to an irreversible increase in the membrane permeability of the muscle.

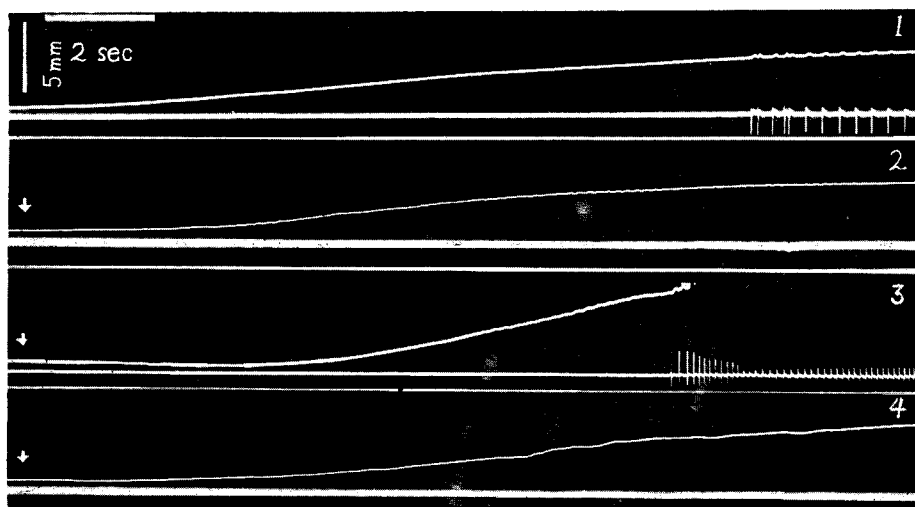


Fig. 6 Repetitive electrical and mechanical responses of the muscle induced by caffeine in the calcium-poor Ringer's fluid. Upper trace: Mechanical response, lower trace: electrical response. Records were obtained from fibres which had been soaked in Ringer's fluids containing (1) 12.5 m.mol caffeine and 0.3 m.mol calcium, (2) 12.5 m.mol caffeine, 0.6 m.mol calcium, (3) 25 m.mol caffeine, 0.6 m.mol calcium, (4) 25 m.mol caffeine, 1.3 m.mol calcium. Arrows indicate the time when caffeine was applied. Note the inhibition of the repetitive responses produced by increasing the level of external calcium contained in the bath solution.

Effect of caffeine injected into the muscle cell. Caffeine dissolved in isotonic potassium phosphate was injected into the muscle cell through a glass micropipette

with a tip diameter of about $1\ \mu$. Kamada and Kinoshita (1943) have reported that potassium phosphate ions applied intracellularly do not produce contraction in the frog muscle. In the present experiment, no contraction of the muscle was produced by injection of caffeine concentrations of 5 m.mol to 50 m.mol in the muscle cell. However, a local contraction was obtained from a part of the muscle where the drug was applied extracellularly through the same microcapillary.

Intracellular recording of transient changes in membrane potential and resistance during the application of caffeine and potassium. The effects of caffeine on the muscle were mediated by the release of calcium ions from the muscle membrane or by prolongation of the period of preliminary immersion in potassium, and caffeine injected into the muscle cell caused no contraction. These facts indicate that caffeine acts upon the cell membrane. Changes with time of the membrane potential during the application of caffeine were followed with the

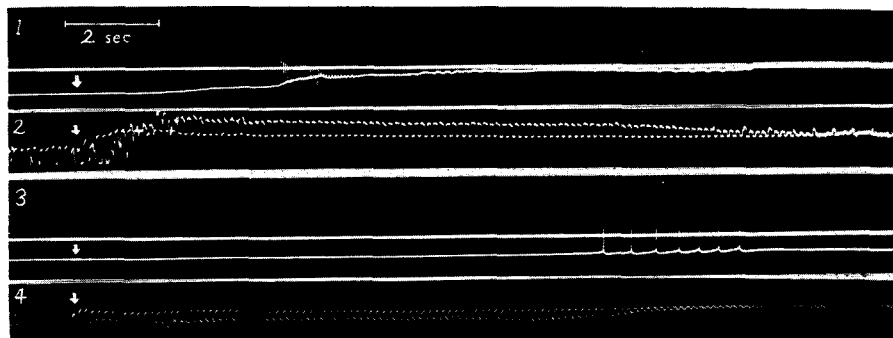


Fig. 7 Changes in the membrane potentials and resistances of isolated muscle fibres during extracellular application of potassium and caffeine. Changes in the membrane potential and membrane resistance of muscle fibres produced by soaking the fibres in the isotonic 128m.mol potassium solution are shown in (1) and (2) respectively. Changes in the membrane potential and resistance with exposures to the calcium-free Ringer's fluid containing 25 m.mol caffeine in (3) and (4) respectively. Arrows indicate the time at which the muscle fibres were soaked in the test solutions. The small deflections at start of each response are artifacts caused by exchanging the bath solution. Further explanations are contained in the text.

microelectrode technique. Recording of the changes in the membrane permeability of the muscle produced by caffeine was also attempted by measuring the an-electrotonic potentials of the muscle, which were induced by applied repeated electrical currents at the frequency of 10 c.p.s. As a control, changes in the membrane potential and resistance of the fibre produced by soaking the muscle in the potassium solution were also determined by the same method. The membrane potential of the muscle soaked in calcium-free Ringer's fluid containing 25 m.mol caffeine decreased to 80% of the initial level within 10 sec after the application

of caffeine, by which a succession of action potentials was initiated. The frequency of the repeated action potentials was usually lower than that of the action potentials induced by potassium. Effective membrane resistance obtained from the anelectrotonic potential was reduced to 40% of the initial value by exposing the muscle to caffeine. From the muscle fibre soaked in the potassium solution, faster changes in time courses of membrane potentials and resistance were obtained compared to the lower changes in time course of caffeine-induced responses. Membrane potential of a muscle soaked in the potassium solution decreased to 15% of the initial level within 10 sec, and a decrease of 84% in the effective membrane resistance was measured in the fibres soaked in the potassium solution for 17 sec. The results obtained with these records clearly show that caffeine can produce a substantial increase in membrane permeability and membrane depolarization, which is sufficient to generate the action potential, when the muscle was placed in a bath solution containing a rudimentary level of calcium.

Discussion

Results obtained from previous investigations allow the following to be said about the processes linking membrane excitation to contraction of a muscle stimulated electrically. It is well known that depolarization of a muscle membrane is caused by stimulation of the muscle with an electrical current which flows outwardly across the cell membrane. A release of calcium ions from the binding site of the cell membrane can be considered as the first step in the elicitation of membrane depolarization (Hisada and Miyamoto, 1961). When membrane depolarization increases to a critical level, an action potential of the all-or-none type is induced by the depolarization. The action potential propagates along the entire length of the muscle, and activates the contractile element of the muscle (Miyamoto, 1962). An increase in the level of free calcium ions in the muscle cell which is produced by membrane depolarization makes it possible to reduce the level of free phosphate ions in the muscle cell, and this process initiates an electro-chemical reaction which activates the contractile element of the muscle (Kamada and Kinoshita, 1943 and Umezawa, 1958).

The fundamental mechanism of elicitation of the response induced by potassium is essentially the same as that described in the above. The muscle membrane is depolarized by the suprathreshold potassium contained in the bath solution surrounding the muscle, and a reversible contracture of the muscle is induced by the changes in the membrane permeability. With increase in concentration of calcium contained in the preliminary treating solution, the following changes have been obtained from the mechanical and electrical responses induced by external potassium. The duration of the relaxation phase of the contraction is prolonged, while the frequency of the initial repetitive action potentials and twitches decreases. These changes in the membrane excitability have also been produced in frog muscle stimulated by electrical current in various concentrations of the

external calcium (Miyamoto, 1962). The membrane resistance and membrane potential of muscles, measured with the microelectrode technique, show a remarkable decrease with the potassium treatment. A decrease of 86% in the effective membrane resistance of the potassium-induced muscle corresponds well with the decrease of 90% in the membrane resistance reported by Tamasige (1951). Tamasige (1952) has also reported that the effect of external potassium on the muscle membrane can be inhibited by an increase in the external calcium. These facts indicate that the excitation of muscle membrane requires a condition in which calcium ions are easily released from the binding sites on the cell membrane. Increases in the outflux and influx of calcium ions have been observed in frog muscle exposed to electrical stimulus or to external potassium in the experiments using radio-calcium (Shanes and Bianchi, 1960).

The contraction induced by caffeine dissolved in Ringer's fluid containing calcium of normal concentration (1.3 m.mol) has never been accompanied by changes in the membrane potential. But caffeine has induced vigorous repetitive mechanical and electrical responses when the muscle is soaked in calcium-poor fluid. Occurrence of repetitive responses, along with the slow decrease in the membrane potential and resistance of the muscle during the application of caffeine, offers direct proof that an enhancement in the membrane permeability can be generated by external caffeine when the external calcium is suppressed. An increase in the permeability attributed to a long immersion of the muscle in potassium solution causes a slight decrease in the sensitivity of the muscle to caffeine. As above stated, a decrease in the membrane sensitivity due to the potassium depolarization has already appeared in muscles stimulated by electrical currents. In contrast to the irreversible change in the muscle membranes, it is thought that the contractile element of the muscle retains its function even if the function of the membrane is completely diminished with the potassium-treatment. Muscle soaked in potassium solution for 24 hr has still responded to caffeine by shortening as much as 2 to 3 mm. Electrical current also affected the potassium-treated muscle, and a local contraction of the muscle could be obtained with it (Tamasige, 1956). These facts suggest that the contractile element of the muscle can be activated without membrane depolarization, and that the mechanism of membrane excitation and muscle contraction are essentially independent. Analysis of the muscle responses induced by agents of these three types, electric current, external potassium and external caffeine leads to the conclusion that the processes involved in the excitation-contraction coupling in these three responses are the same except for differences in their time courses, and that release of the bonded calcium ions from the muscle membrane must be the first step of the process.

Summary

1. Mechanical and electrical responses induced by external potassium and caffeine in an isolated single muscle fibre of the frog, *Rana japonica* were recorded simultaneously.

2. With time, a frequency of the repetitive twitches and action potentials appearing in the initial period of the potassium-induced responses decreases. A decrease in the amount of the contraction and an increase in the duration of the relaxation phase were also observed in the muscle fibre kept in the bath solution for a long period.

3. The sensitivity of the muscle membrane to potassium could be inhibited by previously soaking the muscle in a bath solution containing a high concentration of calcium. A decrease in the level of calcium contained in the preliminary treatment fluid generated vigorous repetitive twitches in the initial period of the potassium-induced response.

4. Caffeine induced a graded contraction without any membrane potential change in muscle kept in Ringer's fluid containing the normal ratio of calcium. The contracture induced by caffeine was not modified by an increase in the level of external calcium, but muscle soaked in calcium poor-fluid responded to caffeine of above 12.5 m.mol with a succession of repeated mechanical and electrical responses.

5. Caffeine still affected the muscle depolarized with isotonic potassium solution, but the sensitivity of the muscle to caffeine decreased with time following the initiation of the potassium-treatment.

6. Caffeine injected into a muscle cell through a micropipette caused no contraction. This fact eliminates the possibility that caffeine may directly activate the contractile element in the muscle cell.

7. Membrane potential and resistance recorded intracellularly with the microelectrode technique showed a remarkable decrease with application of caffeine when the muscle was kept in calcium-poor fluid. This fact clearly indicates that caffeine can generate a depolarization in the muscle membrane if suitable conditions exist in the bath solution surrounding the muscle.

8. The possible role of calcium ions in the mechanism of the excitation-contraction coupling was discussed and it was concluded that a release of calcium ions from their binding sites on the muscle membrane must be the first step in the mechanism of membrane excitation.

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