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EFFECTS OF CHANGES IN EXTRACELLULAR CALCIUM CONCENTRATION ON THE POTASSIUM-INDUCED CONTRACTURE OF FROG'S SKELETAL MUSCLE

BY G. B. FRANK

From the Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg, Canada

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The exact nature of the process or processes which link the electrical and mechanical events in muscle activity remain unknown. Evidence indicating that calcium ions are in some manner involved in this process has been summarized by Sandow (1952). As a working hypothesis, he suggested that an action potential or depolarization of the muscle fibre surface permits or promotes the entrance of calcium ions into the fibre and that these ions then initiate the mechanical events (Sandow, 1952). The demonstration by Hodgkin & Keynes (1957) that an influx of calcium ions accompanies each action potential in the squid giant axon encouraged investigation of this hypothesis. Recently, a similar influx of calcium into frog's skeletal muscle during activity has been demonstrated by Bianchi & Shanes (1959).

In a preliminary note (Frank, 1958), it has been reported that soaking the extensor longus digiti IV muscle of the frog for a few minutes in a calcium-free solution will prevent the development of a potassium-induced contracture in this muscle. Treatment of the muscle with the calcium-free solution did not reduce the depolarization produced by an increased potassium concentration, normally effective in inducing the contracture. Evidence is now presented that the speed with which the potassium-induced contracture is eliminated is determined by the rate at which calcium ions leave the extracellular spaces of the muscle.

METHODS

Preparation. The extensor longus digiti IV muscle of the frog, *Rana pipiens*, was used in most experiments. This muscle was removed with pieces of adjacent muscles attached and dissected free under a dissecting microscope. The loosely fitting sheath surrounding the muscle was also removed. The tendons at each end of the muscle are attached to the sheaths of other muscles. These attachments and adjacent connective tissues were left intact, to permit tying with minimum damage to the muscle. After isolation this muscle has the shape of a cylinder approximately 20 mm long with either a circular cross-section (diameter,

0·26–0·42 mm) or an elliptical cross-section (major axis, 0·27–0·70 mm and minor axis 0·19–0·49 mm). In a few experiments the extensor longus digiti IV muscle of the bullfrog, *Rana catesbeiana*, was used. Unless otherwise noted all results refer to experiments performed on muscles from *Rana pipiens*.

Solutions. The primary solution had the following composition (mM): choline chloride 111·8; KCl 2·47; $CaCl_2$ 1·08; $NaHCO_3$ 2·38; NaH_2PO_4 0·087; glucose 11·1. Except for the absence of the $CaCl_2$, the composition of the calcium-free solution was identical. Solutions with intermediate calcium concentrations were prepared by mixing appropriate amounts of the above two solutions. Solutions with elevated calcium or potassium concentrations were prepared by the addition of solid $CaCl_2$ or KCl to the primary solution. Exceptions were the isotonic potassium chloride solutions, which contained 123 mM-KCl with or without 1·08 mM-$CaCl_2$. In some experiments the solutions contained NaCl in the place of choline chloride. The cases in which sodium solutions were used will be specifically pointed out in the Results. The caffeine (Kahlbaum) was dissolved in a small quantity of solution with the aid of a few drops of concentrated HCl and diluted to the desired concentration. The resulting caffeine solution was buffered to pH 7·2 by the addition of solid $NaHCO_3$. In all solutions D-tubocurarine chloride (Burroughs Wellcome) was added to make a final concentration of 10^{-4} g/ml. The solutions were stored in polyethylene bottles in order to minimize contamination.

Water for the above solutions was prepared by passing distilled water through a Bantam Demineralizer (Barnstead Still and Sterilizer Co.). The conductivity of this purified water was less than that of a 0·0017 mM-NaCl solution. All glass-ware was rinsed thoroughly with this purified water and care was exercised during the experiments to prevent contamination with calcium from any source.

Mechanical recording. The muscle was mounted vertically in a cylindrical bath of 10 ml. capacity. Solutions were introduced into the bath at the top with a syringe and removed via an outlet at the bottom. The lower end of the muscle was fixed and the upper end was attached by means of a nylon thread to the free end of a lever approximately 3 in. (7·5 cm) above the bath. Two strain gauges (Baldwin–Lima–Hamilton Corpn, SR_4 Type A-7) were mounted on both sides of a brass plate near the fixed end of the lever arm. These formed two series arms of a Wheatstone bridge. The output of the latter was fed into a Grass Model 5P1, low-level DC pre-amplifier and recorded with a Grass Model 5 ink-writing oscillograph. A 0·5 g weight displaced the lever arm 0·25 mm. This was equal to a muscle shortening of about 1·25 % and, and since the muscles rarely develop so great a tension, the recordings were considered to be essentially isometric. The resting tension was about 0·5 g in all experiments.

Electrical recording. The muscle was mounted horizontally in a rectangular bath. A bipolar stimulating electrode, a ground electrode and a bipolar recording electrode, all constructed of platinum wire, were placed along the muscle. Before recording, the solution was removed from the bath by suction. Supramaximal rectangular stimulating pulses 1 msec in duration were used. The compound action potential was recorded with DC amplification, displayed on a cathode-ray oscilloscope and photographed. The amplitude of the initial negative phase of the diphasic action potential was taken as a measure of the number of the muscle fibres responding to the stimulus. In one experiment the muscle under one pole of the recording electrode was crushed and a monophasic action potential was recorded.

Procedure. In all but a few of the experiments tests consisted of determining the effects of altering the ionic environment of the muscle on the potassium-induced contracture. After isolation the muscle was kept in the primary solution (see above) for at least 30 min before testing. The contracture (Fig. 1) was produced by replacing this with a solution containing 25 mM potassium. Near the end of the mechanical response the test solution was replaced by the primary solution in which the muscle was kept for at least 10–15 min before another test was performed. After an initial equilibrium period of about 1 hr reasonably

reproducible contractures were obtained. However, in a few muscles the response gradually increased for several hours.

To determine the effects of altering the ionic environment on the contracture, the muscle was soaked in the altered solution for a predetermined time (pre-test soaking period) and then tested with the altered solution containing 25 mM potassium. In order to eliminate, if possible, all sources of calcium contamination, when soaking the muscle in a low calcium or a calcium-free solution the bath was emptied approximately 10 sec after the initial exposure to the altered solution and fresh altered solution was placed in the bath. Another replacement of the altered solution was carried out approximately half way through the pre-test soaking period. Between each test with altered solutions, a control contracture of the muscle was obtained using the primary solution containing 25 mM potassium. The response immediately preceding a test in an altered solution was used as control. Whenever a control response reasonably similar to previous controls was not obtained the experiment was abandoned. However, as judged from the control contractures, this preparation almost always survived in good condition for 5–6 hr of testing.

THEORETICAL

When a cylinder containing an initial concentration of a substance, C_0, is placed in an infinitely large volume of solution having a zero concentration of the substance, the concentration, C', remaining in the cylinder at time t, will depend upon the diffusion coefficient, D, of the substance, the dimension of the cylinder and conditions at the surface of the cylinder (Crank, 1956). Two limiting conditions occur. One when the surface concentration is kept at 0 and the other when the cylinder and the surrounding solution is undisturbed. The actual conditions of the present experiments lay somewhere between these two extremes, because it was necessary to disturb the system in order to reduce calcium contamination and to produce a contracture and vigorous stirring of the external solution would have made it impossible to record small responses.

The actual conditions of the experiments came closer to the undisturbed system. A solution for the diffusion equation for this condition and for large values of t is

$$\frac{C'}{C_0} = \frac{R^2}{4Dt}, \tag{1}$$

where R is the radius of the cylinder. This relation is plotted in Fig. 3. Also under either condition

$$\frac{C'}{C_0} = f(R^2/Dt). \tag{2}$$

Using this relation it can be shown that

$$\frac{\log t_2 - \log t_1}{\log (c_2/A_2) - \log (c_1/A_1)} = -2, \tag{3}$$

where the subscripts 1 and 2 refer to different muscles; t_1 and t_2 are the diffusion times at $C'_1/C_{01} = C'_2/C_{02}$. c is the circumference of the muscle;

and A is the cross-sectional area. Since the toe muscles used in this work often had elliptical cross-sections, the value c/A was used rather than the value R. These are related by $c/A = 2/R$.

Thus, provided the percentage inhibition of maximum contracture tension is related to the change in calcium concentration in various muscles, the time that toe muscles need to be kept in a calcium-free solution in order to produce a certain percentage inhibition would be linearly related to their c/A's when these values are plotted on log-log. scales (Fig. 5).

RESULTS

Effects of calcium-free solutions. In a previous communication (Frank, 1958) it was reported that soaking the toe muscle of the frog in a calcium-free solution consistently inhibited the potassium-induced contracture of the muscle. A typical experiment is shown in Fig. 1. The rectangular artifact at the start and end of each response was produced by emptying the bath. It took approximately 0·5 sec to fill the bath with the new solution and thus the filling was completed during the downstroke at the end of the rectangular artifact. Each test involving the use of calcium-free solutions was preceded and followed by a control response, the only exception being the final test response in a series. Although in the experiment shown in Fig. 1 complete elimination of the response was not followed by a control response, this was not the usual case. In all the muscles so tested a normal control response was obtained when the muscle was kept in the primary solution for a sufficient length of time following a test response. It should also be pointed out that between the responses in Fig. 1 *F* and *G* another experiment was carried out using this same muscle.

Although in all the experiments presented below the contractures were induced by a solution containing 25 mM potassium, contractures induced by an isotonic (123 mM) potassium chloride solution also were eliminated by first treating the muscle with a calcium-free solution. An experiment in which an isotonic potassium chloride solution was used is shown in Fig. 2. Eight experiments with isotonic potassium chloride have been performed and as far as could be determined the time course for the inhibition produced by calcium-free solutions did not differ from experiments in which the contracture was induced by 25 mM potassium.

It should be pointed out that in the experiment illustrated (Fig. 2) the maximum tension of the control responses averaged about 1 g. The cross-sectional area of this muscle was 0·041 mm² and thus the maximum tension of the contracture induced by isotonic potassium chloride averaged 2·44 kg/cm². This is quite close to the maximum tetanic tension of the frog's sartorius muscle, reported by Hajdu (1951) to be 2·5 kg/cm². Thus there can be little doubt that all the muscle fibres were maximally activated during the contracture and that the response of all fibres was eliminated by treating the muscle with a calcium-free solution.

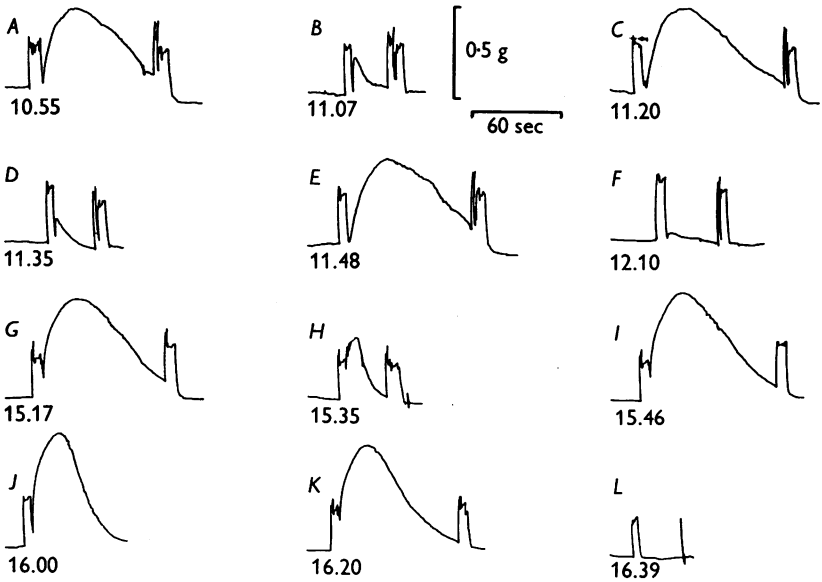

Fig. 1. Inhibition of contractures produced by 25 mM potassium in the frog's toe muscle by soaking the muscle in a calcium-free solution. *A*, *C*, *E*, *G*, *I*, and *K* control responses. Time (min) that muscle kept in a calcium-free solution before testing with a calcium-free solution containing 25 mM potassium *B*, 2; *D*, 5; *F*, 10; *H*, 1; *J*, 0; and *L*, 15. Rectangular artifacts at start and end of each response caused by emptying the bath. Bath filled during the downstroke at the end of the artifact. Time of day recorded below each test record. Muscle cross-section 0·10 mm².

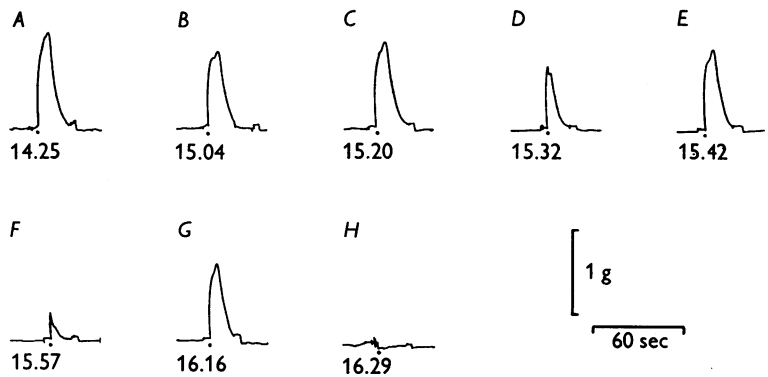

Fig. 2. Inhibition of contractures produced by isotonic potassium chloride in the frog's toe muscle by soaking the muscle in a calcium-free solution. *A*, *C*, *E*, and *G* control responses produced by a solution containing only 123 mM potassium chloride and 1·08 mM calcium chloride. Time (min) that muscle was kept in a calcium-free solution before testing with a 123 mM potassium chloride solution, *B*, 0; *D*, 1; *F*, 3; and *H*, 6. Dots immediately below each record indicate point at which potassium chloride solutions put in bath. Time of day recorded below each test record. Muscle cross-section 0·041 mm².

In all of five small toe muscles (cross-sectional areas less than 0·10 mm²) tested the maximum tension of the contracture induced by isotonic potassium chloride was at least as large as the maximum tetanic tension of the same muscle.

In order to eliminate the complicating effect on excitability of altering the calcium concentration, all experiments with potassium-induced contractures were performed with solutions which contained choline rather than sodium. However, the potassium-induced contracture also was eliminated by soaking the muscle in a calcium-free solution when the solutions contained sodium in place of choline. In the latter case the complete elimination of the response took about one third longer than when choline solutions were employed. When using sodium solutions it was essential that they should contain adequate concentrations of tubocurarine. This may account for the differences in the results reported here and those previously reported by Denton (1948). The small residual response in the absence of curare is dependent upon the cations present in the bathing solution. It is being investigated and results will be reported later.

In different frog toe muscles, a 50% inhibition of the contracture produced by 25 mM potassium required from 0·5 to 7·8 min of pre-test soaking in the calcium-free solution. The marked differences in the time course of the inhibition in various muscles could be eliminated if, as is shown in Fig. 3, the percentage inhibition is plotted against the time (t) divided by the pre-test soaking period needed for 50% inhibition of the response ($t_{\frac{1}{2}}$). Also included in Fig. 3 is the theoretical curve for the diffusion of a substance from an infinitely long cylinder into an unstirred surrounding medium of infinite dimensions having initially an 0 concentration of the substance (see Theoretical). This curve was fitted to the data on the assumption that the contracture was 50% inhibited when 94% of the calcium, which would have left the muscle at the time the response was 100% inhibited, had diffused out of the muscle (Fig. 8). Despite the differences in the conditions on which the theoretical curve is based and in the experimental conditions, the experimental points fit the theoretical curve reasonably well. This result would seem to suggest that the time course of the inhibition is determined by the rate at which calcium ions diffuse out of the muscle.

Further support for the idea that the time course of the inhibition is determined by a simple diffusion process is provided by the similarity of the time courses of the inhibition and of the recovery from inhibition. This similarity is shown in Fig. 4. In the experiment illustrated, it was first found that the maximum tension was 96% inhibited if the muscle was kept for 1 min in the calcium-free solution before testing. Recovery was then determined in subsequent tests by first soaking the muscle for 1 min in the calcium-free solution and then placing it in the primary

solution for various times before inducing a contracture with potassium. The difference between the inhibition observed and 96% inhibition is plotted as percentage recovery.

As mentioned above, the speed with which the inhibition developed varied greatly in muscles from different frogs. If the time course for the inhibition is determined by the rate at which calcium ions leave the extracellular space when the muscle is placed in a calcium-free solution, the time course would be expected to be related to the dimensions of the muscle. Thus the larger the diffusion area (or the circumference of the

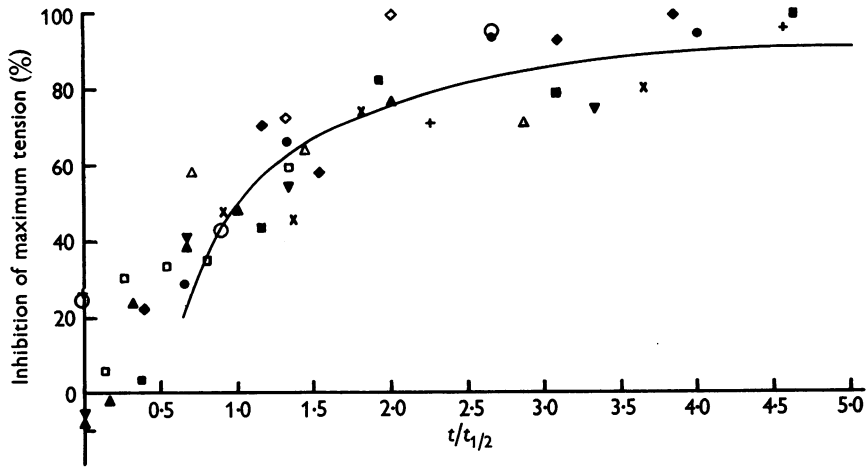

Fig. 3. Reduction in the maximum tension of the potassium-induced contracture of the frog's toe muscle produced by soaking the muscle in a calcium-free solution. Experiments with 11 muscles represented by different symbols. t, time muscle kept in the calcium-free solution before testing with 25 mM potassium; $t_{\frac{1}{2}}$, time for a 50% inhibition of maximum tension (range, 0·5–7·8 min). The line is the theoretical diffusion curve for the change in the concentration of a substance in a cylinder bathed in an infinitely large volume of a solution having a zero concentration of the substance. See text for further details.

muscle, c) the faster the calcium would leave the muscle and therefore the faster the inhibition would develop; whereas the larger the volume of the muscle (or the cross-sectional area, A) the slower the inhibition would develop. It can be shown (Theoretical) that when the time course of the inhibition is related to the dimensions of the muscle, the time for 50% inhibition ($t_{\frac{1}{2}}$) is linearly related to the circumference of the muscle divided by its cross-sectional area (c/A) with a slope of -2 when these values are plotted on a log-log. scale. Since it proved difficult to obtain *Rana pipiens* toe muscles having a low $c:A$ ratio, it was necessary to use the toe muscle of the bullfrog (Fig. 5, open circles) to cover an adequate range for analysis. The results with both species are shown in Fig. 5, where the solid line is

the expected line (slope = -2) and the interrupted line is the regression line of log $t_{\frac{1}{2}}$ on log c/A. The slope of the experimental line is significantly different from 0 ($P < 0{\cdot}01$) and is not significantly different from the theoretical line ($P > 0{\cdot}5$; student's t test).

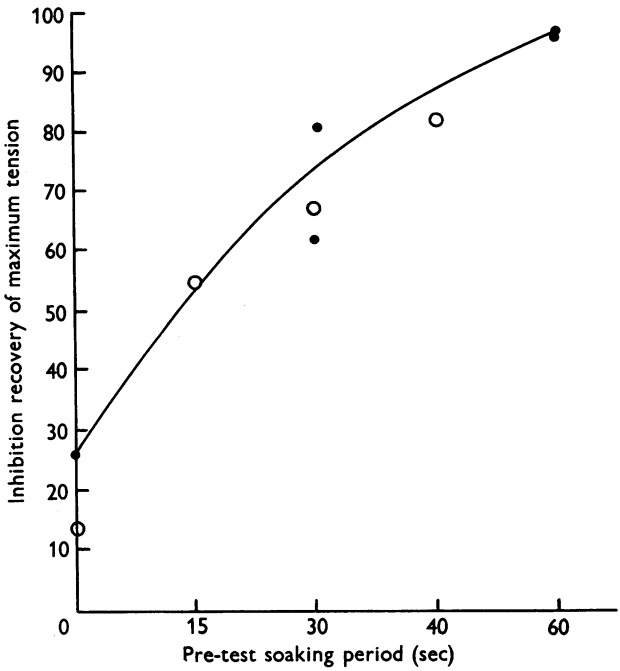

Fig. 4. Comparison of the development of the inhibition of potassium-induced contractures produced by placing a toe muscle in a calcium-free solution (●) with the recovery produced by placing the muscle in a solution having 1·08 mM calcium following 1 min in a calcium-free solution (○).

Onset of the inhibition. When the toe muscle is placed in the calcium-free solution, one would expect the fluid surrounding the fibres at the surface of the muscle to be rapidly depleted of calcium ions, while the calcium concentration of the extracellular fluid in the centre of the muscle would decrease more slowly. It was therefore of interest to determine how soon an inhibition of tension could be seen after the muscle was placed in the calcium-free solution, in order to get some estimate of the speed with which the inhibition develops in the individual muscle fibres. In four of the experiments the maximum tension was inhibited by 10–26 % when the contracture was produced by a high-potassium, calcium-free solution without previously soaking the muscle in the calcium-free solution. These inhibitions developed during the 10–15 sec necessary for the contracture to reach its maximum (Fig. 6*A*).

However, more commonly, an increase of the maximum tension of the contracture was observed when the muscle was tested after a short exposure (less than 1 min) to the calcium-free solution (Fig. 6*B*). This increase was always accompanied by a decrease in the duration of the

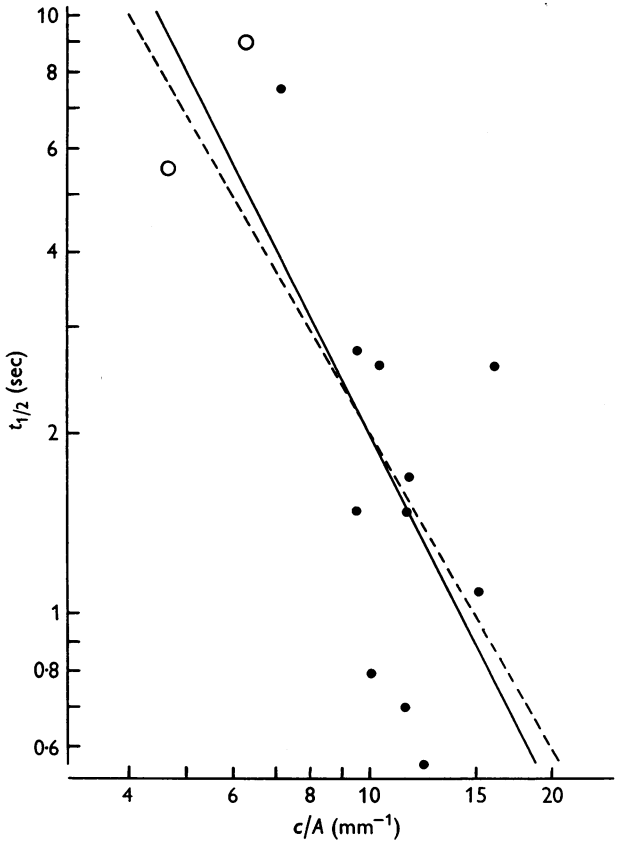

Fig. 5. Relation between the cross-sectional dimensions of toe muscles and the time ($t_{\frac{1}{2}}$) that they had to be kept in a calcium-free solution in order to produce 50% inhibition of the maximum tension of the potassium-induced contracture. Muscles from *Rana pipiens* (●); muscles from *Rana catesbeiana* (○); solid line, theoretically expected on basis of diffusion relations (see Theoretical); interrupted line, regression line of log $t_{\frac{1}{2}}$ on log c/A; circumference/cross-sectional area, (c/A); log-log. scale.

rising and falling phases of the contracture. With longer pre-test soaking periods in the calcium-free solution the contracture tension was always either reduced or completely suppressed.

Effects of changes in the external calcium concentration on the potassium-induced contracture. The observations just described clearly show that as the calcium ion concentration in the fluid surrounding the muscle fibres is

reduced the fibres pass through a phase in which their mechanical response is potentiated. In order to appreciate the changes which occur when the toe muscle is soaked in a calcium-free solution, it is necessary to know the changes in tension developed by the muscle fibres when the calcium ion concentration in the fluid surrounding the individual fibres is altered.

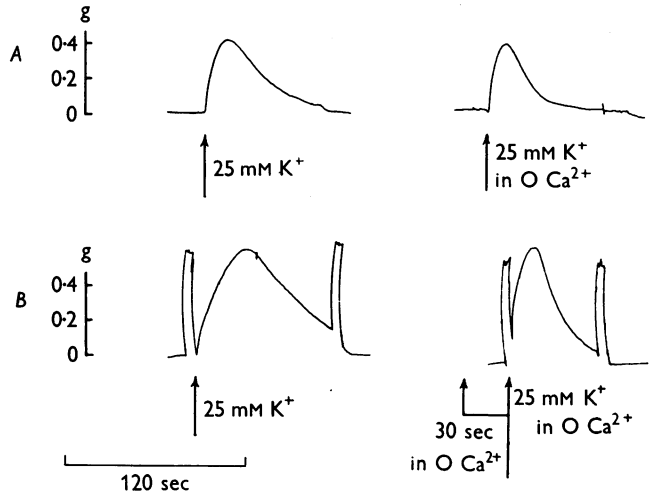

Fig. 6. Typical changes in the potassium-induced contracture of toe muscles produced by short exposures to calcium-free solutions. Control responses on the left. *A* and *B* different muscles. Square-wave-like traces at the start and end of the contractures in *B* are artifacts produced by changing the solution in the bath.

In the experiment presented in Fig. 7, it was found that keeping the muscle for 1·5 min in a solution containing only 0·054 mM calcium inhibited the contracture by about 55 %. It took the same length of time to eliminate the mechanical response completely when this muscle was treated with the calcium-free solution. Soaking the muscle for more than three times this length of time in the 0·054 mM calcium solution did not produce a significant further reduction in maximum tension. Thus it would seem that an equilibrium was achieved between the Ca^{2+} ion concentrations of the bathing solution and some location in the muscle resulting in a change in the tension of the contracture.

The above observation made it possible to study the mechanical response of the muscle after equilibration with a solution of altered calcium concentration had been achieved. The time that the toe muscle had to be kept in the calcium-free solution in order to obtain 100 % inhibition was first determined. Next, the muscle was placed in a solution having an altered calcium concentration for the same length of time and then the potassium-induced contracture was tested. The results from experiments

of this type were variable (Fig. 8). However, as is shown by the line drawn through the means, a consistent pattern appeared. As the calcium concentration of the bathing solution was reduced there was a phase in which the maximum tension of the contracture was increased. From 0·108 to 0 mM calcium, the maximum tension decreased in a regular fashion and even at 0·01 mM calcium a mechanical response was still present. The durations of all contractures induced with the muscle bathing in a solution

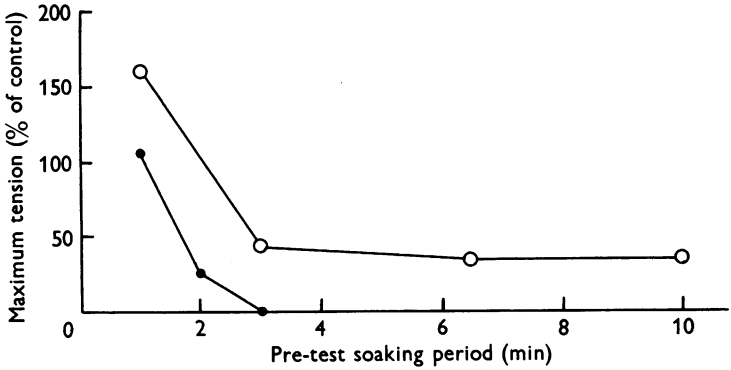

Fig. 7. Changes in the maximum tension of the potassium-induced contracture of a toe muscle produced by exposing the muscle to either a calcium-free solution (●), or to a solution containing 0·054 mM calcium (○).

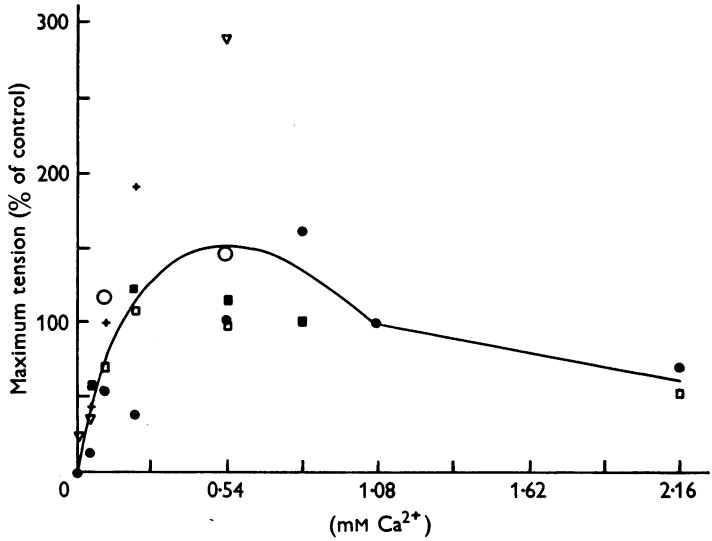

Fig. 8. The maximum tension of potassium-induced contractures after equilibrium with various calcium concentration in the bathing solution. Six toe muscles represented by different symbols. Line drawn through the means.

having less than the usual calcium concentration (1·08 mM-Ca^{2+}), were reduced.

When the calcium concentration was doubled (2·16 mM-Ca^{2+}) there was a small reduction in the maximum tension of the contracture. However, the most striking change produced by this treatment was a large increase in the duration of the response. Contractures lasting more than three times as long as the controls were obtained in this manner.

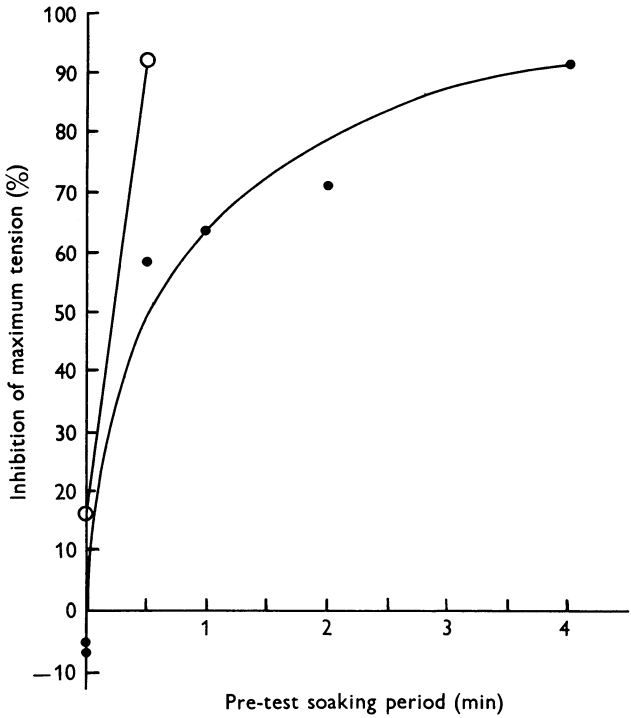

Fig. 9. Effect of ethylenediamine tetraacetic acid (EDTA) on the inhibition of the potassium-induced contracture produced by treating a toe muscle with a calcium-free solution. (●), calcium-free solution; (○), calcium-free solution containing 1·08 mM-EDTA.

Effect of EDTA on the inhibition. The disodium salt of ethylenediamine tetraacetic acid (EDTA) is a chelating agent which rapidly and firmly binds calcium ions (Chenoweth, 1956) and there is evidence that its distribution is limited to the extracellular space (Leckie & Tompsett, 1958). It was found that the addition of a small amount of EDTA to the calcium-free solution greatly accelerated the rate with which the inhibition could be produced (Fig. 9). In the experiment illustrated, 92 % inhibition was obtained eight times faster when 1·08 mM-EDTA was added to the calcium-free solution.

Inhibition of the action potential. Recently it has been demonstrated that placing nerves (Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser, 1957) or skeletal muscles (Ishiko & Sato, 1957; Edwards, Ritchie & Wilkie, 1956) in a calcium-free solution quickly renders these tissues inexcitable. Although information concerning this phenomenon is meagre, there is little doubt that it is the result of a loss of calcium ions from a location at or very near the surface of the cells. For this reason it was of interest to compare the time course of the development of inexcitability (of the muscle) with the time course of inhibition of the contracture.

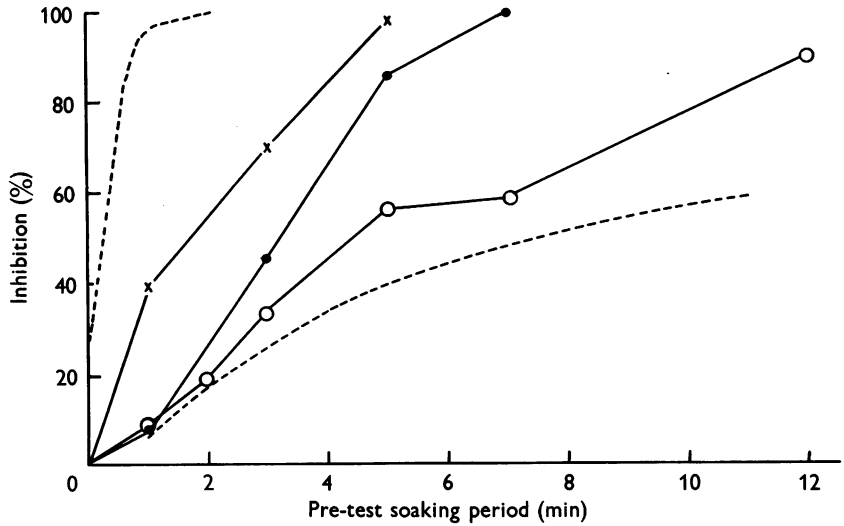

Fig. 10. Comparison of the rate of inhibition of the compound action potential of toe muscles (solid lines), with that for inhibition of the maximum tension of the potassium-induced contracture (interrupted lines) both produced by keeping the muscles in a calcium-free solution for various periods of time. The compound action potential was studied in three toe muscles represented by different symbols. The interrupted lines represent the extremes observed during the course of this investigation. Each symbol represents a separate test preceded and followed by a control test in the primary solution.

Such a comparison is given in Fig. 10. The procedure adopted for this experiment was the same as that described above, with the exceptions that sodium replaced choline in all the solutions, supramaximal electrical stimuli replaced the 25 mM potassium, and the electrical rather than the mechanical response was recorded. Thus control responses were obtained between successive tests in the calcium-free solution. In two of the three muscles the controls, following the tests which resulted in 100 % inhibition, were reduced in amplitude; all other control responses were unmodified. The time courses for the two phenomena (Fig. 10) are remarkably alike.

This would indicate that both time courses are determined by the rate at which calcium ions are lost from the same location in the muscle.

The caffeine contracture. Axelsson & Thesleff (1958) have shown that caffeine contractures can be produced in a skeletal muscle depolarized with potassium. This would indicate that caffeine activates the contractile mechanism of the muscle cell by acting on some process which normally occurs after depolarization or by initiating some process which normally does not occur during contraction. It also suggests the possibility that caffeine contractures can be produced in a calcium-free solution at a time

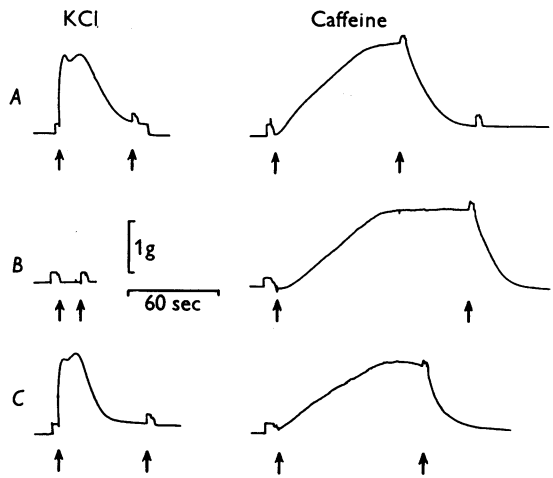

Fig. 11. Comparison of the effects of soaking a frog's toe muscle in a calcium-free solution on the potassium-induced and on the caffeine-induced contractures. *A*, control responses. *B*, tests after muscle kept in a calcium-free solution for 28 min, KCl; and for 31 min, caffeine. *C*, control responses with muscle in primary solution for 40 min after *B*. KCl = 123 mM potassium chloride. Caffeine solution containing caffeine $0{\cdot}5 \times 10^{-4}$ g/ml. Rectangular artifacts caused by emptying the bath. Test solutions in bath between arrows.

when the potasssium contracture is completely inhibited. The effects of soaking a toe muscle in a calcium-free solution on the two types of contractures are shown in Fig. 11. In Fig. 11 *B* it can be seen that an essentially unmodified caffeine contracture can be produced in a calcium-free solution, whereas the potassium-induced contracture is completely suppressed. This result, which was obtained in each of four experiments, shows that the response of the contractile mechanism to an appropriate stimulus can be unchanged although the potassium-induced contracture is completely eliminated by bathing the muscle in a calcium-free solution.

DISCUSSION

The idea that calcium ions play an essential part in muscular contraction is not new. In cardiac and skeletal muscles an action potential normally precedes and initiates the mechanical response. It is possible, and indeed probable, that calcium ions play an essential part in several of the steps leading to contraction. For simplicity we can divide this process into three stages: the action potential, the mechanical response, and the link between the action potential and the mechanical response. We are here concerned with the role of calcium ions as an essential part of the link between electrical and mechanical events in contraction.

In 1883 Ringer demonstrated that the frog heart fails to contract when calcium ions are absent from its perfusion fluid. Later it was shown that under this condition the rhythmic spontaneous action potentials of this tissue are still present in an only slightly modified form (Mines, 1913). The latter observation has been confirmed by using intracellular microelectrode recording techniques (Ware, Bennett & McIntyre, 1955).

One obvious explanation of these observations is that the action potential permits or promotes the movement of calcium ions from the surface to the interior of the cardiac fibres and that these ions then initiate the contraction. This explanation is strongly supported by the work of Niedergerke (1956*a*, *b*, 1957; Niedergerke & Lüttgau, 1957; Lüttgau & Niedergerke, 1958) who studied the effects of altering the ionic environment of cardiac muscle on its electrical activity and mechanical responses.

The striking effects of altering the external calcium concentration on the electrical activity of skeletal muscles have tended to obscure the role of calcium ions as a link between electrical and mechanical events in contraction. Thus, although the tension developed by a skeletal muscle in response to an action potential is markedly reduced when the muscle is bathed in a solution having 5 % of the usual calcium concentration, this reduction has been ascribed to a form of fatigue caused by the spontaneous repetitive action potentials which occur under the same conditions (Bülbring, Holman & Lüllmann, 1956). Similarly, the suppression of action potential formation in a calcium-free solution is sufficient to account for loss of the mechanical response to electrical stimulation which simultaneously occurs (Edwards *et al.* 1956). It is for these reasons that in the present study the potassium-induced contracture has been used as a model for the events which occur during a normal contraction. It was assumed that the events leading to the mechanical response of a skeletal muscle during an action potential or a depolarization type of contracture are the same. This concept was probably first suggested by Biedermann (1896) and a considerable body of evidence supporting it has

since accumulated (Gasser, 1930; Kuffler, 1946; Sandow, 1947, 1952; Sten-Knudsen, 1954).

In the present study an attempt has been made to break the link between electrical and mechanical events in contraction by soaking a skeletal muscle in a calcium-free solution. Under the specified conditions it has been shown that mechanical response soon disappears although the depolarization remains undiminished (Frank, 1958). From the results presented above, it has been concluded that the rate at which the mechanical response disappears is determined by the rate at which calcium ions leave the extracellular space when the muscle is placed in a calcium-free solution.

The latter conclusion is based on three lines of evidence. First, it was found that the rate at which the inhibition develops is determined by the dimensions of the muscle. Since the size of the corresponding muscle in different animals is determined by the number of cells in the muscle rather than the size of the cells, we should not expect to find a relation between muscle size and rate of development of inhibition if the rate was determined by the speed with which calcium ions left the intracellular space of the individual cells. In this respect it is interesting that average muscle fibre diameter often varies less in homologous muscles from animals of different species (Emerson & Emerson, 1959) than in different muscles from the same animal (Katz, 1948). Secondly, the acceleration in the rate of inhibition produced by the addition of a small amount of EDTA to the calcium-free solution shows that the speed of inhibition development is limited by the rate at which unbound calcium ions are removed from the extracellular spaces of the muscles. Finally, though our understanding of the cause of the inexcitability produced by placing a nerve or muscle in a calcium-free solution is incomplete (Edwards *et al.* 1956; Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser, 1957; Ishiko & Sato, 1957), our knowledge of the processes involved in the generation of action potentials in these tissues (Hodgkin, 1951; Shanes, 1958*a*, *b*) leaves us with little reason to expect that calcium ions must be removed from the centre of the cells in order to prevent the production of an action potential. Therefore, the similarity in the time courses for the two effects (Fig. 10) suggests that the inhibition of contracture tension is dependent upon the rate at which calcium ions are lost from the extracellular space.

Niedergerke (1957) has presented evidence indicating that the loss of calcium ions from a strip of cardiac muscle bathing in a calcium-free solution is too slow to be accounted for solely on the basis of free diffusion out of the extracellular space. The free solution diffusion coefficient for calcium is $7{\cdot}8 \times 10^{-6}$ cm^2/sec (Wang, 1953), and the apparent diffusion coefficient, D', out of the extracellular space would be about 3 or 4 times

slower. because of obstruction by the fibres, if the loss of calcium from the muscle were determined by diffusion alone. Using the data available in Figs. 5 and 8, one can estimate the apparent diffusion coefficient D' for the loss of calcium ions when the toe muscle is placed in a calcium-free solution. Since the maximum tension of the contracture is inhibited by 50 % when the extracellular calcium concentration is reduced to 6 % of its initial value (Fig. 8), if the concentration at the surface had been held constant at 0 D' would be calculated by

$$D' = \frac{0{\cdot}372R^2}{t_{\frac{1}{2}}}.$$

By using values from Fig. 5 in this equation, one gets a value for D' of $1{\cdot}24 \times 10^{-6}$ cm^2/sec. By making a similar calculation based on the assumption of an undisturbed system (eqn. 1, p. 520), one obtains a value for D' of $13{\cdot}9 \times 10^{-6}$ cm^2/sec. Since the actual experimental conditions lay somewhere between these two theoretical extremes, the true value for D' is between the two calculated values. Such calculations are compatible with the assumption that the inhibition is determined by the free diffusion of calcium ions out of the extracellular spaces of the toe muscles.

There are at least two likely sites in the muscle where deprivation of calcium might account for the observed inhibition. If, as suggested here, calcium ions serve as a link between electrical and mechanical events in contraction, it would be sufficient to remove calcium ions from the outer surface of the muscle fibres in order to inhibit the mechanical response. On the other hand, it is conceivable that inhibition results from the removal of calcium from an intracellular location resulting in an inability of the contractile proteins to respond to their normal stimulus. The dependence of the time course of the inhibition on the rate at which calcium ions are removed from the extracellular spaces of the muscle provides strong suggestive evidence for the former possibility. Furthermore, production of unchanged caffeine contractures in a muscle bathed in a calcium-free solution (Fig. 11) renders the latter explanation of the inhibition unlikely.

The results presented above were easily and consistently obtained as long as described procedures were followed scrupulously. In this regard the possibility of contamination of the calcium-free solutions with slight quantities of calcium is worthy of special mention. This problem has been discussed in some detail by Frankenhaeuser (1957), but a few additional words are warranted. From the results presented in Fig. 8 we should expect that as little as 0·01 mM calcium in the calcium-free solution would prevent a complete elimination of the contracture, and in the one case that a toe muscle was equilibrated with this calcium concentration the contracture tension was only 76 % inhibited. This degree of contamination would be obtained if as little as 0·1 ml. of the primary solution clung to the sides of the bath and to the muscle when the solutions were changed. Such contamination can also occur unless special precautions are observed in preparing the water and in selecting the reagents for making the solutions.

Since Kuffler & Vaughan Williams (1953) have listed the extensor longus digiti IV as a muscle containing slow fibres, it is of interest to examine the evidence showing that the inhibition of the contracture produced by placing the toe muscle in a calcium-free solution is not limited to these slow fibres. It has been shown that the contracture produced by using isotonic potassium chloride, which activates all muscle fibres, can be eliminated by prior treatment of the muscle with a calcium-free solution (Fig. 2), and the time course of this inhibition is identical to that reported here when using 25 mM potassium, despite the considerably greater tensions developed during the isotonic potassium-chloride-induced contractures. Further, the isometric recording techniques used here would tend to favour the responses produced by twitch fibres and since the duration of the contractures produced by 25 mM potassium chloride rarely exceeded 60 sec, this would identify the response as resulting mainly from twitch fibre activation (Kuffler & Vaughan Williams, 1953). Finally, direct anatomical observation has shown that only 6–20 % of the fibres in the frog's toe muscle can be considered slow fibres (Gray, 1958), and it would seem most unlikely that this small number of fibres could be responsible for the tensions recorded in this work.

The concept of the role of calcium ions in contraction as proposed here has been vigorously supported in the writings of Heilbrunn (1943) and Sandow (1952). Some time ago Heilbrunn & Wiercinski (1947) found that calcium was the only physiologically occurring cation which would cause a shortening when injected into skeletal muscle fibres in low concentrations. Recently Bianchi & Shanes (1959) demonstrated an enhanced influx of calcium ions into frog sartorius muscle fibres during a twitch or potassium-induced contracture. Further, when the twitch was increased by replacing chloride ions with nitrate ions in the bathing solution, the extra influx of calcium ions per twitch was also increased.

A major criticism of the concept of the influx of calcium ions acting as a link between electrical and mechanical events in contraction of skeletal muscle has been voiced by Hill (1949). On the basis of his work showing that the contractile mechanisms is fully activated throughout the cross-section of the cell during the latent period, he has calculated that there is not sufficient time to permit a substance released at the surface of the cell to diffuse to its centre. Although theoretical models have been proposed to overcome this objection (Sandow, 1952; Shanes, 1958*b*), it need only be pointed out that this objection does not necessarily invalidate the role proposed here for the influx of calcium ions during contraction. It may be necessary for calcium ions only to reach the inner surface of the muscle membrane to initiate some other process which eventually leads to a mechanical response.

SUMMARY

1. The potassium-induced contracture of the extensor longus digiti IV muscle of the frog is reduced and eventually eliminated when the muscle is kept in a calcium-free solution for various periods of time before testing.

2. That the time course for this inhibition is determined by the rate that calcium ions leave the extracellular spaces of the muscle is shown by

(*a*) a relation between the speed with which the inhibition develops and the size of the muscle,

(*b*) increased speed of inhibition development by the addition of a small quantity of EDTA to the calcium-free solution, and

(*c*) a similar time course for the inhibition of the compound action potential of the same muscle when it is placed in a calcium-free solution.

3. Caffeine contractures can be obtained with the muscle in a calcium-free solution at a time when potassium-induced contractures have been completely eliminated, indicating that the contractile mechanism is still able to respond provided it receives an adequate stimulus.

4. These results, along with previous results showing that under the same conditions the potassium-induced depolarization is not reduced, support the idea that during an action potential or depolarization of a skeletal muscle fibre there is an influx of calcium ions which act as a link between the electrical and mechanical events in contraction.

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