INTERNAL CITRATE IONS REDUCE THE MEMBRANE POTENTIAL FOR CONTRACTION THRESHOLD IN MAMMALIAN SKELETAL MUSCLE FIBERS

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ABSTRACT An effect of internal citrate ions on excitation-contraction coupling in skeletal muscle is described. The threshold for contraction was measured in rat extensor *digitorum longus*, (EDL), and soleus muscle fibers using a two microelectrode voltage clamp technique with either KCl-filled or K_3 citrate-filled current electrodes. Contraction thresholds were stable for many minutes with KCl current electrodes. In contrast, thresholds fell progressively towards the resting membrane potential, by as much as -15 mV over a period of 10 to 20 min of voltage–clamp with citrate current electrodes. In addition, prepulse inhibition was suppressed, subthreshold activation enhanced and steady-state inactivation shifted to more negative potentials. Fibers recovered slowly from these effects when the citrate electrode was withdrawn and replaced with a KCl electrode. The changes in contraction threshold suggest that citrate ions act on the muscle activation system at an intracellular site, since the citrate permeability of the surface membrane is probably very low. An internal citrate concentration of 5 mM was calculated to result from citrate diffusion out of the microelectrode into the recording area for 20 min. 5 mM citrate added to an artificial cell lowered the free calcium concentration from 240 to 31 μM. It is suggested that citrate modifies excitation-contraction coupling either by acting upon an anion-dependent step in activation or by reducing the free calcium and/or free magnesium concentration in the myoplasm.

INTRODUCTION

The aim of this investigation was to discover how the threshold for contraction was altered when citrate-filled current electrodes were used to voltage-clamp skeletal muscle fibers. Contraction threshold has been found to be unstable in the presence of citrate electrodes (Adrian et al., 1969; Dulhunty, 1982) and this could have been due either to the current-passing properties of the electrodes or to an effect of citrate ions on excitation-contraction coupling. During impalement, potassium and citrate ions enter fibers by diffusion of potassium citrate from the low resistance current electrodes (Fromm and Schultz, 1981) and by ion injection during the passage of current. Therefore citrate could alter intracellular steps in muscle activation. There are well documented cases of foreign anions potentiating contraction in skeletal muscle. Nitrate (Hodgkin and Horowicz, 1960) and perchlorate (Foulks et al., 1973; Gomolla et al., 1983) ions in the external media shift the threshold for contraction to more negative potentials and two anions with high calcium affinities, EGTA (Luttgau et al., 1985; Luttgau et al., 1987) and Fura2 (Hollingworth and Baylor, 1987), potentiate excitation-contraction coupling when applied internally.

Reproducible and reversible changes in contraction threshold in the presence of citrate-filled electrodes are reported in this paper. The experiments show that citrate can be added to the list of foreign anions which shift skeletal muscle activation to more negative potentials. Citrate may act either at an intracellular anion-sensitive step, a calcium-sensitive step, or a magnesium-sensitive step in excitation-contraction coupling.

METHODS

Biological Preparations and Solutions

The experiments were done on extensor digitorum longus (EDL) and soleus muscles from male wistar rats (240–280 gm body weight). The muscles were dissected into thin sheets of fibers, two to five fibers thick, for adequate visibility. The preparation was stretched at 1.2 times rest length across a perspex bridge for support during microelectrode penetration. The fibers were bathed in a Krebs solution containing (mM): NaCl 120; KCl 3.5 mM; $CaCl_2$ 2.5; $MgCl_2$ 1.0; $NaHCO_3$ 25; glucose 11; and TES (N-tris-[hyroxy-methyl]-methyl-2-aminoethanesulphonic acid), 2. The solution was bubbled with 95% O_2 and 5% CO_2 (pH = 7.4) and maintained at 30°C. The low chloride solution used in some experiments contained (millimolar): NaCl 16, Na_2SO_4 32.25; K_2SO_4 1.75; $MgSO_4$ 1; $CaSO_4$ 7.6; sucrose, 170, glucose, 11; and TES 2 (pH = 7.4). All solutions contained tetrodotoxin 2×10^{-6} M to prevent action potentials. Temperature was controlled by a water jacket surrounding the tissue bath and was monitored by an immersible thermistor probe. A Radiometer (ion 83) ion

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meter was used to measure free calcium ion concentrations in the 120 mM potassium propionate solutions used to mimic an intracellular solution.

Microelectrodes and Voltage Clamp

Glass microelectrodes filled with 2.5 M KCl (resistances of 2 to 5 MΩ) were used to monitor membrane potential and electrodes filled with 2.5 M KCl, 2.5 M K_3 citrate, or 0.83 M K_3 citrate (resistances = 1 to 3 MΩ) were used to pass current. Details of the recording setup have been described (Dulhunty, 1982). Briefly, the microelectrodes were inserted into opposite edges of a fiber with a separation of 0.8 to 1.0 times the fiber diameter (50 to 80 μm). This arrangement was chosen to minimise errors from nonuniformities of potential around the current electrode (Eisenberg and Johnson, 1970). Membrane potential was recorded as the difference between the intracellular voltage electrode and a reference (KCl/agar) electrode located in the solution within 0.5 cm of the intracellular electrode. A conventional voltage-clamp circuit was used to control potential.

Determination of the Membrane Potential for Contraction Threshold

The term "membrane potential for contraction threshold" will be abbreviated to "contraction threshold" in the remainder of the paper. The methods used to determine contraction threshold were essentially the same as those described by Adrian et al. (1969) except that the fibers were observed on a videomonitor attached to a dissecting microscope with a final magnification of ×500. Contraction was observed as movement of the edge of the fiber opposite the current electrode. A depolarizing test pulse was increased in 2 mV steps until movement of the fiber edge was detected. Then the pulse was reduced in 0.2 mV steps until contraction was no longer visible. The membrane potential during the pulse to just below visible movement was recorded and referred to as "contraction threshold." Problems associated with the control of membrane potential in the two microelectrode voltage clamp situation are discussed fully elsewhere (Dulhunty, 1982).

Two Pulse Experiments

The two pulse protocols used to measure subthreshold activation, prepulse inhibition, and steady-state inactivation have been described before (Dulhunty, 1982). The pulse protocols are summarised schematically in Figs. 2 and 4 in the Results section and the terms "subthreshold activation," "prepulse inhibition," and "steady-state inactivation" are defined.

RESULTS

The use of citrate-filled current electrodes led to time-dependent changes in the four parameters of contractile activation that were measured using the two microelectrode voltage clamp, i.e., contraction threshold, prepulse inhibition, subthreshold activation, and steady-state inactivation.

Effect of the Current Electrode on Contraction Threshold

The contents of current electrodes were varied between 2.5 M KCl, 2.5 M K_3 citrate, and 0.83 K_3 citrate. The term "citrate electrode" refers to the 2.5 M K_3 citrate-filled electrodes. Voltage electrodes were filled with 2.5 M KCl in all experiments.

Contraction threshold was stable for many minutes with KCl current electrodes as illustrated with the two fibers in Fig. 1 *A* (filled symbols). Similar stable thresholds with KCl current electrodes have been routinely obtained in many hundreds of EDL, soleus and sternomastoid fibers over the past few years. In contrast the threshold fell progressively in all fibers voltage clamped with citrate electrodes (5 EDL and 12 soleus fibers), from values close to those recorded with KCl electrodes, to values closer to the resting membrane potential. The threshold fell by −12 mV in the EDL fiber (squares), and by −15 mV in the soleus fiber (circles), shown in Fig. 1 *A*. As previously reported (Dulhunty and Gage, 1983), contraction thresholds in the soleus fibers were closer to the resting membrane potential than in EDL and this relationship was not influenced by the type of current electrode used in the experiment.

Contraction threshold was recorded in a single soleus fiber when the current electrode was changed from a KCl electrode to a citrate electrode and then back to a KCl electrode (Fig. 1 *B*). There was a progressive fall in contraction threshold of −12 mV during the first 10 min

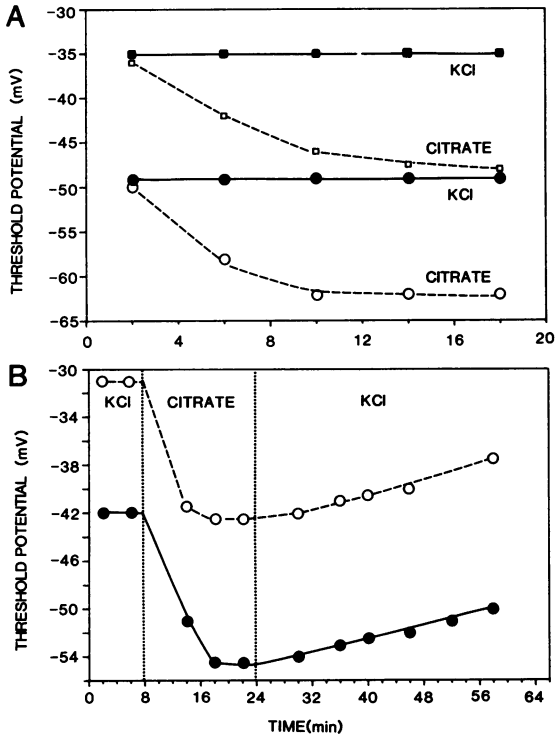

FIGURE 1 Contraction thresholds recorded in individual fibers using KCl-filled or citrate-filled microelectrodes. Contraction threshold in mV is plotted as a function of time in minutes. *A* shows contraction threshold for 10 ms test pulses in two EDL (*squares*) and two soleus fibers (*circles*). The data shown with filled symbols was obtained with KCl-filled electrodes and the open symbols show data obtained with citrate-filled electrodes. *B* shows contraction thresholds measured in one soleus fiber with 2 ms (*open circles*) and 10 ms (*filled circles*) test pulses. The current electrode was changed from a KCl-filled to a citrate-filled electrode at the first vertical dotted line and back to a KCl-filled electrode at the second vertical dotted line. The holding potential for fibers in *A* and *B* was −80 mV.

after insertion of the citrate electrode. The threshold initially remained at this negative value when the citrate electrode was removed and then gradually recovered towards its original value. Immediate recovery would have been expected if the fall in threshold had been due to rectification in the citrate current electrode.

Two facts suggest that the fibers were perturbed by the citrate electrodes rather than the KCl electrodes: (*a*) contraction thresholds measured with KCl electrodes were similar to potassium contracture thresholds (Dulhunty, 1980; Dulhunty and Gage, 1983) and (*b*) the changes in threshold with citrate electrodes were progressive. The results could be explained by citrate diffusing into the fiber from the microelectrode (Fromm and Schultz, 1981). Diffusion of citrate away from the recording site would allow recovery when the electrode was removed.

The Effect of Citrate Electrodes Was Not Due to Potassium Accumulation

Since the concentration of potassium ions in the citrate-filled electrode was three times greater than the concentration in the KCl-filled electrode it was possible that the effects of the electrode were due to the intracellular accumulation of potassium ions rather than citrate ions. Electrodes filled with 0.83 mM K_3 citrate (i.e., with a potassium ion concentration of 2.5 mM, equivalent to that in the KCl-filled electrode) were used to voltage clamp 5 soleus fibers and there was a fall in the average contraction threshold of 4.5 mV ± 0.78 mV after 10 min. Therefore the effects of the K_3 citrate electrode can be attributed entirely to an effect of citrate ions.

Effect of the Current Electrode on Resting Membrane Potential

An increase in internal potassium ion concentration would hyperpolarize the muscle fibers. However, the change in membrane potential with a realistic increase in internal potassium of 14 mM (calculated to result from K_3 citrate diffusion into the fiber, see below) would be very small in the solutions used in these experiments, where chloride conductance is high (Dulhunty, 1978). A 14 mM increase in internal potassium concentration was calculated to hyperpolarize the fiber by only 0.5 mV, using a Goldman, Hodgkin, and Katz equation with constants derived for mammalian skeletal muscle (Dulhunty, 1979). Indeed, in the experiment shown in Fig. 1 *B*, the resting membrane potential was −59 mV when the electrode was withdrawn after the first impalement with a KCl electrode and −57 mV when the citrate electrode was withdrawn.

In separate experiments, membrane potential was measured in a low chloride solution, and consequently reduced chloride conductance. In this situation a 14 mM increase internal potassium concentration was calculated to produce a 2.0 mV hyperpolarization. 11 fibers were impaled with citrate electrodes and the resting membrane potential followed for 20 min. The average resting potential was −80.2 ± 2.1 mV at the start of recording and −79.5 ± 2.9 mV after 20 min. Therefore, either the hyperpolarization due to increased potassium concentration was too small to record, or potassium accumulation was minimal because potassium diffused out of the fiber across the surface and T-tubule membranes. The surface membrane potassium permeability is high in skeletal muscle (Hodgkin and Horowicz, 1959), in both the external and T-tubule membranes (Eisenberg and Gage, 1969).

Prepulse Effects on Contraction Threshold are Modified by Citrate Electrodes

Contraction threshold is altered by just subthreshold prepulses (Adrian et al., 1969; Costantin, 1974) in either an excitatory way by prepulses that are close to contraction threshold, or in an inhibitory way by prepulses that are further from threshold (Dulhunty, 1982). Examples of these phenomena are given in Fig. 2 and the method used to quantitate the effect of prepulses is described.

Prepulses that were very close to contraction threshold produced "subthreshold activation": the prepulse itself did not elicit contraction but had the effect of reducing the depolarization required to reach threshold with the test pulse. The threshold test pulse, P (Fig. 2 *C*), was smaller than the threshold test pulse delivered alone, P_0 (Fig. 2 *A*), and the test pulse ratio, P/P_0, <1. The phenomena was called subthreshold activation because it was assumed that the conditioning pulse released a subthreshold amount of calcium (or other activator substance), some of which remained in the myoplasm (or in an active form) during the test pulse. As a consequence, the total calcium (or activator) concentration after a test pulse of amplitude P exceeded contraction threshold and a smaller test pulse of amplitude P_0 was required to release just threshold amounts of calcium (or activator).

If the duration (or amplitude) of the prepulse was reduced so that it was no longer very close to contraction threshold, but still of significant amplitude and duration (Fig. 2 *B*), the excitatory effect was replaced by "prepulse inhibition" and the threshold test pulse shifted to a more positive potential, i.e., the test pulse ratio used to quantitate the prepulse effects was >1. The mechanism of prepulse inhibition is not understood.

The degree of activation or inhibition varied with the duration (or amplitude) of the prepulse as shown by comparing the graphs of test pulse ratio against prepulse duration in Fig. 2 *D* and Fig. 3. The same effects of prepulses on contraction threshold were seen using 10 ms test pulses (Fig. 2 *A*, *B*, and *C*), or 1 ms test pulses (Fig. 2 *D*), although the threshold potentials were more positive with the briefer test pulse. The actual amplitude and duration of prepulses required to elicit subthreshold activation or inhibition differed in the four fibers described in Figs. 2 and 3 (see Figure legends) and depended on the

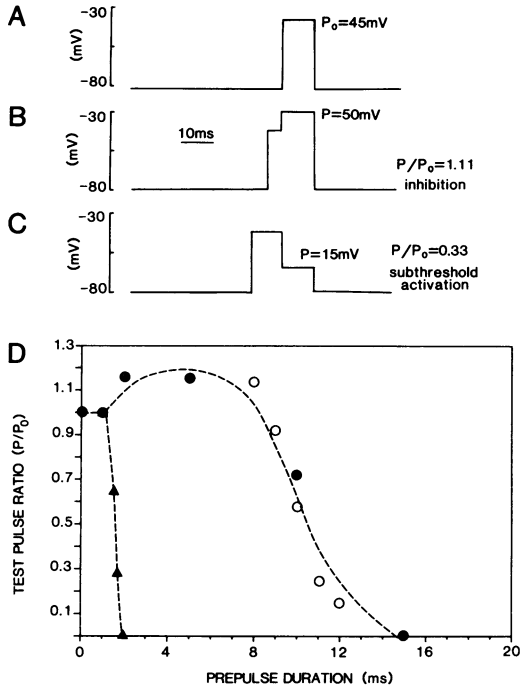

FIGURE 2 Examples of prepulse inhibition and subthreshold activation recorded under voltage clamp with KCl-filled current electrodes and expressed as the ratio P/Po. Po is the threshold amplitude of a test pulse delivered alone (*A*) and P is the amplitude of the test pulse with a conditioning prepulse (*B* and *C*). *A*, *B*, and *C* show pulse patterns that elicited threshold contraction in one EDL fiber: (*A*), a 10 ms test pulse alone elicited contraction with depolarization to a membrane potential, Vm, of −35 mV (Po = 45 mV); (*B*), a 5 ms prepulse of 40 mV (to Vm = −40 mV) caused prepulse inhibition and contraction threshold increased to Vm = −30 mV (P = 50 mV) and the ratio of test pulses, P/Po, was 1.11; (*C*), an increase in prepulse duration to 10 ms caused subthreshold activation and a reduction in contraction threshold to Vm = −65 mV (P = 15 mV) and P/Po = 0.33. Data obtained with a KCl-filled current electrode from a second EDL fiber is shown in *D*. The test pulse ratio (defined in *B* and *C*) has been plotted against prepulse duration (ms). A 1.0 ms test pulse, eliciting contraction at +29 mV (Po = 99 mV), was used. The fiber was held at −80 mV. The circles indicate data obtained with 60 mV prepulses (to Vm = −20 mV). The filled circles show data obtained during the first run and the open circles show the results of a second run, 5 min later. The two sets of data fall about the same broken line drawn through the points. The filled triangles show that increasing the prepulse amplitude to 90 mV (to Vm = +10 mV) caused subthreshold activation without inhibition.

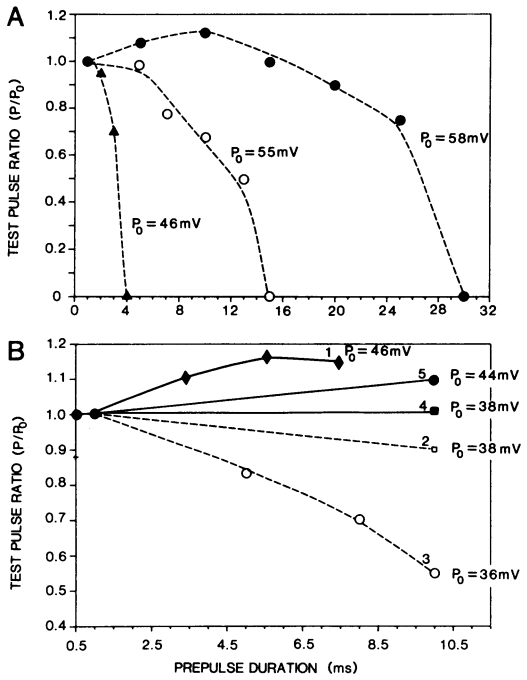

FIGURE 3 The effect of citrate-filled current electrodes on prepulse inhibition and subthreshold activation. Test pulse ratio, P/Po (defined in Fig. 2), has been plotted against prepulse duration (ms). The results in *A* were obtained in an EDL fiber with a citrate-filled current electrode. Results are shown for three runs: the first (*filled circles*) immediately after impalement with a citrate electrode; the second (*open circles*) obtained one minute later and the third (*triangles*) recorded after a further 5 min. The holding potential was −80 mV and the prepulse was 40 mV (to Vm = −40 mV) for each run. A 2 ms test pulse was used and Po measured before each run. Po was: 58 mV (Vm = −22 mV) for run 1; 55 mV (Vm = −25 mV) for run 2; 46 mV (Vm = −34 mV) for run 3. The results in *B* were obtained from a soleus fiber with a holding potential of −80 mV. Run 1 (*diamonds*) was obtained after the initial impalement with a KCl electrode. Run 2 (*open squares*) was obtained 5 min after the fiber had been impaled with a citrate electrode and run 3 (*open circles*) were obtained 6 min later. The prepulse was 18 mV (to Vm = −62 mV) for each run. The test pulse was 2 ms and Po was 46 mV (Vm = −34 mV) for run 1, 38 mV (Vm = −42 mV) for run 2 and 35 mV (Vm = −45 mV) for run 3. The current electrode was then changed to a KCl electrode and the prepulse amplitude maintained at 18 mV. Run 4 (*filled squares*) was recorded after 5 min and Po for the 2 ms test pulse increased to 38 mV (Vm = −42 mV). After a further 15 min Po had increased further to 44 mV (Vm = −36 mV) and run 4 was recorded (*filled circles*).

strength–duration curve for contraction threshold in the individual fibers. Therefore both subthreshold activation and prepulse inhibition occurred with shorter duration, or lower amplitude, prepulses in soleus fibers than in EDL. In all other respects, the results were identical in fibers from EDL and soleus muscles (Dulhunty, 1982) and the examples in Fig. 2 serve as controls for subsequent observations in fibres from both types of muscle.

The relationship between the threshold test pulse amplitude and prepulse duration was constant with time in at least 150 fibers when KCl current electrodes were used and one example is shown in Fig. 2 *D*. In contrast, the relationship changed during the recording period in all 9 fibers examined under voltage clamp with citrate electrodes and the curves in Fig. 3 *A* show progressive abolition of inhibition and shifts in subthreshold activation to shorter prepulse durations. Curves 2 and 3 in Fig. 3 *B* were also obtained with a citrate electrode and show that subthreshold activation was stronger in a second run (curve 3). The citrate effect on the fiber in Fig. 3 *B* was reversible when the electrode was replaced with a KCl electrode: subthreshold activation was first abolished (curve 4) and then replaced by inhibition (curve 5) as the fiber recovered towards the control situation (curve 1). The effects of citrate on inhibition or subthreshold activation were the same as the effect of increasing prepulse amplitude (as

shown in Fig. 2 *D*, filled triangles), or progressively reducing contraction threshold, so that the prepulse was closer to contraction threshold.

The contraction thresholds (P_0 values) in Fig. 3 show that there was a parallel effect of the citrate electrode on contraction threshold and subthreshold activation or prepulse inhibition: as contraction threshold became more negative, prepulse inhibition became less pronounced and subthreshold activation more pronounced. A deviation from this parallel relationship is seen in curves 2 and 4 (Fig. 3 *B*) which differ slightly despite the identical P_0 values in both runs. Since curve 2 was recorded about 15 min before curve 4, the difference can probably be attributed to a small time-dependent change in the fiber.

Effect of Citrate Ions on Contractile Inactivation

Inactivation (Fig. 4 *B*) differs from inhibition in that it is a steady-state phenomenon which continues for as long as the fiber is depolarized. Contraction threshold changed after a change in holding potential and reached a steady value after 2 to 3 min. The contraction thresholds shown in Fig. 4 *C* were recorded between 3 and 5 min after establishment of the holding potential shown on the horizontal axis. A sharp increase in threshold, as a result of partial inactivation, is apparent at more positive holding potentials. The average increase in threshold occurred at −30 mV in 6 soleus fibers voltage-clamped with KCl electrodes (filled circles, Fig. 4 *C*) and at −50 mV in 9 soleus fibers clamped with citrate current electrodes (open circles). The citrate results were obtained 20 min after impalement with the citrate electrode, at a time when the control threshold at a holding potential of −80 mV had reached a constant value (see results above).

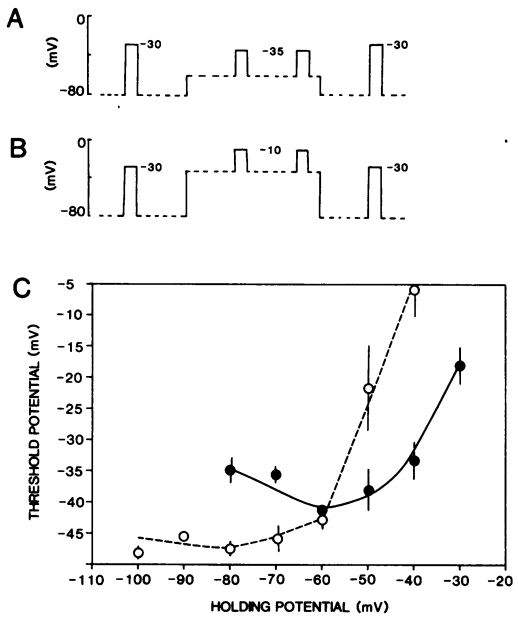

FIGURE 4 The effect of a citrate-filled current electrode on steady-state inactivation in soleus fibers. *A* and *B* illustrate the effect of partial inactivation on the threshold for contraction measured with a brief (2 ms) test pulse. The single arrows indicate test pulses and the double arrows indicate changes in holding potential. In *A*, contraction was elicited with a 50 mV test pulse (to Vm = −30 mV) when the holding potential was −80 mV. Reduction in the holding potential to −60 mV for 5 min did not produce inactivation, in fact there was a slight decrease in threshold to V_m = −35 mV. A further reduction in the holding potential to −40 mV for 3 min (shown in *B*) did produce inactivation and the threshold increased to Vm = −10 mV. Average data for inactivation measured in the same way is shown in *C*. The filled circles show data obtained with KCl-filled current electrodes from 6 soleus fibers and the open circles show data obtained 20 min after impalement with citrate-filled current electrodes in 9 soleus fibers. The vertical bars indicate ±1 sem where this is greater than the dimensions of the symbol.

Predicted Internal Concentration of Citrate and Potassium Ions

A rough estimate of the internal concentration of citrate and potassium ions after a period of impalement with a K_3 citrate electrode can be made by assuming that the ions diffuse from the electrode into a given volume of the fiber immediately around the electrode. This calculation is obviously only approximate because the true concentration would be reduced by ion diffusion away from the recording site and across the surface membrane in the case of potassium ions, and would be enhanced by iontophoresis during voltage clamp. The concentration of K_3 citrate in a fiber 20 min after insertion of a citrate-filled microelectrode was calculated to be of the order of 5 to 10 mM by extrapolation of data given by Fromm and Schultz (1981). Diffusion of K_3 citrate from a 1 to 3 MΩ microelectrode is 15 fmol/s i.e., 1.8×10^{-11} mol in 20 min into 3.8 nl (in a 100 μm length of an 70 μm fiber), giving a concentration of 4.7 mM of citrate ions and 14.1 mM of potassium ions. These numbers probably (*a*) underestimate the citrate concentration since hyperpolarizing holding currents were routinely used and additional citrate would enter by iontophoresis and (*b*) overestimate the potassium concentration since the surface and T-tubule membranes are permeable to potassium (Gage and Eisenberg, 1969) but not citrate (see Discussion) so that the potassium concentration would be selectively reduced by diffusion out of the cell.

The Effect of Citrate on Free Calcium Ion Concentrations

The affinity constants of citrate for calcium ions, determined under a variety of conditions (Sillen and Martell, 1964) suggest that citrate should reduce the free calcium ion concentration in the myoplasm. However the affinity constants vary considerably depending on the conditions of measurement. Therefore the reduction in free calcium concentration was measured in a "myoplasmic-like" potassium propionate solution (Table I). 5 mM citrate reduced the free calcium concentration from 240 to 31 μM.

1 mM $CaCl_2$ added to the propionate solution resulted in 240 μM-free Ca^{2+}, an amount equivalent to the 200

TABLE I
THE EFFECT OF K CITRATE ON THE FREE CALCIUM CONCENTRATION IN AN ARTIFICIAL CELL CONTAINING 120 mM K PROPIONATE AND 1 mM ADDED $CaCl_2$

K-citrate (mM)	0	0.5	1.0	5.0	10.0
[Ca] (μM)	240	200	150	31	15

μM-free calcium that can be calculated to surround the terminal cisternae during threshold activation, assuming that (*a*) the threshold concentration of free calcium ions in the myofilament space is 1 μM (Stephenson and Williams, 1981) and (*b*) calcium is released into 0.5% of the fiber volume (surrounding the terminal cisternae) before it diffuses into the myofilaments (which occupy 85% of the volume, Eisenberg and Kuda, 1975). For simplicity the calculation ignores the fact that calcium binds to myplasmic buffers and is thus most accurate for soleus fibers which contain essentially no parvalbumin (Heizmann et al., 1982). However the calculation probably underestimates the concentration of calcium that must be released to raise the free calcium in the myofilament space to 1 μM. The conclusion of the experiment remains the same since citrate has been shown to be effective in reducing free calcium and magnesium ion concentrations when the cations are present at 1 to 10 mM range (Hastings et al., 1934).

It is clear that citrate at 0.5 to 10 mM would reduce the free calcium concentration in the myoplasm of resting fibers and during activation.

DISCUSSION

The changes in contraction threshold, subthreshold activation, and prepulse inhibition observed during voltage clamp with citrate-filled current electrodes suggest that internal citrate ions shift the voltage dependence of excitation-contraction coupling to more negative membrane potentials. This action cannot be attributed to a simple change in surface charge since addition of citrate and/or removal of calcium from the inside of the surface membrane would effectively hyperpolarize the membrane and cause an apparent shift in the opposite direction, to more positive potentials.

The effects of citrate are unlikely to be caused by changes in the calcium affinity of the contractile proteins since organic anions are largely excluded from the myofilament lattice (Stephenson et al., 1981). If citrate is excluded from the myofilament space, which occupies 85% of the fibers' volume (Eisenberg and Kuda, 1975), the true concentration of citrate in areas involved in excitation-contraction coupling could be considerably greater than the 5 mM calculated assuming that the ions were uniformly distributed throughout the myoplasm.

It has been assumed that citrate acts at an intracellular site. Although the author could find no reference to the citrate permeability of the surface membrane in vertebrate skeletal muscle, other similar organic anions of lower molecular weight, lactate, and acetate are essentially impermeable (Woodbury and Miles, 1973) and the surface membrane of crustacean muscle fibers is relatively impermeant to internally perfused citrate ions (Lakshminarayanaiah and Rojas, 1973).

Citrate could act on an anion-sensitive step in excitation-contraction coupling. Evidence for such a step is provided by the effects of foreign anions on contraction, but it is not clear (*a*) whether the anions act on a single site and (*b*) whether the site, or sites, are external or internal. Nitrate in the external medium potentiates contraction (Hodgkin and Horowicz, 1960), possibly through an effect on external surface charge. Perchlorate ions on either side of the membrane potentiate contraction and shift the voltage dependence of asymmetric charge movement, a step in muscle activation (Schneider and Chandler, 1973), to more negative potentials (Luttgau et al., 1983). The impermeant anions EGTA and Fura2 act at intracellular sites (Luttgau et al., 1985; Hollingworth and Baylor, 1987) without altering charge movement (Horowicz and Schneider, 1981). It is likely that citrate acts at the same site as EGTA and Fura2.

It is interesting to speculate about the internal anion-sensitive step in excitation-contraction coupling. One possible site might be the sarcoplasmic reticulum membrane if charge transfer during calcium release from the sarcoplasmic reticulum involves an anion flux. Chloride ions might be involved in an anion flux (Somlyo et al., 1981) but other anions must be equally effective since contraction in mammals and amphibia is unaffected by reduced external chloride concentrations. The chloride permeability in the surface membrane is high (Hodgkin and Horowicz, 1959; Eisenberg and Gage, 1969; Palade and Barchi, 1977; Dulhunty, 1978) and low external chloride concentrations lead to low internal chloride, even in mammals, where the chloride pump is more active (Dulhunty, 1978). Hydrogen/hydroxyl ion fluxes may be equally important and interchangeable with chloride fluxes (Baylor et al., 1987). An anion channel in the sarcoplasmic reticulum could be modified by foreign anions such as EGTA, Fura2, or citrate.

An alternative explanation for the potentiating effects of EGTA, Fura2, and citrate is that they act via their ability to reduce myoplasmic calcium ion concentrations. Indeed, it has been suggested that EGTA and Fura2 potentiate contraction by reducing the effect of a "calcium inhibition of calcium release" (Luttgau et al., 1985, 1987; Hollingworth and Baylor, 1987). Other evidence suggests that calcium can block calcium release: (*a*) Mezler et al. (1984) describe an early inhibition of calcium release which is faster when calcium release is greater; (*b*) prepulse inhibition (Dulhunty, 1982) could be explained if subthreshold amounts of calcium released by the prepulse inhibited contraction and (*c*) reduced extracellular calcium concen-

trations cause an increase in twitch and submaximal potassium contracture tension (Dulhunty and Gage, 1988). Calcium inhibition of calcium release has not been reported in skinned muscle fibers and is therefore associated with the normal sequence of depolarization-induced excitation-contraction coupling. Calcium inactivation of calcium release is in apparent conflict with the idea of calcium-activated calcium release seen in skinned muscle fibers (Endo, 1977; Stephenson, 1981) but the two phenomena could well reflect the dependence of different steps in the activation sequence on internal calcium ion concentration.

The shift in contraction to more negative membrane potentials caused by reductions in external calcium concentration can be explained in terms of a simple model in which calcium ions must be removed from a voltage activated molecule before activation can proceed and calcium must be replaced before the molecule can be reactivated (Dulhunty and Gage, 1987). The same model could be used to explain the effects of internal EGTA, Fura2, and citrate if the voltage-sensitive molecule were influenced by bulk internal as well as external calcium concentrations. This could happen if, for example, the voltage sensor was in contact with a calcium pool close to the T-tubule membrane that was isolated from the extracellular space but replenished from the bulk solution by restricted diffusion, and from the myoplasm by calcium pump activity (Malouf and Meissner, 1979). Depletion of the pool calcium concentration after depletion of either source would facilitate the calcium dissociation necessary for activation.

Finally citrate could act by reducing the internal magnesium concentration, if normal excitation-contraction coupling depends on a calcium activated calcium release mechanism (Endo, 1977). The affinity constants of citrate for calcium and magnesium are similar (Sillen and Martell, 1969) and Endo (1975) has shown that a 10-fold reduction in free magnesium concentration causes a 10-fold reduction in the threshold calcium concentration needed to induce calcium activated calcium release. Endo (1977) suggested that the minimal effective concentration of calcium is proportional to the level of free magnesium. Both EGTA and Fura2 (Ashely and Moisescu, 1977; Grynkiewicz et al., 1985) have relatively low affinities for magnesium ions and it is unlikely that their actions can be attributed to a reduction in magnesium concentration. If the actions of the three anions depend on a common mechanism then the mechanism is probably not a reduction in magnesium concentration.

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