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ON THE ROLE OF CALCIUM IN THE EXCITATION-CONTRACTION PROCESS OF FROG SARTORIUS MUSCLE

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There are reasons to believe that calcium is functionally relevant in several different mechanisms involved in the excitation-contraction process in muscle. It is known that the resting potential of skeletal muscle fibres starts to fall immediately after removal of calcium from the extracellular medium and continues to decrease until the membrane is no longer electrically excitable (Ishiko & Sato, 1957). In view of calcium's significance for the membrane excitability in other tissues, e.g. heart muscle (Brooks, Hoffman, Suckling & Orias, 1955; Weidmann, 1955) and frog myelinated nerve fibres (Frankenhaeuser, 1957), calcium may also be expected to play a part in the mechanisms governing the production of the action potential in skeletal muscle fibres. Furthermore, there are experimental findings which suggest that the function of the contractile system inside the cell is critically dependent on the calcium concentration in the intracellular medium. As originally demonstrated by Heilbrunn & Wiercinski (1947) and later confirmed with refined technique by Niedergerke (1955) and Caldwell (1961), intracellular micro-application of calcium produces local contracture in the intact skeletal muscle fibre. Evidence has been obtained in many recent investigations favouring the hypothesis that release of cellular calcium is an essential step in the initiation of contraction of skeletal muscle (Frank, 1960, 1962; Bianchi & Shanes, 1961; Shanes, 1961; Bianchi, 1961*a*, *b*; Edman & Grieve, 1963; Curtis, 1963), cardiac muscle (Niedergerke, 1956; Winegrad, 1961; Winegrad & Shanes, 1962) and smooth muscle (Robertson, 1960; Edman & Schild, 1961, 1962; Durbin & Jenkinson, 1961).

The present investigation is an attempt to elucidate further the actions of calcium in the excitation-contraction process of skeletal muscle by correlating the effects of calcium lack on resting potential, action potential and mechanical output of the frog sartorius muscle in response to electrical

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stimulation. Measurements of mechanical as well as electrical changes have been made on individual surface fibres of the muscle, to provide a more detailed analysis of the effects. It has recently been reported (Frank, 1960; Curtis, 1963) that reduction of calcium in the bath may produce mechanical failure of the frog toe muscle in response to depolarization by potassium at a stage where there has been no substantial change of the resting potential of the muscle fibre membrane. As will be shown in the present work the diminution of mechanical response of the sartorius muscle to electrical stimulation in calcium-deficient Ringer's solution is closely paralleled by lowering of the resting potential of individual fibres. By the progressive depolarization the fibres are eventually brought into an inexcitable state, but before this stage is reached there is a gradual decline of the twitch output of the muscle fibres. The diminution of contractility of the muscle as a whole in response to electrical stimulation has been found to be a complicated phenomenon, which involves both inexcitability of individual fibres and reduction of the intensity of response of still excitable fibres. The mechanism of the gradual decrease of the twitch response of the individual fibre has been analysed further, in order to find out to what extent the mechanical failure is due to impairment of the electrical activity of the cell membrane. A preliminary report of this work has already been given (Edman & Grieve, 1961).

METHODS

Mounting of the muscle. The sartorius muscle of Summer *Rana temporaria* was used. When measurements of whole-muscle tension were required, the muscle was dissected together with the pelvic bone and held by means of a screw clamp on the pelvis. The tibial end was attached by a short ligature and a hook to the arm of a photo-electric transducer. The muscle was free in the solution and rested lightly upon one of the external stimulating electrodes (see below).

When single-fibre tensions were required the muscle was dissected free of the pelvis and the pelvic end secured by clamping two ligatures attached to the corners of the pelvic tendon. In this situation the muscle rested upon a slightly convex Perspex surface and the tibial end was attached by a ligature and hook to the RCA transducer extension arm (see below).

In both cases the ventral convex surface of the muscle was uppermost. Single-fibre tensions and membrane potentials were always measured from fibres in the ventral surface. This is mentioned because smaller fibre cross-sections are to be found on the ventral surface than on the more commonly used dorsal surface (Grieve, 1961).

Tension measurements. Whole-muscle tensions were measured by means of a photo-electric transducer in which the small movement of a lever arm mounted on a torsion strip is used to interrupt the light falling upon a Mullard OCP 71 phototransistor which was incorporated in a bridge circuit. Single-fibre tensions were recorded by means of an RCA 5734 transducer mounted vertically and fitted with a thin 2 cm long glass extension tube. The output was linear up to 180 mg. A single stage of amplification was used between the RCA bridge circuit and the Cossor oscilloscope.

Stimulation. The stimulator delivered square pulses of 1 msec duration. The whole muscle

was stimulated by passing current through two external wire electrodes passing transversely across the dorsal and ventral muscle surfaces in the pelvic half of the muscle. The mechanical output of single fibres in response to electrical stimulation via an inserted micro-electrode was recorded.

Membrane potential measurements. Micro-electrodes were used with resistances in the range 10–20 MΩ and tip potentials less than 5 mV. They were filled by boiling under reduced pressure in 3 M-KCl solution. The electronic arrangement used for recording consisted of a double-sided cathode follower, a d.c. amplifier and a cathode-ray oscilloscope. The amplifier output was also used to modulate an audio frequency generator. A good indication of successful micro-electrode insertion was given by a sharp change of frequency. With a micro-electrode of 10 MΩ the time constant of the recording device was about 80 μsec.

Muscle bath and temperature. The muscle bath contained about 40 ml. solution. The temperature of the solution in most of the experiments was held in the range 0·5–2·0° C by placing ice-water in the jacket surrounding the thin-walled inner bath. Efficient stirring was achieved by bubbling oxygen through the solution except when measurements were being made.

Solutions. De-ionized water was used for washing and making up the solutions. All glassware, after ordinary washing, was treated with 6 M-HCl, immediately followed by de-ionized water. All metal surfaces, except for the external stimulating electrodes, were coated with Perspex cement. The muscle chamber was washed with EDTA solution before each experiment.

The sodium methylsulphate was provided by Hopkins & Williams, Ltd. All other chemicals used were of analytical grade. The composition of the Ringer's solution (1) was as follows (mM): KCl 2·0, NaCl 115·5, $CaCl_2$ 1·8, Na phosphate 2·0, pH 7·0. The following solutions were used for stepwise depolarization of the muscle cell membrane in the presence of calcium (mM): (2), KCl 4·0, NaCl 52·95, $NaCH_3SO_4$ 60·55, $CaCl_2$ 1·8, Na phosphate 2·0, pH 7·0; (3), KCl 6·0, NaCl 30·77, $NaCH_3SO_4$ 80·73, $CaCl_2$ 1·8, Na phosphate 2·0, pH 7·0. 'Calcium-free' solutions were of the same composition as solution 1 above except for the omission of 1·8 mM-$CaCl_2$ and the addition of 0·1 mM dihydrogen EDTA.

RESULTS

The effect of calcium lack upon the whole muscle

The effects of calcium lack upon the tetanic response and the twitch: tetanus ratio of the whole muscle and on the membrane potentials of surface fibres are illustrated in Fig. 1. Tetanic responses and membrane potentials showed gradual decline. Complete inexcitability was found after 3 hr, at which stage the mean membrane potential of surface fibres was about 50 mV, with a scatter of individual values between 25 and 60 mV. The twitch: tetanus ratio also fell slightly, by approximately 20 %. It was of interest to find out to what level the calcium concentration could be lowered without loss of contractility. A decline of contractility associated with a decrease of membrane potential occurred in 0·01 mM calcium with a similar time course to that in calcium-free solution. In 0·1 mM calcium solution only a partial loss of mechanical responses (and membrane potentials) occurred, as shown in Fig. 1.

Reintroduction of calcium to an almost inexcitable muscle restored both

the mechanical responses and the membrane potentials substantially. Complete mechanical recovery of the muscle as a whole was never obtained, however. Many fibres repolarized completely, although others remained

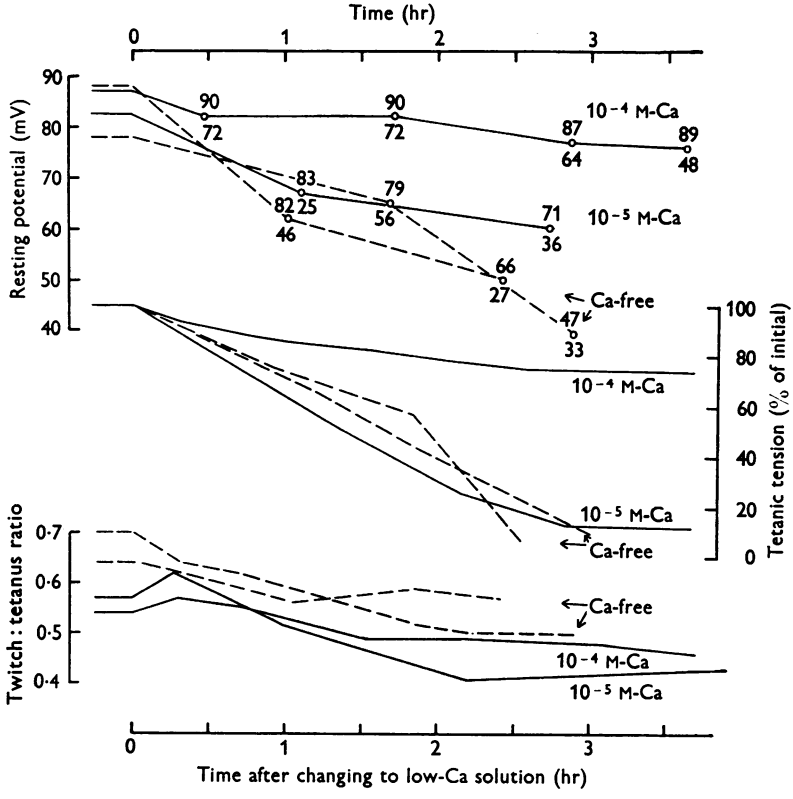

Fig.1. Effect of lowering the calcium concentration in the muscle bath upon the mean resting potentials (upper curves, total range of potentials also shown), tetanic tensions (middle curves) and the twitch:tetanus ratios (lower curves) of frog's sartorius muscle. The muscles were initially in Ringer's solution containing 1·8 mM calcium. At time zero the solution was changed to either 0·1 mM, 0·01 mM calcium or calcium-free Ringer's solution. The calcium-free solution contained 0·1 mM EDTA. Temperature 0–1° C.

depolarized and inexcitable over a period of 2 hr after replacing the calcium. This pattern of recovery was found after treatment with 0·01 mM external calcium as well as after treatment with calcium-free Ringer's solution containing 0·1 mM EDTA.

It can be seen from Fig. 1 that during the fall of mechanical responses some fibres depolarized to such an extent that they can be presumed to have become inexcitable. The reduction of the mechanical response of the

muscle by calcium lack can therefore be partly accounted for by a fall of membrane potentials of some fibres below the level at which those fibres are excitable. This is demonstrated by experiments in the following section but it will be shown that a decrease in the number of excitable fibres is not sufficient to account for all the observed fall of mechanical responses in the whole muscle.

The effect of calcium lack on single fibres

Twitch tension and resting potential. In order to test whether there is a gradual decrease in the contractility of individual fibres before they become completely inexcitable in calcium-free solution, surface fibres of the frog sartorius were individually stimulated intracellularly and the mechanical response to the single current pulse was recorded. The membrane potential was always measured immediately before stimulating through the same electrode. In the experiments in which intracellular action potentials were recorded, a stimulating pulse was applied to small groups of fibres in the ventral surface by passing current through small platinum bipolar electrodes. The results are illustrated in Fig. 2.

When the muscle was immersed in ordinary calcium-containing Ringer's solution, twitch tensions in the range 9–115 mg were found, consistent with the range of fibre cross-sections in the ventral surface of the frog sartorius muscle. The mean tension exerted by an individual fibre was 33 mg, when the whole muscle had been set up with an initial resting tension of about 50 mg.

As was found in the whole-muscle experiments, resting potentials fell progressively after the omission of calcium. Many fibres became inexcitable after a certain time because resting potentials were too low for the development of propagated impulses. However, as is evident from Fig. 2, it is not simply an all-or-nothing failure of individual fibres. The individual fibres, while still excitable, were not able to exert as much tension as they did in the calcium-containing Ringer's solution. Thus after $\frac{1}{2}$–$1\frac{1}{2}$ hr in calcium-free Ringer's solution 10 % of the fibres were inexcitable and the mean peak twitch tension of the individual excitable fibres fell from 33 to 29 mg. In the period $1\frac{1}{2}$–$2\frac{1}{2}$ hr 55 % of the fibres were found inexcitable, and the mean peak tension of the excitable fibres was only 14 mg. The probability that this value differs by chance from the mean twitch output before the omission of calcium (33 mg/fibre) is $< 0{\cdot}001$, according to the t test.

The results show that the time course of the decay of active tension pertaining to the surface-fibre layer was not markedly different from that found for the muscle as a whole. As demonstrated in Fig. 1 the twitch tension of the whole muscle (the change of tetanic tension corrected for

the change of twitch:tetanus ratio) was reduced by 70–85 % of the initial output 2 hr after the omission of calcium. According to the above data referring to measurements of individual surface fibres about 55 % of the initial twitch tension was lost owing to inexcitability of fibres after 2 hr

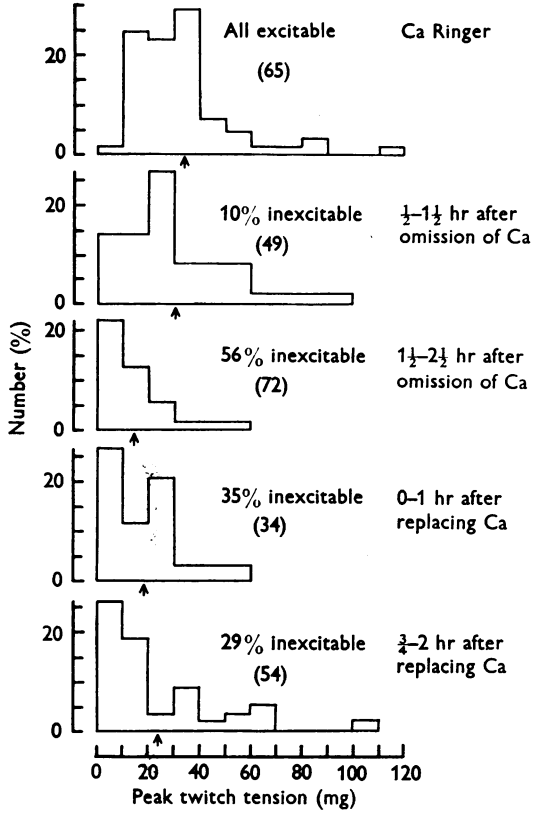

Fig. 2. Histograms of the distribution of twitch tensions recorded from ventral-surface fibres of frog's sartorius muscle (3 experiments) in ordinary calcium-containing Ringer's solution, after omission of calcium and after re-introduction of calcium to the bath. The mean twitch tensions of the excitable fibres is indicated by arrows. The number of fibres measured is given in parentheses and the percentage of this number of fibres that were inexcitable is shown. Temperature 0–1° C.

in calcium-free medium, and another 25 % by the decline of contractile response in the remaining excitable fibres, i.e. a total loss of twitch response in the superficial fibre layer of about 80 %. The changes in contractility as recorded in individual surface fibres may therefore, in essence, be regarded as representative for the rest of the fibres in the muscle.

Figure 3, which summarizes the results obtained with three different muscles, shows the occurrence of twitch responses or inexcitability of

individual surface fibres in relation to their resting potentials in calcium-free Ringer's solution. There is a substantial overlap between the ranges of excitability and inexcitability. The resting potential, below which the fibres were inexcitable, averages about 60 mV, essentially the same level as was found to be critical for the maintenance of electrical excitability

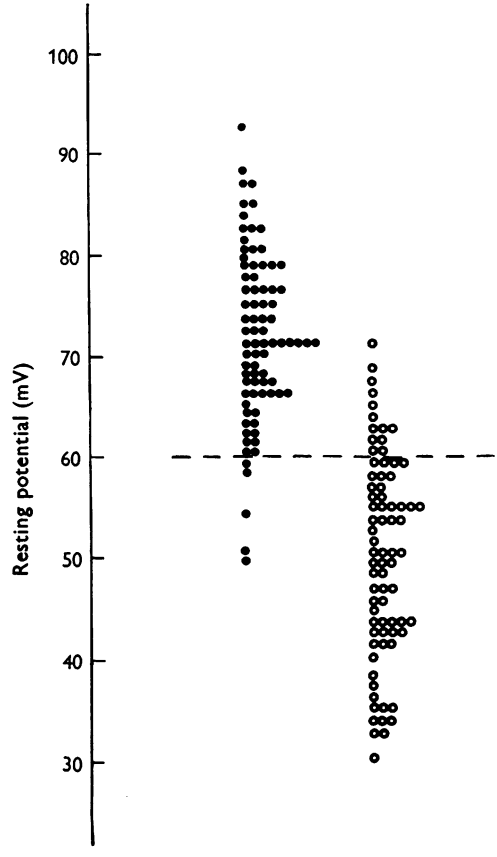

Fig. 3. The relation between resting potential and occurrence of twitch response (filled circles) or inexcitability (open circles) of individual surface fibres of frog's sartorius muscle after half an hour or longer in calcium-free Ringer's solution. Intracellular stimulation. Summary of 3 experiments. Temperature 0–1° C.

under similar conditions in the presence of calcium (Jenerick & Gerard, 1953). Results similar to those illustrated in Fig. 3 have been obtained in experiments where instead of twitch responses the occurrence of action potentials of individual surface fibres in response to external electrical stimulation was recorded. In a brief communication Jenden & Reger (1962) have reported a complete failure of excitability and mechanical

response below 75 mV resting potentials in frog sartorius muscles; information about temperature and time of year was not given.

Re-introduction of calcium restored the resting potential of most fibres and increased the number of excitable fibres. As is shown in Fig. 2, the mechanical responses of the excitable fibres are also restored. A few fibres showed low membrane potentials and were inexcitable even 2 hr after the restoration of calcium, and at this time the peak twitch tension of the excitable fibres was generally somewhat smaller than before the omission of calcium. The mean peak twitch tension per excitable fibre was 24 mg in the period $\frac{3}{4}$–2 hr after re-introduction of calcium to the bath, as compared with 33 mg before the removal of calcium from the intracellular fluid.

The relation between mechanical and electrical response. It was relevant at this stage to see whether the decrease in the mechanical response of individual fibres in the absence of calcium was related to a change in the action potential. Figure 4 shows a series of action potentials recorded intracellularly 1 *cm from the point of stimulation* after removing calcium from the bath. As can be seen, there was a progressive diminution in the size of the action potentials. Overshoots were gradually abolished and finally, after 1 hr or more, small propagated responses occurred with approximately 30 mV magnitude. After this stage no propagated electrical response to stimulation was observed. Action potentials may, however, have existed between the point of stimulation and the recording electrode. It will be shown in a later paper that after deprivation of calcium the action potential may be blocked on its way along the fibre.

To test whether the changes of action potential and mechanical response were due simply to the fall of resting potential produced by the calcium lack, action potentials and single-fibre twitch tensions were recorded when the muscle was immersed in solutions with varying potassium concentrations in the range 2–6 mM and with normal calcium concentration. These solutions all had the same $[K]_0 \times [Cl]_0$ product and total ionic strength, so that changing from one to another caused no movements of potassium chloride across the membrane (Boyle & Conway, 1941). In order to fulfil these requirements the increase of the potassium was made at the expense of the extracellular sodium (see Methods). These solutions were used to reduce the resting potential by steps within the range of excitability. The small differences of the total sodium ion concentration in the solutions used would have very little effect upon the overshoot of the action potential. The results are illustrated in Fig. 5. It was found that all fibres that were excitable gave fully developed action potentials with overshoots. Moreover, as can be seen from Table 1, the mean twitch tension of ventral-surface fibres did not decrease on lowering of the resting potential in the presence of

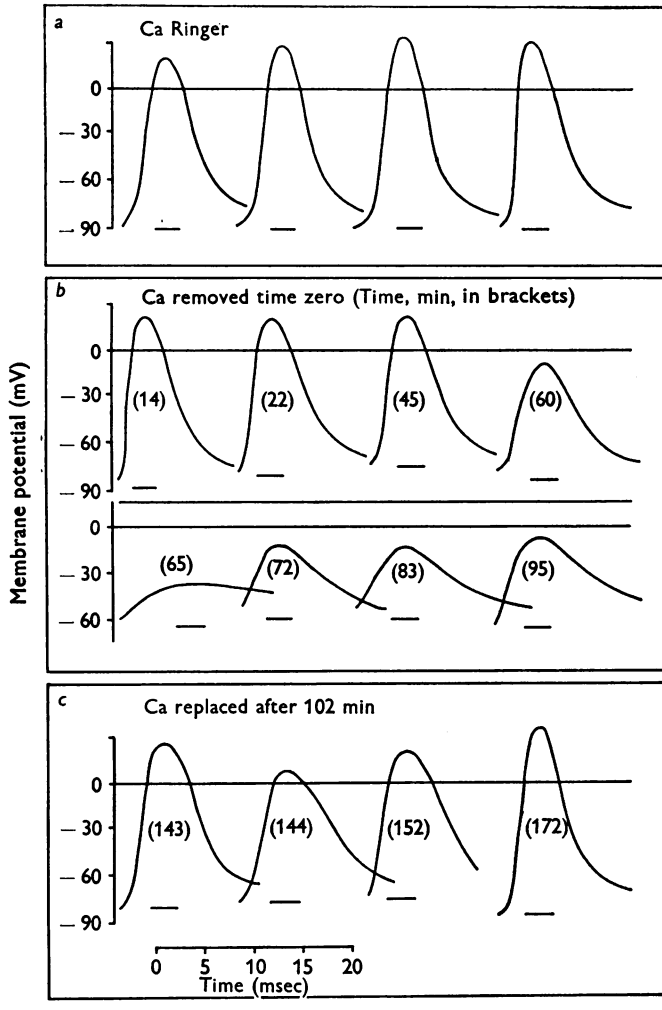

Fig. 4. Tracings from photographs of action potentials recorded intracellularly at 0–1° C from ventral-surface fibres of frog's sartorius muscle, 1 cm from the point of stimulation. *a*, Recorded in ordinary Ringer's solution. *b*, Recorded in calcium-free solution; time in minutes after changing the solution is shown in parentheses. *c*, Recorded after calcium was replaced (after 102 min.). The resting potentials are represented by short horizontal lines. Note: (1) Reduction of resting potentials; (2) disappearance of overshoot with marked reduction in amplitude of action potentials in the calcium-free medium; (3) restoration of resting potentials and return of overshoot of the action potentials after replacement of calcium.

calcium. Even in the lowest range of resting potentials the mechanical response of excitable fibres to electrical stimulation was at least as high as in the range of 90–100 mV.

Increase of the potassium ion concentration in the external fluid to 4·5–6 mM from the original 2 mM concentration was found by Sandow & Kahn (1952) to produce a small (about 10 %) potentiation of the mechanical response of the frog sartorius muscle to 'massive' electrical stimulation. As is seen in Table 1, the mean twitch output of the fibres was slightly

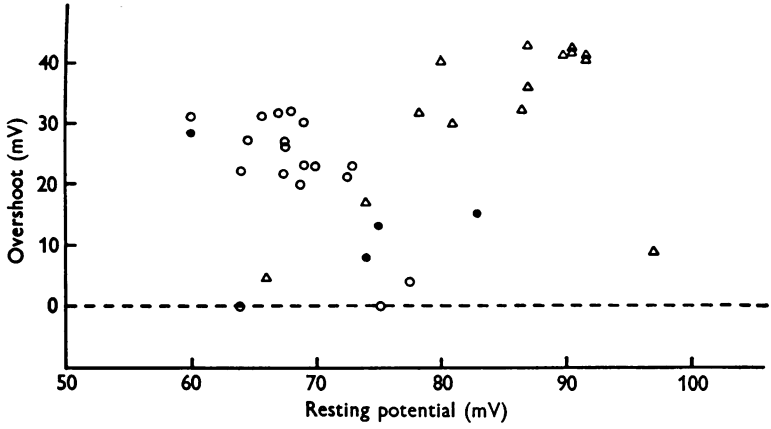

Fig. 5. The relation between the resting potential of individual surface fibres of frog's sartorius muscle and the overshoot of the action potential in the presence of 1·8 mM calcium. Different symbols represent different experiments. The resting potential was modified by changing the extracellular potassium concentration ($[K]_0 \times [Cl]_0$ and ionic strength constant). Temperature 0–1° C.

TABLE 1. Mean peak twitch tension (±S.E. of the mean) of individual surface fibres in various ranges of resting potentials
Calcium-containing Ringer's solution with varying concentrations of potassium (see text)

Resting potential range (mV)	Mean twitch tension as % of that in 90–100 mV range	No. of fibres measured
90–100	100±12·4	30
80– 90	85±10·5	45
70– 80	108±10·0	63
60– 70	140±17·5	16

increased in the lowest range of resting potentials, which mainly represents recordings done in 6 mM external potassium. The increase, with the twitch output in the range of 90–100 mV as a reference, was not, however, statistically significant.

It is concluded that the reduction of the action potential and contractility of the individual fibre in the absence of calcium is not produced

simply by a lowering of the resting potential. Calcium lack is having a more direct effect upon the mechanisms governing the excitability of the muscle cell membrane and the mechanical output of the contractile system.

DISCUSSION

The reduction of mechanical output of both individual fibres and the whole frog sartorius muscle in response to electrical stimulation after deprivation of calcium is closely associated with changes of excitability of the fibre membrane. The removal of calcium from the bath produces a steady fall of the resting potential, sufficiently pronounced to bring all fibres in the muscle into an inexcitable state. The resting potential level at which inexcitability occurs in the absence of calcium is about 60 mV, which is not significantly different from the level at which electrical and mechanical activity is lost in the presence of calcium when depolarization is produced by potassium (Jenerick & Gerard, 1953). The *complete* mechanical failure of the muscle fibres in the preparation used can thus be fully explained by loss of excitability of the membrane.

However, while in the presence of calcium there is an abrupt loss of electrical and mechanical activity of the fibre when the resting potential is lowered below a certain level, calcium lack produces a gradual decline of both the mechanical response and the amplitude of the action potential. It is reasonable to assume that the progressive change of excitability of the membrane is of relevance in the development of mechanical failure. Hodgkin & Horowicz (1960) have shown that the contractile output (contracture) of single frog semitendinosus fibres in response to stimulation with potassium is proportional to the degree of depolarization of the cell membrane beyond the threshold potential, about 50 mV. Because of the difficulty in producing a similar selective change of the action potential it is still not clear to what extent the mechanical output of the fibre is dependent on the size of the action potential. There is evidence, however, that the action potential induces contraction of the muscle cell by virtue of depolarization of the membrane (Kuffler, 1946; Sandow, 1952, 1955; Sten-Knudsen, 1954; Hodgkin & Horowicz, 1960). It therefore seems probable that reduction in size of the propagated impulse beyond a certain level will gradually reduce its ability to activate the subsequent steps in the excitation-contraction process in a way equivalent to that found in the depolarization contracture induced by potassium. Impairment of some more intimate calcium-dependent link in the contraction coupling may also contribute to the decrease in contractility. The present results support the view, however, that failure of the electrical excitability of the membrane

is an essential cause of the gradual decline of contractile output of the individual fibre in a calcium-free medium.

As will be treated in more detail in a forthcoming paper the process of excitation of the muscle cell membrane may be further complicated in a few fibres by deficient propagation of the action potential after removal of the extracellular calcium. The action potential is thus blocked in certain fibres before it reaches the end, which implies that only a portion of the contractile system of such fibres is activated.

Considering the muscle as a whole the decline of mechanical response in calcium-free solution is a composite phenomenon, which involves both inexcitability of individual fibres and diminution of contractile output of excitable fibres. The results have shown the approximate contributions of these two factors to the mechanical failure of the whole muscle. About two-thirds of the reduction of active tension of the muscle 2 hr after removal of the extracellular calcium is due to loss of excitable fibres, the rest of the failure being caused by diminished contractility of the fibres remaining excitable.

It can be concluded that calcium is required for the excitability of the muscle cell membrane, and that loss of excitability is the principal cause of the mechanical failure of the frog sartorius muscle in response to electrical stimulation after removal of calcium from the extracellular medium. It is clear that calcium is essential for the maintenance of the resting potential, and that it is needed for the production of the action potential. A similar dependence of resting potentials and the production of action potentials upon calcium has also been demonstrated in other excitable tissues, e.g. heart muscle (Weidmann, 1955), frog myelinated nerve fibres (Frankenhaeuser, 1957), lobster axon (Adelman & Adams, 1959) and squid axon (Frankenhaeuser & Hodgkin, 1957). The progressive decrease in amplitude of the action potential in frog sartorius fibres has also recently been observed by Koketsu & Noda (1962): it was furthermore found by them that the action potential, after being reduced in amplitude or completely lost by deprivation of calcium, is restored by anodal polarization of the fibre membrane, even after the preparation has been soaked in calcium-free solution containing EDTA for 24 hr. This is a very interesting finding in view of the fact, shown in the present work, that the decrease in amplitude of the action potential after removal of the extracellular calcium is not simply due to decrease of the resting potential. It indicates that the calcium ion does not play an immediate part in the production of electrical activity of the membrane. The calcium ion may instead maintain the integrity of the systems which are responsible for the maintenance of resting potential and the production of the action potential.

SUMMARY

1. The effects of calcium lack on the mechanical output of whole sartorius muscle and of individual surface fibres of the frog have been correlated with changes of the resting potentials and the electrical activity of the individual fibres.

2. Removal of the extracellular calcium produced a progressive decline of the resting potentials of individual fibres to equilibrium values of about 40 mV.

3. The twitch response was lost after the resting potential had been reduced to about 60 mV, i.e. not markedly different from the level at which electrical inexcitability occurs in the presence of calcium.

4. Instead of an all-or-nothing failure of mechanical and electrical activity, as occurs in the presence of calcium, there is a gradual decline of both the amplitude of the action potential and the height of the twitch response of the individual muscle cells in the absence of calcium, in parallel with the lowering of the resting potential. After 2 hr in calcium-free Ringer's solution 55 % of the fibres were inexcitable and the mean peak twitch tension per excitable fibre had been lowered to half the original value in the calcium-containing medium. Overshoots of the action potentials were gradually abolished, and after 1 hr or more, before the fibres became inexcitable, propagated impulses of approximately 30 mV amplitude were recorded.

5. The complete loss of mechanical output of individual fibres in response to electrical stimulation in the absence of extracellular calcium can be fully explained by inexcitability of the cell membrane caused by reduction of the resting potential. The progressive decline of the twitch response of the single fibre which precedes the complete mechanical failure may to a great extent be accounted for by deficient activation of the contractile system by the diminished action potential.

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