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THE RELATION BETWEEN EXTERNAL POTASSIUM CONCENTRATION AND THE RELAXATION RATE OF POTASSIUM-INDUCED CONTRACTURES IN FROG SKELETAL MUSCLE

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SUMMARY

1. Frog toe muscles and isolated fibres from frog semitendinosus muscles were allowed to develop maximum K-contractures, and after reaching peak tension were transferred to media containing intermediate $[K]_0$ (20–40 mM-K). Under these circumstances, relaxation displayed an early rapid phase, and a subsequent slower phase whose magnitude and rate varied with $[K]_0$. The removal of activator (calcium ion) from the sarcoplasm appears to have a potential-dependent component.

2. The relation between relaxation and $[K]_0$ suggests that, with progressive depolarization, membrane sites from which calcium is initially released become converted into sites which are again capable of binding calcium and removing it from the sarcoplasm.

3. The rate of relaxation of frog toe muscle after maximum K-contractures can be either accelerated or retarded by abrupt alterations in $[Ca]_0$ or by sudden replacement of the major extracellular anion. These effects are attributed to shifts in the relation between calcium rebinding and membrane potential.

INTRODUCTION

In frog twitch muscle, maximum potassium-induced contractures are followed promptly by relaxation even though depolarization persists (Kuffler & Vaughan Williams, 1953). Hodgkin & Horowicz (1960*a*) described the time course of potassium-induced contractures in single frog muscle twitch fibres. As a tentative explanation for their observations they postulated that depolarization results in the production and release into the sarcoplasm of a hypothetical activator and that this activator subsequently is destroyed in a first-order reaction with a rate constant of about 30 sec^{-1}. The increase in the rate of both contraction and relaxation with increased $[K]_0$ could be accounted for by assuming that the activator is

released from a limited supply of its precursor at a rate which is proportional to the degree of membrane depolarization. In this scheme, the shortening of the time constant of relaxation which accompanies increasing degrees of depolarization could result from more rapid exhaustion of the precursor rather than from an increase in the rate of activator destruction. The limited period of activator production during a single action potential could readily explain the brief time course of a muscle twitch without requiring any change in the rate constant which was used to account for activator elimination during persistent depolarization.

A great deal of evidence has now accumulated which indicates that calcium ion is the activator of the contractile process, and that the tension developed by the muscle reflects the sarcoplasmic concentration of this ion (Shanes, 1958; Ebashi, 1961; A. F. Huxley, 1964). Podolsky & Constantin (1964) found that isolated segments of single frog skeletal muscle twitch fibres which had been denuded of their surface membrane relaxed quickly following contractions produced by the local application of calcium ions. These authors concluded that relaxation is controlled by intracellular structures rather than by the state of the cell membrane. They suggested that the accumulation of calcium by the sarcoplasmic reticulum furnishes a 'calcium sink' which removes added calcium ions rapidly, thereby terminating contraction. A similar mechanism has been proposed to explain the ability of certain particulate fractions which can be separated from muscle homogenates by centrifugation, to bring about (*a*) the relaxation of glycerinated muscle fibres, (*b*) the inhibition of myofibrillar ATP-ase, (*c*) the removal of bound calcium from actomyosin, and (*d*) the prevention of actomyosin superprecipitation (Hasselbach & Makinose, 1961; Weber, Herz & Reiss, 1963). Ohnishi & Ebashi (1963, 1964) used ingenious spectrophotometric procedures for measuring the rate at which fragments of the sarcoplasmic reticulum lower the calcium concentration of the medium, and concluded that this process was sufficiently rapid to account for the rate of relaxation of muscle twitches. Calculation of the rate of calcium uptake by isolated reticular fragments led Hasselbach (1964*a*, *b*) to the same conclusion. However, these observations do not preclude potential-dependent variation in the rate at which the activator (calcium) is removed from the sarcoplasm. The persistence of K-induced contractures in cardiac muscle and in slow (tonic) skeletal muscle clearly shows that relaxation is potential-dependent in these tissues.

Lee has reported that the efficacy of reticular fragments of fast muscle in accumulating calcium *in vitro* is altered by passing current through the medium (Lee, 1965; Lee, Tanaka & Yu, 1965). A considerable body of evidence from studies of twitch potentiation in fast skeletal muscle suggests that delayed onset of relaxation during a twitch may be associated with

prolongation of the terminal part of the action potential (Sandow & Preiser, 1964; Sandow, Taylor, Isaacson & Seguin, 1964), although these events are not simultaneous (Falk, 1961). Procedures which potentiate twitches in skeletal muscle usually prolong the duration of the contractile state (Hill & Macpherson, 1954; Ritchie, 1954; Lammers & Ritchie, 1955; Hutter & Noble, 1960; Sandow, 1964). The very rapid onset of the effects of twitch potentiating procedures has been attributed to a superficial (membrane) site of action (Kahn & Sandow, 1950; Hill & Macpherson, 1954; Hodgkin & Horowicz, 1960*b*; Sandow, 1964).

The curve which relates contracture tension in single phasic muscle fibres to membrane potential can be shifted along the voltage axis by changes in the ionic composition of the external medium (Hodgkin & Horowicz, 1960*b*; Luttgau, 1963). Hodgkin & Horowicz (1960*b*) noted that after subthreshold depolarization (20 mM-K), contractures in single fibres could be elicited merely by shifting the position of this curve toward the membrane resting potential level (by replacement of external chloride with nitrate). Luttgau (1963) reported that increased $[Ca]_0$ produced an immediate change in the pattern of relaxation following K-contractures in single muscle fibres. Sudden changes in the ionic environment also can produce marked changes in the pattern of relaxation of maximum K-contractures in frog toe muscle, whose small size reduces (but does not entirely eliminate) limitations resulting from diffusion (Frank, 1960). This technique has been employed in the present investigation in an effort to provide more direct evidence concerning the degree of dependence of relaxation rate upon membrane potential in this tissue. Additional experiments have been carried out with isolated twitch fibres dissected from frog semitendinosus muscle in order to exclude, to a greater extent, diffusional delay as a factor of importance in determining the response to abrupt alteration in the composition of the medium.

METHODS

Experiments with the long extensor of the fourth toe of the frog (*Rana pipiens*) were carried out at room temperature *in vitro*. Frogs were obtained from College Biological Supply House, Seattle, during all seasons of the year. Experiments on toe muscles were confined to preparations which relaxed rapidly and completely after maximum K-contractures and which therefore were presumed to consist predominantly of fast (twitch) fibres (Kuffler & Vaughan Williams, 1953). Contractile tension was recorded isometrically, using Sanborn transducers, amplifiers and direct-writing oscillographs. The relaxation rate was estimated by careful measurement of the slope of the major phase(s) of tension decline. The various media usually used were isotonic derivatives of a conventional frog Ringer fluid containing 0·1 mg/ml. (+)-tubocurarine chloride. The normal extracellular concentration of calcium was taken to be 1·08 mM (Boyle & Conway, 1941; Frank, 1960). The other components of the solution were (mM): Na^+, 114·3; K^+, 2·47; Mg^{2+}, 1·2; HCO_3^-, 2·38; SO_4^{2-}, 1·2; PO_4^{3-}, 0·087; glucose, 11·1. All solutions were adjusted to pH 7·1 immediately before use. The muscles

were suspended vertically in a 6 ml. acrylic bath. Solutions were abruptly changed by draining and refilling the bath. This procedure took 4–5 sec.

In other experiments, one or two twitch fibres were dissected from frog semitendinosus muscles and suspended horizontally in a closed cylindrical bath resembling that employed by Hodgkin & Horowicz (1959). This bath had a capacity of about 1 ml. The contents of the bath were expelled at one end when a new solution was introduced into the other. This procedure was executed in about 1 sec. In these experiments, the output of the transducer was displayed on an oscilloscope and photographed on polaroid film.

A 15 min rest period in Ringer solution was allowed between successive contractures Muscles were placed in a solution in which all Na was replaced with choline for a short interval (1–2 min) before each contracture in order to eliminate Na influx as a source of depolarization. To induce contractures, $[K]_0$ was increased by substitution of potassium for choline in this Na-free medium. When $[Ca]_0$ was to be varied, an osmotically equivalent part of the choline salt in the various media used was replaced with the corresponding calcium salt. Hypertonic solutions were required when the combined concentration of K and Ca ions exceeded 118 mM. When chloride was replaced with nitrate, solutions of the choline salt were prepared by mixing equimolar portions of choline hydroxide with nitric acid.

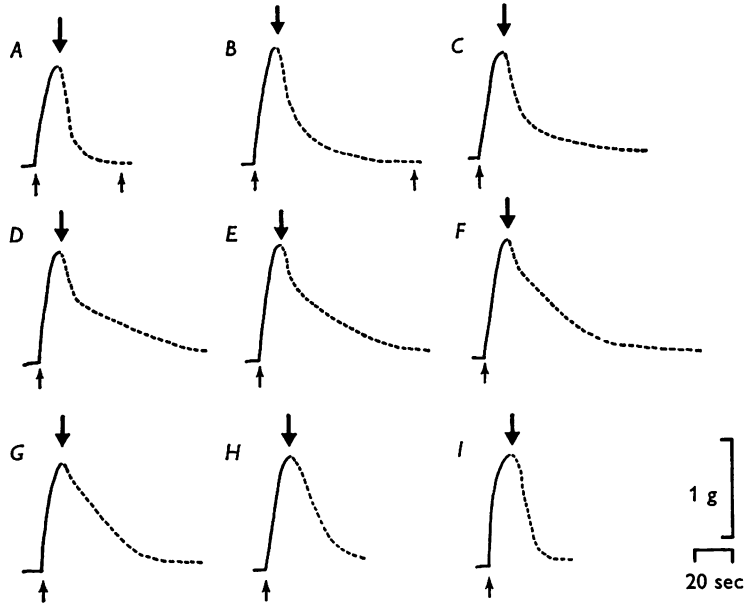

Fig. 1. Effect of varying $[K]_0$ on the relaxation of maximum K-contractures in frog toe muscle. All contractures were produced at a $[K]_0$ of 60 mM. When peak tension was reached, the bath was emptied and refilled (↓) with a medium whose potassium concentration was: *A*, 12 mM; *B*, 17 mM; *C*, 22 mM; *D*, 27 mM; *E*, 31 mM; *F*, 36 mM; *G*, 41 mM; *H*, 60 mM; *I*, 94 mM. External chloride concentrations remained constant throughout. Similar results were obtained with contractures produced initially at 94 mM-K. (In this and subsequent figures, direct tracings of contractures have been made from original oscillograph recordings of tension. These have been retouched to eliminate the artifacts which accompany emptying and refilling of the muscle bath. In each instance, the small arrows beneath the records indicate the introduction and subsequent removal of the contracture-inducing solution.)

RESULTS

Changes in relaxation rate produced by variation in $[K]_0$. Toe muscles subjected to maximum K-contractures relaxed rapidly whether $[K]_0$ was maintained at a high level or restored to its normal level. However, when peak tension was reached in such contractures, an abrupt reduction in $[K]_0$ to an intermediate level slowed the time course of relaxation (Fig. 1). Retarded relaxation was most prominent when $[K]_0$ was reduced to 20–40 mM. Relaxation from contractures after reduction of $[K]_0$ to an intermediate level usually was divisible into two stages, the first being considerably more rapid than the second. The relaxation rate in the second phase often was similar to that observed during contractures initially developed at the same $[K]_0$. Both the rate of relaxation during the slower second phase, and the level of tension at which this phase began,

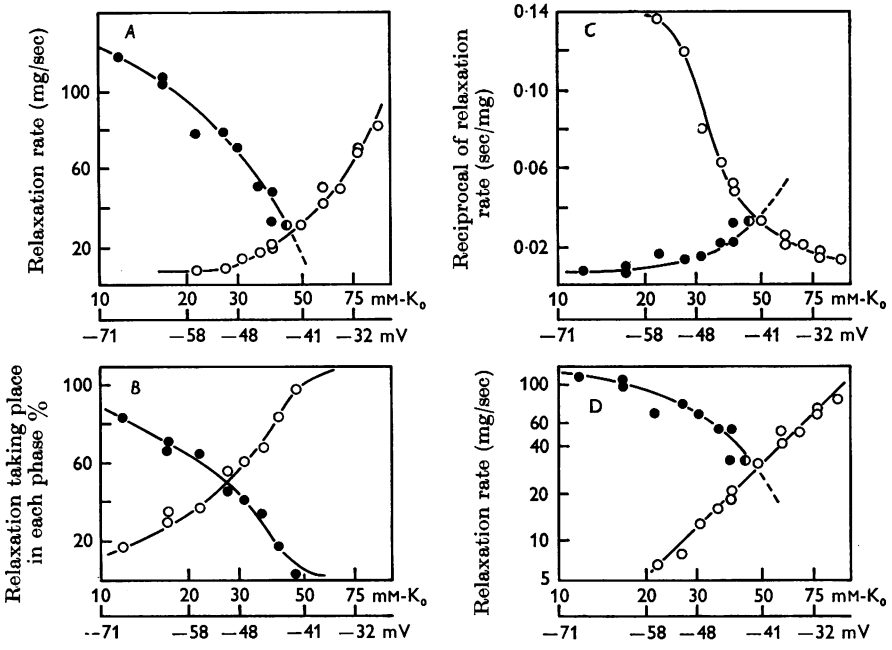

Fig. 2. Relation between log $[K]_0$ and various aspects of relaxation after maximum K-contractures in frog toe muscle. Log $[K]_0$ is plotted against: *A*, the relaxation rate of the early (●) and late (○) phases of relaxation; *B*, the percentage of relaxation taking place in each phase; *C*, the reciprocal of the relaxation rates of each phase; and *D*, the logarithm of the relaxation rates of each phase. The slope for each phase of relaxation was carefully drawn and extrapolated on the original records in order to estimate the relaxation rates. The point of intersection of the slopes of the two phases was used to estimate the percentage of relaxation taking place in each phase. The estimated membrane potential scale has been taken from Hodgkin & Horowicz (1960*a*).

varied with $[K]_0$. With successively greater reductions in $[K]_0$, the transition to the slower phase of relaxation occurred at progressively smaller levels of residual tension, and the rate of relaxation during this phase was decreased. The relaxation rates for the two phases, when plotted against log $[K]_0$, appear as parts of S-shaped curves which are approximate mirror images of one another. For the faster initial phase, the steep portion of this curve lay between 20 and 40 mM-K (Fig. 2*A*). The amount of relaxation taking place in this phase displayed a similar relation to log $[K]_0$ (Fig. 2*B*). The change taking place in the relaxation rate of the second phase in the 20–40 mM range of $[K]_0$ was relatively small in comparison

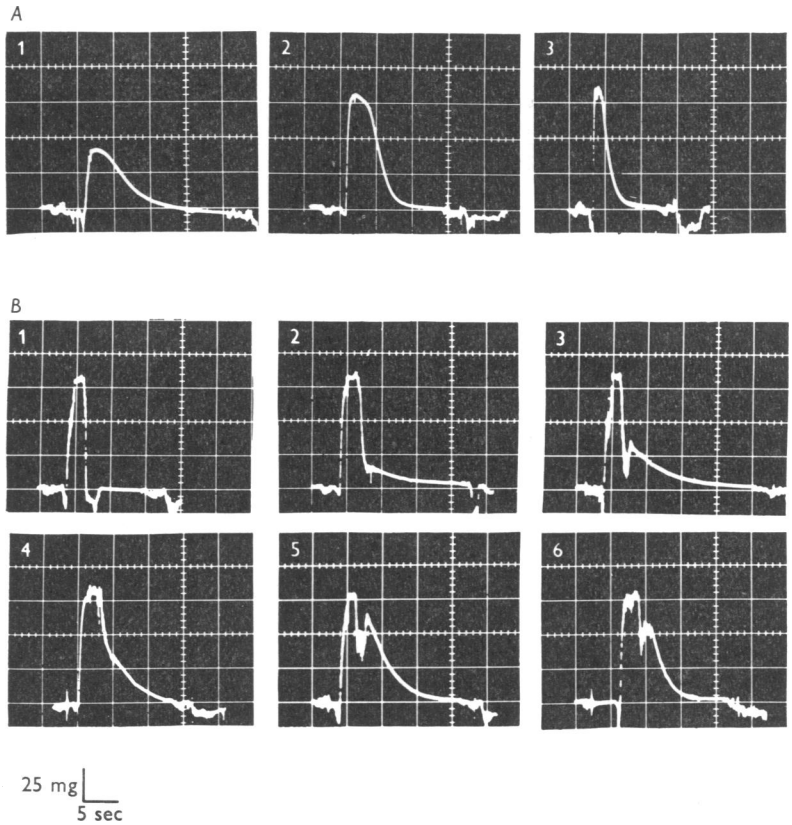

Fig. 3. *A*. Oscilloscope records of contractures produced in a pair of isolated fibres of frog semitendinosus muscle by the following K concentrations: 1, 31 mM; 2, 60 mM; 3, 94 mM. *B*. Oscilloscope records of the effect of varying $[K]_0$ on the relaxation of maximum K-contractures in this pair of isolated muscle fibres. All contractures were produced initially at a $[K]_0$ of 60 mM. When peak tension was reached, the solution in the bath was quickly changed to a medium whose potassium concentration was: 1, 2·5 mM; 2, 31 mM; 3, 36 mM; 4, 41 mM; 5, 50 mM; 6, 60 mM. External chloride concentrations remained constant throughout.

with that which occurred at higher $[K]_0$. However, when the reciprocal of the relaxation rate (an index of the time required for unit tension decline) was plotted against log $[K]_0$ the changes occurring in the 20–40 mM range of $[K]_0$ assumed greater prominence (Fig. 2*C*). The progressive increase in relaxation rate at higher $[K]_0$ seemed to represent augmentation of the process responsible for the slower second phase of relaxation at lower $[K]_0$. This conclusion was strengthened by the observation that all the points representing these particular phases lay along the same straight line when the logarithm of their relaxation rates was plotted against log $[K]_0$ (Fig. 2*D*). Essentially similar results were obtained with isolated fibre preparations from semitendinosus muscles (Fig. 3).

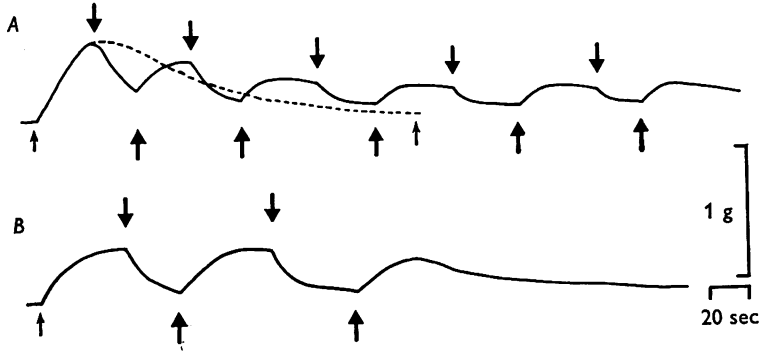

Fig. 4. Successive suppression and renewal of tension in submaximum (31 mM) K-contractures in frog toe muscle as the result of alternate increases and decreases in $[Ca]_0$. In *A* the initial contracture was 55 % of maximum contracture tension (in 60 mM-K), and the time course of a control contracture at normal $[Ca]_0$ is shown by the interrupted line. In *B*, the initial contracture was 38 % of maximum contracture tension (in 60 mM-K). (↓) indicates points at which the $[Ca]_0$ was increased 10-fold (to 10·8 mM). (↑) indicates points at which the $[Ca]_0$ was returned to its normal level (1·08 mM).

Changes in relaxation rate produced by variation in $[Ca]_0$. During submaximum K-contractures tension develops and subsides more slowly than during maximum contractures (Hodgkin & Horowicz, 1960*a*). Luttgau (1963) showed that relaxation after such contractures was accelerated by abrupt elevation of $[Ca]_0$. We have confirmed this observation. During half-maximum contractures, tension was suppressed and renewed repeatedly through several cycles by alternately increasing and decreasing $[Ca]_0$ while $[K]_0$ remained constant at 31 mM (Fig. 4). (Raising and lowering $[Ca]_0$ produces a similar cycle of tension changes during the submaximum contractures which accompany chloride withdrawal (Foulks, Pacey & Perry, 1965).) On the other hand, at $[K]_0$ sufficiently high to produce maximum contractures, a sudden increase in $[Ca]_0$ after peak tension had

been reached led to retardation rather than acceleration of subsequent relaxation. An experiment in which this effect was particularly marked is illustrated in Fig. 5. In this preparation, when relaxation was interrupted by a sudden increase in $[Ca]_0$ tension sometimes actually increased slightly before relaxation was resumed, even when elevation of $[Ca]_0$ was delayed until relaxation was well under way (Fig. 5*E*). Thus the direction of the effect of increased $[Ca]_0$ on the rate of relaxation after K-contractures depends upon the $[K]_0$ at which this manoeuvre is carried out.

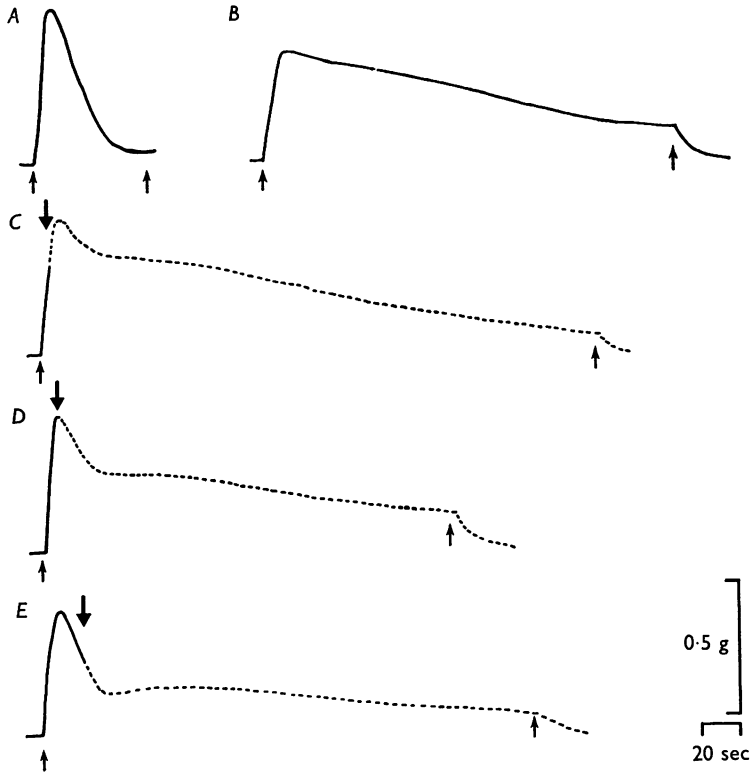

Fig. 5. Interruption and slowing of the rate of relaxation of maximum K-contractures in frog toe muscle (interrupted lines) by an abrupt increase in $[Ca]_0$ to (10·8 mM). All contractures were produced at 60 mM-K. *A*, control contractures at 1·08 mM $[Ca]_0$. In *B* $[Ca]_0$ was increased at the time the contracture was initiated. In the other experiments illustrated, $[Ca]_0$ was increased (↓) at various intervals after the onset of contracture as follows: *C*, 5 sec; *D*, 10 sec; *E*, 20 sec.

Hodgkin & Horowicz (1960*a*) called attention to the fact that the rate of relaxation of single muscle fibres continued to increase as $[K]_0$ was raised above the level necessary for maximum contracture tension. The rate of relaxation after K-contractures may be expressed as the percentage of the

rate observed when depolarization is complete (150 mM-$[K]_0$). When this value is plotted against log $[K]_0$ (Fig. 6) the steep portion of the curve lies at considerably higher K concentrations than the contracture tension curve. Both tension (see also Luttgau, 1963) and relaxation curves were shifted toward higher $[K]_0$ by a 5-fold increase in $[Ca]_0$ (Fig. 6), the curves being displaced to about the same degree. At higher $[Ca]_0$ (11–16 mM) the shift in the position of the relaxation rate curve appeared to be greater than that of the contracture tension curve, and in isolated fibres this effect

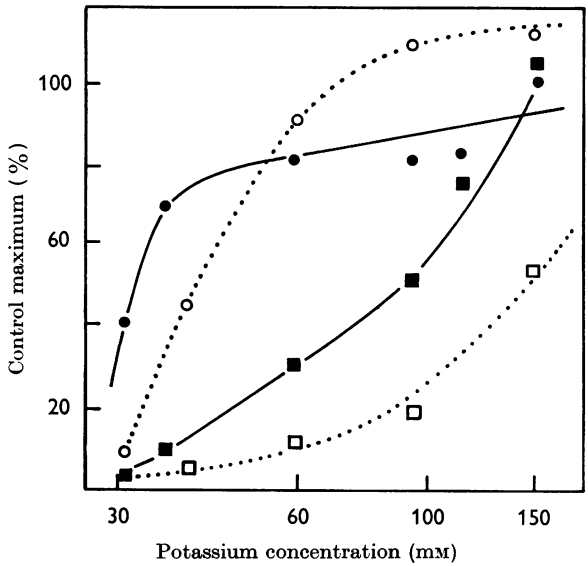

Fig. 6. The effect of increased $[Ca]_0$ on the relation between log $[K]_0$ and (1) contracture tension (●, ○), and (2) the rate of relaxation (■, □), in frog toe muscle, both being expressed as per cent of the maximum value observed during control contractures in media containing 1·08 mM calcium. At normal (1·08 mM) $[Ca]_0$ (continuous lines), the curves for both contracture tension (●) and relaxation rate (■) rise steeply at lower $[K]_0$ (less depolarization) than corresponding curves (○, □) in media containing an increased (5·4 mM) $[Ca]_0$ (interrupted lines). Exposure to increased $[Ca]_0$ began 1–2 min before the onset of each contracture and continued until relaxation was complete.

was evident with $[Ca]_0$ as low as 2–3 mM (Fig. 7). For contractures of similar magnitude, sufficiently high $[Ca]_0$ may retard the rate of both tension development and relaxation (Fig. 8).

Changes in relaxation rate produced by variation in external anion composition. The replacement of external chloride with more polar anions of the lyotropic series (e.g. nitrate) permits the development of maximum K-contractures at a considerably reduced $[K]_0$ (Hodgkin & Horowicz, 1960*b*; Frank, 1961; Foulks & Perry, 1965). However, the rate of relaxa-

tion after K-contractures of similar magnitude is slower in the presence of these anions than in chloride-containing media (Frank, 1961) (see Fig. 9). The onset of this effect in the case of nitrate was evident within 2 min after transfer from chloride-containing media (Fig. 9*B*, *D*). Brief (2 min) exposure to nitrate also produced an accentuation of the plateau phase of maximum or near-maximum contractures. At $[K]_0$ just adequate to produce maximum contractures in nitrate-containing media, when peak

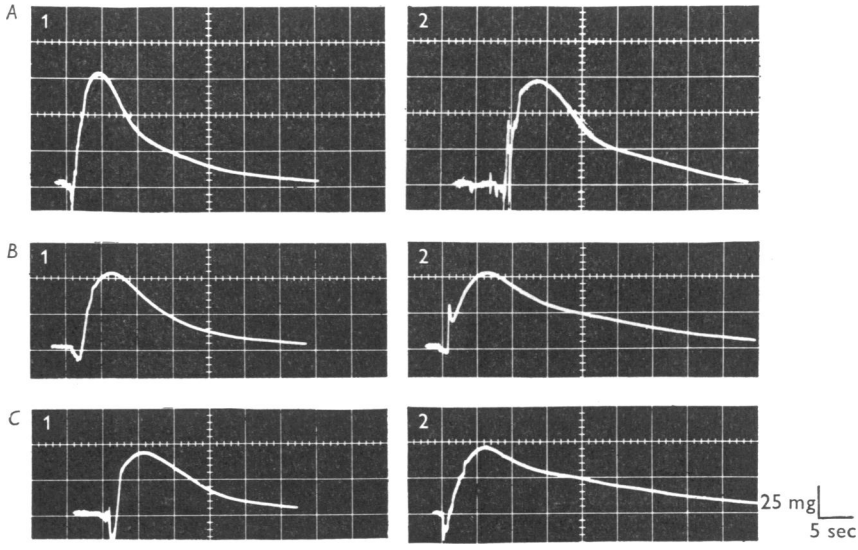

Fig. 7. Oscilloscope records showing the effect of increased $[Ca]_0$ on the time course of submaximum K-contractures in a pair of fibres isolated from frog semitendinosus muscle. The records at the left in each series (A_1, B_1, C_1) were obtained at normal $[Ca]_0$ (1·08 mM), those on the right (A_2, B_2, C_2) at elevated $[Ca]_0$ (3·24 mM). Exposure to high $[Ca]_0$ was limited to the contracture interval and a brief (30 sec) period preceding each contracture. Contracture magnitude expressed as per cent of maximum contracture tension (at 94 mM-$[K]_0$) was: *A*, 60 %; *B*, 40 %; *C*, 33 %. The $[K]_0$ used to produce these contractures was: A_1, 32·5 mM; B_1, 31 mM; C_1, 30 mM; A_2, 65 mM; B_2, 55 mM; C_2, 50 mM.

tension was reached the sudden replacement of nitrate with chloride usually resulted in accelerated relaxation (Fig. 10*A*). However, with longer exposures to nitrate, the rate of relaxation during the major phase of tension decline was similar to that observed in chloride at the same $[K]_0$ (Fig. 10*B*, *C*, *D*). Thus, in muscles which were exposed to nitrate-containing media, the tension curve was shifted toward lower $[K]_0$ (as noted by Hodgkin & Horowicz, 1960*b*), whereas the relaxation rate curve remained in approximately the same position with respect to $[K]_0$ (Fig.11) or was shifted to slightly higher $[K]_0$. When peak tension had been reached during maximum K-contractures in chloride-containing media, the abrupt

replacement of chloride with nitrate retarded the onset of relaxation, but the subsequent rate of relaxation was either unchanged or slightly retarded (Fig. 10*E*).

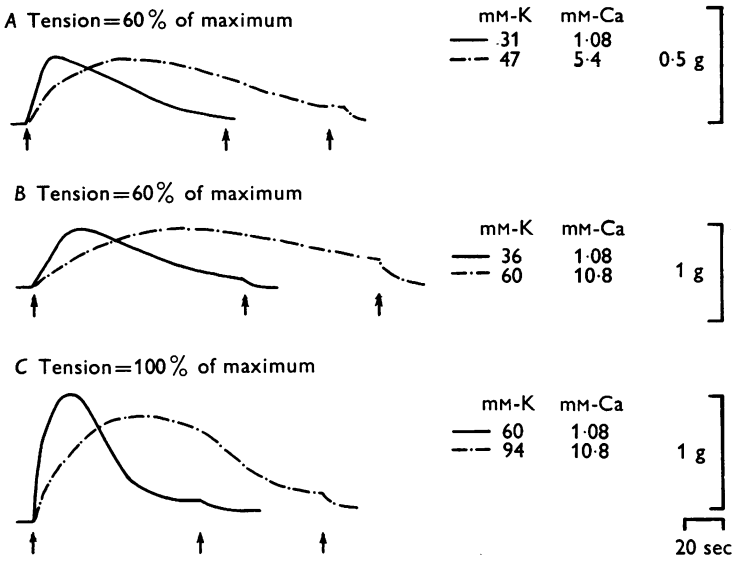

Fig. 8. The effect of increased $[Ca]_0$ (interrupted line) on the time course of submaximum and maximum K-contractures in frog toe muscle. In each instance tracings of contractures in high calcium media were superimposed on tracings of contractures occupying corresponding positions on the tension–log $[K]_0$ curve at normal (1·08 mM) $[Ca]_0$ (continuous line). Experimental conditions are indicated on the figures. In each instance where elevated $[Ca]_0$ was used, the increase was accomplished 1–2 min before contracture induction. Longer exposures to high $[Ca]_0$ did not produce greater effects.

DISCUSSION

The ideas advanced by Hodgkin & Horowicz (1960*a*) to explain the time course of K-contractures require some modification to account for the observations reported here. Their assumption that tension reflects the rates of two opposing processes—(*a*) intracellular release of activator (calcium ion) from a limited source; and (*b*) removal of this activator from the sarcoplasm—is an attractive one. In this view, tension development requires that the rate of calcium release exceed its rate of removal, and relaxation requires the reversal of this relationship, presumably as the result of a decline in the rate of calcium release as its limited source is depleted.

Our observations indicate that relaxation cannot be explained by a single exponential process of calcium removal which is independent of membrane

potential. In toe muscles it is difficult to exclude diffusional delay as a factor contributing to the time course of changes in tension following sudden alterations in the composition of the medium. The fact that the tension reached during maximum K-contractures in toe muscles often equals or exceeds that observed during maximum tetanus (Frank, 1960) suggests that activation in the superficial fibres is maintained until most of the more centrally located fibres also have been fully activated. Moreover, the similarity in the pattern of relaxation of toe muscles and isolated

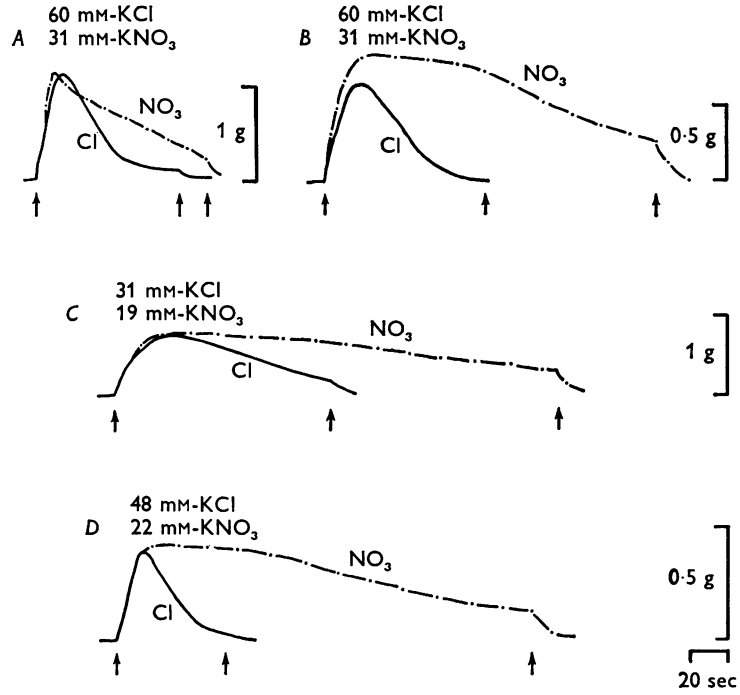

Fig. 9. Superimposed tracings of frog toe muscle contractures of similar magnitude in nitrate- (interrupted lines) and chloride- (continuous lines) containing media. The duration of exposure to nitrate was: *A* and *C*, 30 min; *B* and *D* 2 min. The $[K]_0$ used to produce contractures was: *A* and *B*, 60 mM in Cl, 31 mM in NO_3; *C*, 31 mM in Cl, 19 mM in NO_3 (contracture tension 45 % of maximum); *D*, 48 mM in Cl, 22 mM in NO_3 (contracture tension 75 % of maximum).

muscle fibres indicates that delay due to diffusion is not primarily responsible for the retarded phase of relaxation which appears following abrupt reduction of $[K]_0$ to an intermediate level. After maximum contracture tension has been attained and rapid relaxation already has begun it seems unlikely that partial repolarization would lead to a further increase in the rate of calcium release, since the initial rate of calcium release

appears to increase with increasing degrees of depolarization (Hodgkin & Horowicz, 1960*a*). Retarded calcium removal from the sarcoplasm presumably is responsible for slowed relaxation under these circumstances. Therefore, it seems necessary to postulate that the rate of calcium removal varies with $[K]_0$ and should be considered a potential-dependent process. Similarly, after maximum K-contractures, the immediate changes in the rate of relaxation which are produced by sudden alterations in $[Ca]_0$ or

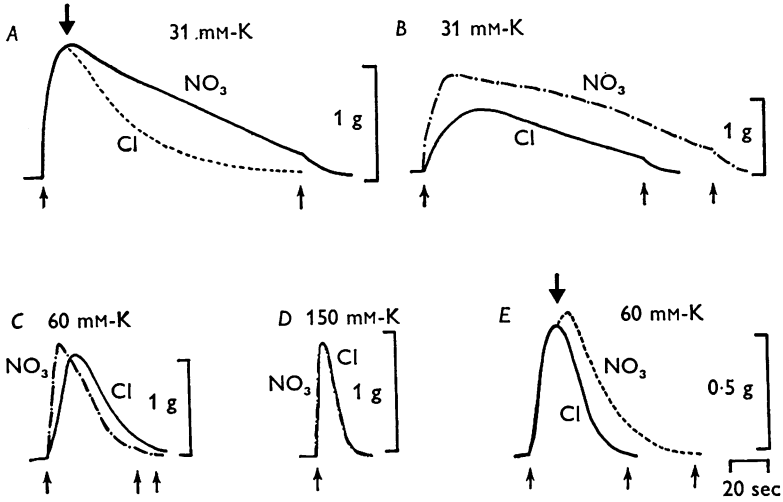

Fig. 10. *A*: maximum K-contractures (31 mM) in frog toe muscle after 30 min exposure to nitrate (continuous line) and the acceleration of relaxation (interrupted line) upon abrupt replacement of external nitrate with chloride (↓) when peak tension was reached. *B–D*: superimposed tracings of contractures produced by the same $[K]_0$ in the presence of chloride (continuous lines) and following 2 min exposure to nitrate (interrupted lines). $[K]_0$ used to produce contractures was: *B*, 31 mM; *C*, 60 mM; *D*, 150 mM. *E*: resumption of tension development and delayed onset of relaxation (interrupted line) during a maximum K-contracture (60 mM) upon substitution of nitrate for chloride when peak tension was reached (↓). The rate of relaxation in nitrate (interrupted line) resembled that of similar contractures which remained in chloride-containing media until tension completely subsided (continuous line).

by abrupt replacement of one external anion with another appear to be primarily the result of changes in the rate of calcium removal rather than in the rate of calcium release. These effects can be explained most readily as the result of changes in the position of the relaxation rate curve with respect to the membrane potential axis (Figs. 6, 11). Thus, slowing of relaxation after maximum K-contractures can be accomplished by a shift either in the level of membrane potential, or in the relation between membrane potential and the potential-dependent calcium removal process which is responsible for rapid relaxation.

Several processes occurring in the membranes of excitable cells are known to be potential-dependent. These include the processes responsible for the rising and falling phase of the action potential (increase in sodium conductance (G_{Na}), reduction in G_{Na}, and increase in G_K) in squid axon (Hodgkin & Huxley, 1952) as well as the processes in frog muscle which are responsible for the activation of contraction and the restoration of contracture capacity (Hodgkin & Horowicz, 1960*a*). Influences which

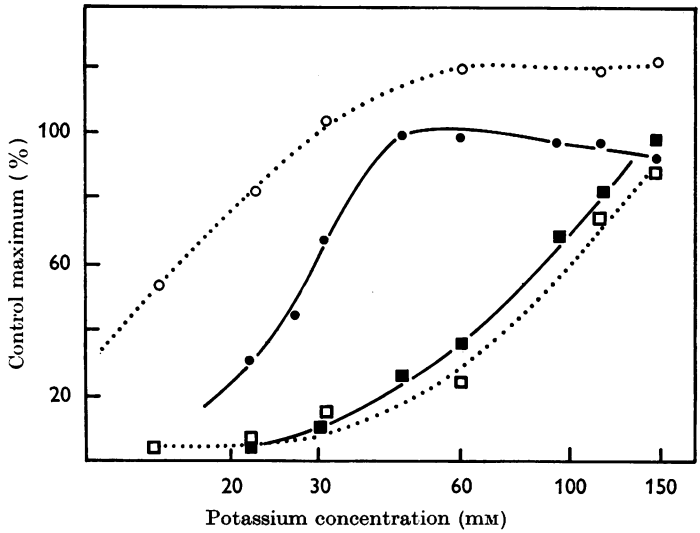

Fig. 11. The effect of replacement of external chloride with nitrate on the relation between log $[K]_0$ and (1) contracture tension (●, ○) and (2) rate of contracture relaxation (■, □), in frog toe muscle, both being expressed as per cent of maximum values observed during control contractures in chloride-containing media. In the presence of nitrate, the contracture tension curve (○) is shifted to lower $[K]_0$ than the corresponding curve in chloride-containing media (●). However, in nitrate-containing media, the curve describing the rate of contracture relaxation (□) either remained unchanged or shifted to slightly higher $[K]_0$ than the corresponding curve in media containing chloride (■). In this experiment, exposure to nitrate began 2 min before the onset of each contracture induction and continued until relaxation was complete.

modify one of these processes may change others. For example, changes in $[Ca]_0$ alter the potential level at which rapid increases in G_{Na} take place in muscle (Ishiko & Sato, 1957) and in nerve (Frankenhaeuser & Hodgkin, 1957) and also alter the relation between membrane potential and contracture activation and re-activation (Luttgau, 1963) as well as between log $[K]_0$ and relaxation rate.

It seems reasonable to assume that potential-dependent processes, which generally respond quite promptly to alterations in the composition of the external medium, are located in the surface membrane or its

internal appendages. In twitch muscles, the transverse tubular system has been shown to be a continuation of the sarcolemma (H. E. Huxley, 1964; Franzini-Armstrong & Porter, 1964; Endo, 1964), penetrating throughout the fibre and encircling each myofibrillar sarcomere. The rapid removal of added calcium in denuded segments of muscle fibre (Podolsky & Constantin, 1964) may represent binding by the transverse tubules whose membranes must be in a non-polarized (depolarized) state under these experimental conditions. Our observations suggest that if an energy-using pump plays a significant role in the rapid calcium removal process which is responsible for relaxation of muscle twitches, this pump also must be potential-dependent and hence may be situated in the walls of the transverse tubules or in the surface membrane. If the longitudinal components of the sarcoplasmic reticulum participate in this process, their activity must be under the control of the transverse tubules and the membrane potential across them. The special structural arrangements at the point of contact of these two structures to form dyads and triads may furnish a basis for such control (Franzini–Armstrong, 1964). Alternatively, the calcium pump may play a subsidiary role, maintaining a generally low intracellular calcium ion concentration, whereas rapid relaxation may depend entirely upon the potential-dependent binding properties of the membranes lining the fibre surface and the transverse tubular system. The possibility that the transverse tubules are the major site of calcium binding during persistent depolarization is suggested by the marked retardation of relaxation after K-contractures in slow (tonic) muscle fibres (Kuffler & Vaughan Williams, 1953) which have a poorly developed transverse tubular system (Peachey & Huxley, 1962).

The rate of calcium removal from the sarcoplasm may depend upon the number of available sites in these membranes at which calcium ions can be bound. After maximum K-contractures, the rapidity of relaxation at the extremes of $[K]_0$ as compared with the marked slowing of relaxation at intermediate $[K]_0$ (Figs. 1, 2, 3) suggests that two discrete sorts of calcium binding sites may be involved, the availability of one site being favoured by polarization of the membrane, while the other site becomes more abundant or accessible as the result of depolarization. This idea is further strengthened by the relation between the degree to which $[K]_0$ is lowered after maximum K-contractures and the distribution of relaxation between two phases, one rapid and the other slow (Figs. 1, 2, 3). The observed patterns of relaxation suggest that as the membrane potential is returned toward its resting level, increasing numbers of sites which can bind calcium rapidly are made available (first phase), whereas the sites at which calcium was rapidly bound at lower membrane potentials undergo progressive impairment in their calcium binding ability (second phase) and are finally

eliminated. This could account for the progressive reduction in the proportion of relaxation taking place during the slow phase (Fig. 2*B*), as well as for the progressive reduction in the rate of relaxation during this phase (Figs. 1, 2*A*, 3*B*), as $[K]_0$ is lowered. The curve which relates log $[K]_0$ to the proportion of relaxation taking place in the early faster phase at 20–40 mM-$[K]_0$ (Fig. 2*B*) is similar in shape and position to the curve relating log $[K]_0$ to the restoration of contracture capacity (Hodgkin & Horowicz, 1960*a*). Both curves presumably represent different aspects of the same process—the return of calcium from the sarcoplasm to sites from which it can again be released to activate contraction upon subsequent depolarization. Another process, or another type of calcium binding site, seems necessary to explain the progressive increase in relaxation rate with rising $[K]_0$, a process which is represented by the slower late phase of relaxation in the 20–40 mM range of $[K]_0$. It seems possible that the calcium-binding sites which remove calcium from the sarcoplasm when the membrane is adequately polarized release their bound calcium as the membrane potential is lowered, and after sufficient depolarization are converted into sites which can again bind calcium. If this view is correct, these sites seem to be restored to their repolarized state without release of the calcium bound while depolarized. This hypothesis could explain the apparent inverse relation between the degree of availability of the two proposed kinds of calcium binding sites. If this interpretation is correct, fewer calcium-binding sites will be available after smaller depolarizations, when the amount of calcium released is small. Some such device seems necessary to explain the fact that just perceptible K-contractures at near-threshold $[K]_0$ develop tension and relax at a very slower rate, whereas a return to this same $[K]_0$, after the rapid and complete release of calcium at a higher initial $[K]_0$, permits a large proportion of relaxation to take place much more quickly (Figs. 1, 2*B*, 3*B*). Apparently an appreciable potential gap must be transversed for the conversion of one type of calcium-binding site to the other during depolarization. This gap is increased or unchanged by increased $[Ca]_0$, and markedly widened when external chloride is replaced with somewhat more polar anions such as nitrate.

These findings support the view (Sandow, 1964; Sandow & Preiser, 1964; Sandow *et al.* 1964) that sufficiently retarded repolarization during the terminal phase of the action potential contributes to prolongation of the contractile state in potentiated twitches. However, our observations suggest that this effect may not result merely from a longer period during which the membrane is depolarized past the 'mechanical threshold' (Sandow, Taylor & Preiser, 1965), but is the result of a more protracted interval during which the membrane potential is at an intermediate level where the removal of calcium from the sarcoplasm is retarded.

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