J. Membrane Biol. 138, 197–207 (1994)

The Journal of
Membrane
Biology

Effects of Membrane Potential on Just Detectable Movement in Rat Skeletal Muscle: Effects of Denervation

M.F. Patterson, A.F. Dulhunty
The Muscle Research Group, JCSMR, Australian National University, P.O. Box 334, Canberra, ACT 2601, Australia

Received: 12 July 1993/Revised: 19 November 1993

Abstract. The potential, *Vt*, at which a brief test depolarization first elicited movement was determined using two-microelectrode point voltage clamp. We expected that inactivation of excitation-contraction coupling at conditioning potentials between −60 and 0 mV would shift *Vt* to more positive potentials, and that fibers would become inactivatable with less conditioning depolarization in EDL than soleus. The curve relating *Vt* to conditioning potential had a negative slope (which was insensitive to addition of 1 mM cobalt or replacement of calcium with 20 mM CaEGTA) between −60 and −35 mV and a steep positive slope with further depolarization. Unexpectedly, fibers became inactivatable with less conditioning depolarization in soleus than in EDL when *Vt* was measured with 50 msec test pulses. However, the positive shift in *Vt* became less steep as test pulse duration lengthened in soleus fibers. When *Vt* obtained with test pulses approaching rheobase (10 msec in EDL and 500 msec in soleus) was compared, EDL fibers became inactive with less conditioning depolarization than soleus fibers. The increase in *Vt* became steeper with 1 mM cobalt or 20 mM CaEGTA and was shifted to more positive potentials by denervation in soleus fibers. We conclude that inactivation (i) does not strongly influence threshold contractions at conditioning potentials between −60 and −40 mV and (ii) influences *Vt* between −40 and 0 mV in a manner that depends on test pulse duration.

Key words: Membrane potential — Movement — Excitation-contraction coupling — Rat skeletal muscle

Correspondence to: A.F. Dulhunty

Introduction

The aim of this investigation was to characterize the effects of conditioning depolarization on small contractions in rat EDL and soleus muscle fibers. Excitation-contraction coupling in skeletal muscle normally occurs when action potentials depolarize the transverse (T) tubule membrane and activate the voltage-sensitive molecules that in turn "gate" ryanodine-receptor calcium release channels in the sarcoplasmic reticulum membrane. The relationship between tension and membrane potential reflects the way in which the voltage-sensor responds to depolarization (*see* Dulhunty, 1992, for review). The effect of conditioning depolarization on contraction provides insight into the state of voltage-sensor and excitation-contraction coupling during membrane depolarization which could occur in damaged muscle fibers, in hyperkalemia, or with potassium accumulation in the T-tubules during repetitive activity (Freygang et al., 1964; Gage & Eisenberg, 1969; Lannergren & Westerblad, 1986).

We have used a two-microelectrode point voltage clamp technique, with visual determination of movement (Adrian, Chandler & Hodgkin, 1969; Costantin, 1974; Chua & Dulhunty, 1988) to measure the most negative membrane potential, *Vt*, at which a brief (10 to 500 msec) test pulse could elicit movement. Since inactivation of excitation-contraction coupling occurs during prolonged depolarization, one aim of the experiments was to examine the effect of inactivation on *Vt*. Maximal test potassium (K^+) contracture tension is reduced as a result of inactivation (Hodgkin & Horowicz, 1960) and this occurs with less conditioning depolarization in fast-twitch *extensor digitorum longus* (EDL)

fibers than in slow-twitch soleus fibers (Chua & Dulhunty, 1988). A starting hypothesis was developed which predicted that inactivation would affect *Vt* in much the same way as it affected maximum 200 mM K^+ contracture tension. Specific predictions of the hypothesis (*see* Hypotheses below) were that (i) *Vt* would become more positive during conditioning depolarization to membrane potentials producing partial inactivation of maximal K^+ contracture tension, (ii) it would not be possible to elicit threshold contractions during conditioning depolarizations to membrane potentials producing full inactivation of K^+ contracture tension and (iii) both effects of depolarization on *Vt* would occur at more negative holding potentials in fast-twitch fibers than in slow-twitch fibers.

A second objective of the study was to examine the effect of denervation on *Vt*. Denervation causes a shift to more negative membrane potentials in the voltage dependence of tension in mammalian skeletal muscles (Dulhunty & Gage, 1985) and in *Vt* measured in fast-twitch fibers at normally polarized holding potentials (Patterson & Dulhunty, 1991). There is a small negative shift in the membrane potential for 50% inactivation of test 200 mM K^+ contracture tension following denervation of fast-twitch fibers (M.F. Patterson and A.F. Dulhunty, *unpublished observations*). If the change in the K^+ contracture inactivation curve in EDL was reflected in the relationship between *Vt* and holding potential, *Vt* should increase at more negative holding potentials in denervated EDL fibers.

We avoid the use of the more conventional term "contraction threshold" in this manuscript because many measurements of minimal contractions in response to the test pulse were made at conditioning potentials at which steady-state contractures or pedestal tensions were observed (Chua & Dulhunty, 1988). At these potentials, the test pulse induced an additional contraction, seen as a flicker of movement. Thus, we prefer to define *Vt* as the most negative test potential at which movement could be elicited.

The results confirmed the predicted effect of inactivation on *Vt* for brief test pulses (<100 msec) at conditioning potentials between −40 and 0 mV, but this effect was less when longer test pulse durations (100 and 500 msec) were used. In general, inactivation had much less effect on *Vt* than on maximum K^+ contracture tension: detectable movement could be elicited when normal and denervated fibers were held at membrane potentials at which K^+ contractures are almost fully inactivated. In addition, and contrary to expectations, depolarization influenced *Vt* at more negative membrane potentials in slow-twitch fibers than in fast-twitch fibers when data obtained with brief test pulses of the same duration were compared. It was concluded that changes in contraction threshold do not necessarily reflect changes in the maximum, or near maximum tension generating capacity of the muscle fiber.

Materials and Methods

Biological Preparations

Fast-twitch EDL or sternomastoid muscles and slow-twitch soleus muscles were removed from adult male Wistar rats following asphyxiation with CO_2. The muscles were pinned at rest length in a Sylgard (Dow Corning, Midland, MI)-lined petri dish containing a modified Krebs solution (*see below*). Thin sheets of intact fibers, 3 to 5 fibers thick and 30 to 50 fibers wide were microdissected from the muscles. Rats to be used for denervation surgery were anesthetized with ether and a long (0.5 to 1.0 cm) segment of the sciatic nerve excised just distal to the sciatic notch. The animals were allowed to recover and experiments were carried out on the denervated muscles four to six weeks after surgery. Muscle atrophy and fibrillatory activity, apparent during dissection of denervated muscles, were used as criteria for assessing successful denervation and the lack of regeneration. Dissections and experiments were performed at room temperature, 23 ± 1 °C.

Solutions

The modified Krebs solution contained (mM): Na_2SO_4, 32.25; NaCl, 16; K_2SO_4, 1.75; $CaSO_4$, 7.6; $MgSO_4$, 1; sucrose, 170; glucose, 11; TES (*N*-tris-(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid) buffer, 2 (adjusted to pH 7.4 with NaOH). Sulfate was used as the major anion to allow us to compare the results with data from previous K^+ contracture experiments and to improve voltage clamp control of the T-tubule membrane potential, since removal of chloride ions increases the T-tubule length constant (Dulhunty, Carter & Hinrichsen, 1984). The use of sucrose in the solutions caused swelling of the T-tubules (A.F. Dulhunty, *unpublished observations*) and thus also contributed to an increase in the electrical length constant of the T-tubules.

Tetrodotoxin, 0.125 μM for normal fibers or 5.0 μM for denervated fibers, was added to all solutions to block action potential activity and ensure that contractions were in response to the applied voltage clamp steps. Where specified in Results, cobalt (1 mM) was added to the modified Krebs solution or $CaSO_4$ was replaced with 10 mM CaEGTA.

Microelectrodes

Glass microelectrodes for injecting current and recording voltages were filled with 2.5 M KCl and had resistances of 2 to 5 MΩ. Current and voltage electrodes were inserted into opposite edges of single muscle fibers, the separation between the electrodes being 50 to 100 μm depending on the diameter of the fiber. This configuration of electrodes is thought to minimize the effects of three-dimensional current spread (Eisenberg & Johnson, 1970). Membrane potential was measured as the difference between an intracellular voltage microelectrode and an extracellular reference microelectrode placed close to the intracellular recording site.

The Two-Microelectrode Voltage Clamp Technique

The two-microelectrode voltage clamp technique was essentially the same as that described by Chua and Dulhunty (1988) and was based on the technique originally described by Adrian et al. (1969) and Costantin (1974). It was assumed that there was a minimal decrement of membrane potential between the fiber surface and the T-tubule membrane associated with myofibrils close to the surface. The minimum test pulse duration used was 10 msec, since time delays within the initial few micrometers of the T-system might limit the degree of depolarization of the T-tubule membrane during brief test pulses.

The holding potential was changed, and *Vt* measured, using one of the three different protocols outlined below. In each case *Vt* was initially established at a holding potential of −80 mV, and the data were accepted only if *Vt* was within 1 mV of its original value at the end of the experiment and the holding current at −80 mV was less than 100 nA.

Protocol 1

The holding potential was changed from −80 mV to a conditioning depolarized potential that was maintained for 10 to 15 min. *Vt* was measured as a function of time at that conditioning potential.

Protocol 2 (Fig. 1A)

The membrane potential was depolarized to −60 mV for 3 min then +5 mV depolarizations of the conditioning potential were imposed at approximately 3 min intervals until conditioning potentials were achieved at which contractions could no longer be evoked by the test depolarizations, i.e., to conditioning potentials between −30 and 0 mV. *Vt* was measured between 2 and 3 min at each conditioning potential.

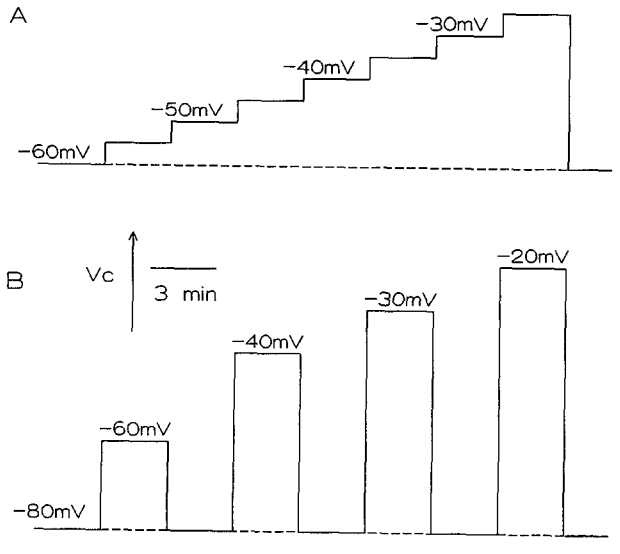

Fig. 1. Depolarization Protocols 2 and 3 (*see text*) used to establish conditioning potentials. The graphs show conditioning membrane potential as a function of time. Detailed descriptions of each protocol are given in the text. Briefly, (*A*) membrane potential was depolarized in progressive 5 mV steps and each potential maintained for 3 min. (*B*) Potential was depolarized for 3 min and then returned to −80 mV for several minutes before the next depolarization.

Protocol 3 (Fig. 1B)

The holding potential was changed from −80 mV to a conditioning potential (more positive than −60 mV) for 3 min and then returned to −80 mV between each subsequent conditioning depolarization. *Vt* was measured between 2 and 3 min after each conditioning potential had been established and between 3 and 5 min after each return to −80 mV.

Determination of Contraction Threshold

Images of the surface sarcomeres, between the intracellular current and voltage electrodes, were captured by a videocamera attached to a Zeiss microscope with an Olympus air objective (6.0 mm working distance; 32×) and a 16× Zeiss eyepiece and were displayed on a videomonitor. The microscope was focused either on sarcomeres on the surface of the fiber or on the edge of fibers.

Movement was detected either as sarcomere shortening (Chua & Dulhunty, 1988), or from rapid flickering of the edge of the fiber (Dulhunty, 1979). The second method was adopted as the standard procedure because most fibers developed contractures with conditioning depolarization to potentials positive to −50 mV. Once the contractures developed, the sarcomeres were less clearly defined and movement was difficult to detect. In contrast, the edges of the fiber remained clearly defined and additional movement could be easily detected in fibers demonstrating maintained contractures. The rapid flicker of movement seen with the brief test pulse was composed of contraction followed by relaxation.

Two possible sources of error were considered. Movement could also have indicated relaxation alone, following a decrease in calcium concentration. This was likely to occur only (i) if the holding potential was returned to more negative values or (ii) with inactivation of excitation-contraction coupling. Such movements would have been slow (holding potentials were manually adjusted and inactivation occurs over a period of seconds), independent of the test pulse, and would not have contributed to the flickering biphasic movement associated with test pulses.

A second possible source of error was that the flickering movement associated with the test pulse did not arise from the area of the fiber that was voltage-clamped. Considerable care was taken to ensure that the movement occurred only between the microelectrodes and was confined to the same small area of fiber surface (usually 5–10 μm^2 or less) throughout the experiment. When these precautions were taken, flickering movement arising outside the voltage-clamped area could be clearly distinguished from that arising within the area. Such extraneous movements were usually only observed with conditioning depolarization to potentials which produced inactivation of excitation-contraction coupling within the voltage-clamped area. All experiments were repeated at least once using observations of sarcomere shortening to determine *Vt*. This was done to ensure that the results were not influenced by the method used to detect contraction. In all cases, identical results were obtained with the two methods.

Depolarizing test pulses were applied every 1.0 sec and increased in 2 mV steps until movement of the fiber edge was detected. The amplitude was then decreased in 0.2 mV steps until the brief movements in response to the test pulse were no longer observed. The potential at which movement was no longer observed, *Vt*, was defined as the most negative membrane potential for just detectable movement elicited by the test pulse.

HYPOTHESES

(a) Voltage-dependent Changes in Contraction Reflect the State of Excitation-Contraction Coupling?

Changes in tension during prolonged depolarization can be interpreted in terms of the voltage dependence of excitation-contraction coupling. Tension (Hodgkin & Horowicz, 1960), myoplasmic calcium concentration (Brum, Rios & Stefani, 1988) and asymmetric charge movement (Chandler, Rakowski & Schneider, 1976) all increase to a peak that is maintained for several seconds and then slowly decay under the influence of the inactivation process. Tension is generated only after a threshold concentration of calcium has been achieved, and the regulatory proteins may become saturated with calcium before calcium reaches maximum concentrations in the myoplasm. Charge movement and tension activation curves both saturate at similar potentials in mammalian fibers (Dulhunty & Gage, 1985), suggesting that the regulatory proteins in these fibers are not saturated with calcium ions before excitation-contraction coupling is maximally activated.

(b) The Strength-Duration Relation for Vt in Terms of Myoplasmic Calcium Concentration

The strength-duration curve for threshold contractions can be interpreted in terms of changes in myoplasmic calcium concentration (Adrian et al., 1969; Costantin, 1974):

(1) A test pulse produces a slow increase in calcium concentration, to a peak or plateau level which is achieved if the test depolarization is maintained for long enough (20 to 1,000 msec). These predicted changes in myoplasmic calcium concentration under voltage clamp conditions have since been recorded in amphibian (Kovacs, Rios & Schneider, 1979; Brum et al., 1988) and mammalian (Eusebi, Miledi & Takahashi, 1985) muscle.

(2) Peak or plateau concentrations of calcium at *Vt* are very much smaller than the concentration achieved with maximal activation.

(3) Rheobase is achieved at pulse durations that are as long or longer than the time taken for calcium to reach plateau concentrations.

(4) A greater test depolarization will be necessary to increase the voltage sensor and calcium to levels that induce movement if the test pulse is brief compared with the time taken to reach the plateau calcium concentration.

(c) What Happens to Vt when the Voltage Sensor Is Partially Converted to the Inactive State?

At a conditioning potential of −40 mV, test 200 mM K^+ contracture tension is reduced to 30% in EDL or to 80% in soleus (Chua & Dulhunty, 1988). Our initial hypothesis was that:

(1) Partial inactivation, at holding potentials between −65 and −20 mV, would cause a proportional reduction in peak or plateau calcium concentration with maximal and submaximal activation. Therefore, a greater test depolarization would be required to release threshold amounts of calcium, i.e., *Vt* at −40 mV would be more positive than *Vt* at −80 mV.

(2) Since inactivation requires less depolarization in EDL fibers than in soleus fibers, the positive shift in *Vt* would be seen with less conditioning depolarization in EDL fibers than in soleus, and fibers should become inactivatable with less conditioning depolarization in EDL.

(3) When the peak or plateau calcium concentration with maximal activation is reduced to levels that are close to minimal values required for contraction, fibers that can just be activated by long test pulses will not be activated by briefer test pulses, i.e., the strongest effect of inactivation will be seen with the briefest pulses.

Results

THE TIME COURSE OF CHANGES IN *VT* DURING A CONDITIONING DEPOLARIZATION

Vt was first measured during prolonged conditioning depolarizations (Protocol 1, Materials and Methods), and the time course of the changes in *Vt* was compared with changes in peak test 200 mM K^+ contracture tension that result from the inactivation process during steady depolarization. The amplitude of test 200 mM K^+ contractures shows a biphasic reduction—a rapid phase occurs within 30 sec and 3 min of depolarization and a slower phase continues for 10 to 15 min in rat soleus muscles (Dulhunty, 1991).

A negative shift in *Vt* was observed with small conditioning depolarizations to potentials between −60 and −40 mV. This reduced *Vt* was either maintained at conditioning potentials around −50 mV (*not shown*) or reversed during conditioning depolarizations to −40 mV (Fig. 2). Conditioning depolarization to more positive potentials resulted in an increase (positive shift) in *Vt,* which was often preceded by a transient reduction

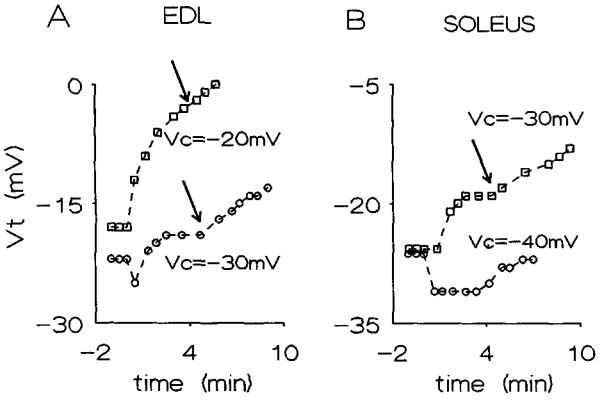

Fig. 2. *Vt* (in mV) is shown as a function of time (in minutes). Control values for *Vt* were taken at a holding potential of −80 mV at times between −1 and 0 min. The conditioning potential was established at zero time and *Vt* was measured at 20 to 60 sec intervals up to 10 min. (*A*) Data from two EDL fibers that were depolarized to −30 mV (circles) and to −20 mV (squares). (*B*) Data from two soleus fibers that were depolarized to −40 mV (circles) and −30 mV (squares). The arrows indicate inflections marking the start of the second phase of the increase in *Vt*.

in contraction threshold (Fig. 2*A*). The increase in *Vt* was, in most cases, biphasic: the arrows in Fig. 2 point to the inflection in the curve marking the onset of the second slow phase. The soleus fiber in Fig. 2*B* demonstrated an initial rapid increase in *Vt*, from −26 to −19 mV, within 3 min of changing the conditioning potential from −80 to −30 mV. *Vt* then increased slowly to −14 mV between 3 and 10 min. These biphasic changes in *Vt* occurred at roughly the same time as the fast and slow phases of K^+ contracture inactivation and may reflect the same processes.

It is clear (Fig. 2) that a steady-state situation could take longer than 10 min to develop and fibers generally deteriorated before a set of data over a range of conditioning potentials could be obtained. Therefore, *Vt* was measured after depolarizations lasting for 3 min, and the data were compared with K^+ contracture inactivation curves also obtained after conditioning depolarizations lasting for 3 min (Chua & Dulhunty, 1988; Dulhunty, 1991).

Conditioning Depolarization Dependence of *Vt* Determined with 50 msec Test Pulses

The conditioning membrane potential was depolarized in +5 mV steps (Protocol 2, Fig. 1*A*, Materials and Methods) and *Vt* was determined using a 50 msec test pulse. As shown in the first experiment (Fig. 2), both EDL and soleus fibers were more easily activated, i.e., *Vt* became more negative by −2 to −5 mV, with small conditioning depolarizations. A minimum *Vt* was recorded at a conditioning potential of −30 mV in the EDL fiber in Fig. 3*A* and at −40 mV in the soleus fiber (Fig. 3*B*). The fibers were harder to activate after further depolarization: *Vt* sharply increased to positive values and many fibers could not be activated with test pulses to +20 mV. The positive shift in *Vt* was usually steeper in soleus fibers.

One factor considered with analysis of the *Vt* data was the maximum amplitude of test pulses and the definition of an "inactivated" fiber. When fibers were well "clamped" during test pulses to potentials of +30 to +40 mV, it was noted that additional activation could not be achieved by increasing the test pulse beyond +20 mV. This was consistent with the tension activation curves obtained with K^+ contractures being well saturated by +20 mV (Chua & Dulhunty, 1988). Therefore, fibers were called "activatable" if $Vt < +20$ mV

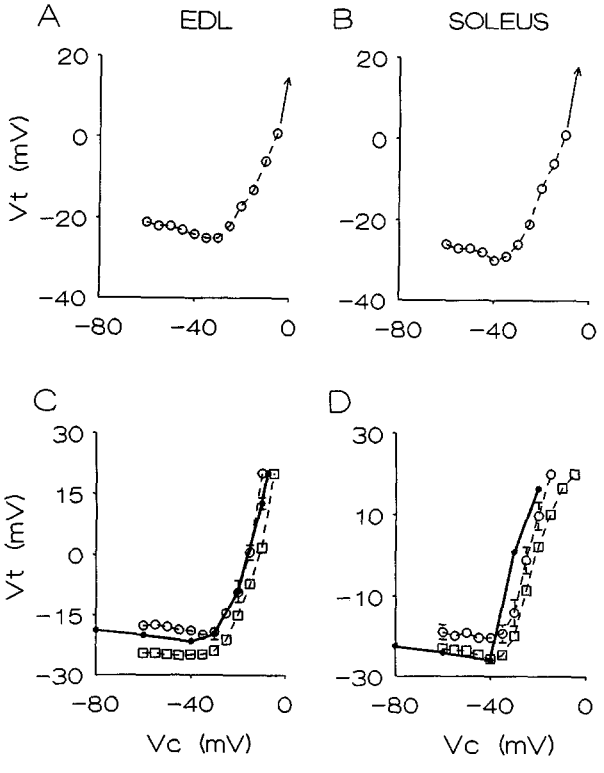

Fig. 3. *Vt* (in mV) is shown as a function of conditioning potential (*Vc*, in mV) after 3 min at each conditioning potential in one EDL fiber (*A*) or one soleus fiber (*B*). *Vt* was measured in both fibers using a 50 msec test pulse with the continuous conditioning depolarization protocol (Fig. 1*A*). The arrows indicate that fibers could not be activated by test pulses to +20 mV. Average data obtained during continuous conditioning depolarization is shown for 10 msec test pulses (open circles) in EDL fibers ($n = 9$) (*C*) and soleus fibers ($n = 8$) (*D*), or with 20 msec test pulses (open squares) in EDL fibers ($n = 8$) (*C*) and soleus fibers ($n = 7$) (*D*). Average data obtained with discrete conditioning depolarizations (Fig. 1*B*) and a 15 msec test pulse (small filled circles and continuous lines) are also shown for EDL fibers ($n = 6$) (*C*) and for soleus fibers ($n = 15$) (*D*). The vertical bars indicate ±1 SEM where this is greater than the dimensions of the symbols.

and "inactivatable" when pulses to +20 mV did not elicit movement.

The second factor was the calculation of average values for *Vt*. *Vt* could not be given a real value when the fibers could not be activated and *Vt* increased to values greater than +20 mV at different holding potentials in different fibers. For example, all seven soleus fibers tested with a 20 msec test pulse could be activated from a holding potential of −25 mV, four fibers could be activated from −20 mV, three from −15 mV and one from −10 mV. To include all fibers in the average data, those that would not be activated were assigned an arbitrary *Vt* of +20 mV. This produced an artificial reduction in the slope of lines through some of the average data at conditioning potentials between −20 and 0 mV (Fig. 3*C* and *D*).

Plots of average *Vt* (open symbols, Fig. 3*C* and *D*) show that fibers were most easily activated at holding potentials (between −30 and −40 mV) at which inactivation produces a significant reduction in test 200 mM K^+ contracture tension (*see* Materials and Methods—Hypotheses). The fact that EDL fibers remained activatable at conditioning potentials at which soleus fibers could not be activated was also unexpected since inactivation of test 200 mM K^+ contracture tension occurs at more negative membrane potentials in EDL (Chua & Dulhunty, 1988, 1989).

Depolarization History Does Not Alter the Relationship between *Vt* and Conditioning Potential

The differences between the changes in *Vt* with conditioning depolarization and inactivation of maximum K^+ contracture tension may have arisen from differences in depolarization protocol. The *Vt* data described thus far were from fibers that were depolarized for many minutes (i.e., 3 min at each of eight to twelve different conditioning potentials). In contrast, fibers were depolarized for only 3 min during K^+ contracture experiments and were then repolarized for 10 to 20 min before subsequent depolarizations. The prolonged depolarization might have produced the observed differences between *Vt* and K^+ contracture data. However, this was not the case because the relationship between *Vt* and conditioning potential (filled circles, Fig. 3*C* and *D*) was unchanged when the potential was returned to −80 mV between each 3 min conditioning depolarization (Protocol 3, Fig. 1*B*, Materials and Methods).

Does *Vt* Depend on External Calcium Concentration?

An influx of extracellular calcium might explain the unexpected effects of conditioning depolarization on *Vt* if inflowing calcium compensated for an inactivation-induced reduction in calcium release from the sarcoplasmic reticulum during a just threshold stimulus, but not during maximal activation. To examine this possibility, either 1 mM cobalt was added to the bathing solution to block calcium channels (*see e.g.*, Sanchez & Stefani, 1978) or calcium in the solution was replaced by 20 mM CaEGTA producing a free calcium concentration of 100 μM (Dulhunty & Gage, 1988). Neither the addition of cobalt to, or the removal of calcium from, the solution produced a significant change in *Vt* at conditioning potentials between −60 and −40 mV in soleus fibers.

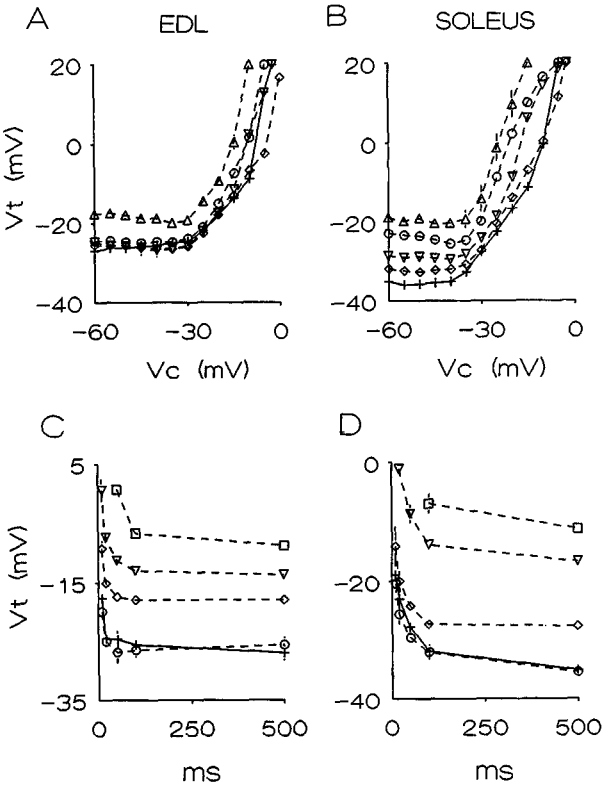

Fig. 4. Effect of test pulse duration on the relationship between *Vt* and conditioning potential (*Vc*). Average data are shown for EDL fibers (*A* and *C*) and for soleus fibers (*B* and *D*). In *A* and *B:* triangles—data for a 10 msec test pulse in 9 EDL fibers (*A*) and 8 soleus fibers (*B*); circles—data for a 20 msec test pulse in 8 EDL fibers (*A*) and 7 soleus fibers (*B*); inverted triangles—data for a 50 msec test pulse in 7 EDL fibers (*A*) and 13 soleus fibers (*B*); diamonds—data for a 100 msec test pulse in 10 EDL fibers (*A*) and 12 soleus fibers (*B*); crosses—data for a 500 msec test pulse in 7 EDL fibers (*A*) and 6 soleus fibers. In *C* and *D*, strength-duration curves for EDL fibers (*C*) and soleus fibers (*D*) were constructed by replotting the average *Vt* data given in Fig. 4*A* and *B* as a function of test pulse duration (in msec). The symbols show data obtained with conditioning depolarizations to: −60 mV (crosses); −40 mV (circles); −25 mV (*C*) or −30 mV (*D*) (diamonds); −20 mV (triangles) and −15 mV (squares). The vertical bars show ±1 SEM where this is greater than the dimensions of the symbols.

The increase in *Vt* at conditioning potentials between −40 and −10 mV either did not change in EDL fibers, or was steeper than normal in soleus fibers, when extracellular calcium influx was reduced.

Therefore, an influx of external calcium was not responsible either for the different results obtained with *Vt* and maximal contractures or for the decrease in *Vt* with conditioning depolarizations between −60 and −40 mV. Indeed, this decrease in *Vt* in EDL fibers was greater than normal in the presence of cobalt ions. The steeper increase in *Vt* at conditioning potentials between −40 and −10 mV in soleus fibers could mean that an influx of external calcium occurred within this range of conditioning potentials. An alternative explanation is that the more rapid increase in *Vt* reflected the inhibitory effects of external calcium deprivation and cobalt on the voltage sensor (Luttgau, Gottschalk & Berwe, 1987; Dulhunty & Gage, 1988, 1989).

The Effect of Test Pulse Duration on Contraction Threshold

Strength-duration relations show that less depolarization is required for contraction when longer test pulses are used, with the relationship approaching a rheobase between 20 and 1,000 msec (Adrian et al., 1969; Costantin, 1974; Chua & Dulhunty, 1988, 1989). It was therefore likely that the effect of conditioning membrane potential on *Vt* might also vary with test pulse duration. To test this possibility, we determined *Vt* using test pulses with durations ranging from 10 to 500 msec and fibers were depolarized in continuous +5 mV steps from −60 to 0 mV.

A strong effect of test pulse duration on *Vt* was observed in soleus fibers (Fig. 4*B*). Each set of data, obtained as the pulse duration was increased from 10 (triangles) to 500 msec (crosses) fell below the preceding data set. Less effect of test pulse duration on *Vt* was seen in EDL fibers (Fig. 4*A*)—*Vt* for 10 msec test pulses was at more positive potentials than *Vt* for the longer pulses and there was considerable overlap between data obtained with 20, 50, 100 and 500 msec test pulses (Fig. 4*A*).

The conditioning potential yielding minimum *Vt* was not much influenced by test pulse duration, although the negative slope between conditioning potentials between −60 and −40 mV was greatest when *Vt* was obtained with 10 or 20 msec test pulses: the curves for 500 msec test pulses were either flat in this range (soleus), or demonstrated small positive increments (EDL). The positive increase in *Vt* at conditioning potentials between −35 and −10 mV was steeper for brief test pulses than for longer test pulses in both types of fiber.

Effect of Test Pulse Duration on the Conditioning Potential at which Fibers Became Inactivatable

Fibers that could not be activated by a brief test pulse at conditioning depolarizations to potentials between −30 and 0 mV would often contract if the duration of the test pulse was lengthened. No EDL or soleus fibers stimulated with a 10 msec test pulse at a conditioning potential of −10 mV contracted. When the test pulse was lengthened to 20 msec, 88% of the EDL and 14% of soleus fibers contracted, while 100% of EDL and 83% of soleus fibers contracted when tested at 100 msec.

The effect of stimulus duration on the ability of a test pulse to activate fibers was seen at all conditioning membrane potentials. As shown previously, *Vt* was less subject to the effect of inactivation in EDL fibers than in soleus—inactivatable soleus fibers were observed at conditioning potentials positive to −35 mV, while conditioning depolarization to −15 mV was required before any EDL fibers became inactivatable.

Strength-Duration Curves at Different Holding Potentials

Strength-duration curves were constructed by replotting the average *Vt* data shown in Fig. 4*A* and *B* as a function of test pulse duration (Fig. 4*C* and *D*). Conditioning depolarizations to potentials between −40 and −10 mV resulted in parallel shifts in the strength-duration curves to more positive values of *Vt*. The curve was shifted by +20 mV in EDL, and +30 mV in soleus, with conditioning depolarization from −60 to −10 mV. The parallel shifts in *Vt* suggest that the time course of calcium equilibration in the myoplasm, and possibly the rate of calcium release from the sarcoplasmic reticulum, were not altered by the conditioning depolarization.

The strength-duration relationship for EDL reached a rheobase between 20 and 50 msec (Fig. 4*C*). In contrast, rheobase was not achieved in soleus at 500 msec (Fig. 4*D*): the average *Vt* fell by −1 to −2 mV with each increment in pulse duration up to 500 msec. The achievement of rheobase in EDL is also reflected in the clustering of data obtained with test pulse durations between 50 and 500 msec in Fig. 4*A*.

Are the Effects of Conditioning Potential on *Vt* the Same in Different Types of Fast-Twitch Fiber?

It was of interest to determine whether the different effects of conditioning depolarization on *Vt* in EDL and

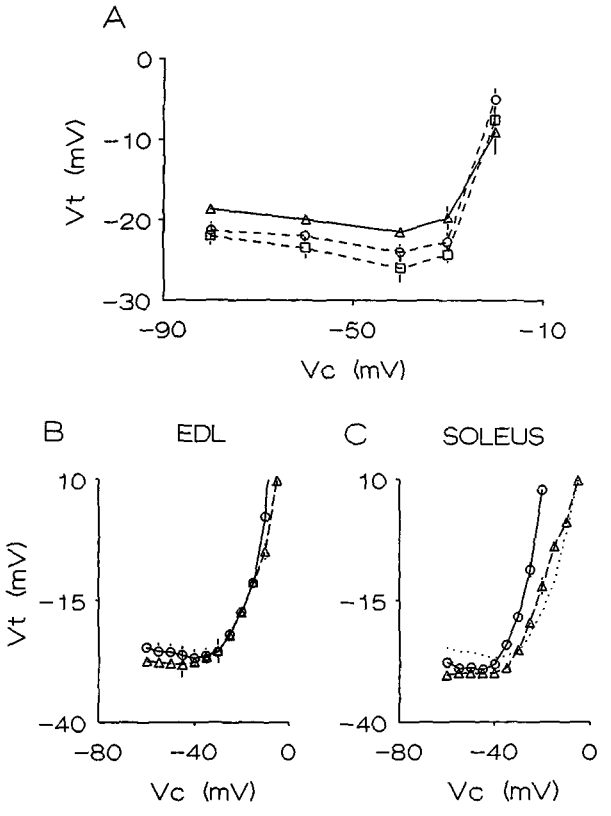

Fig. 5. *Vt* (in mV) plotted against conditioning potential (*Vc* in mV) in fast-twitch fibers from different muscles (*A*) and in denervated EDL (*B*) and soleus (*C*) fibers. In *A*, the triangles show data from 6 EDL fibers, the circles show data from 3 white sternomastoid fibers and the squares show data from 4 red sternomastoid fibers. The data were obtained with discrete conditioning depolarizations (Protocol 3, Fig. 1*B*) and a 15 msec test pulse. In *B*, the circles show data from 7 normal EDL fibers and the triangles show data from 6 denervated EDL fibers. In *C*, the circles show data from 13 normal soleus fibers, the triangles show data from 8 denervated soleus fibers and the dotted line reproduces the line through the normal EDL data in *B*. Data in *B* and *C* were obtained with continuous conditioning depolarization (Protocol 2, Fig. 1*A*) and 50 msec test pulses. All symbols show average data and the vertical lines indicates ±1 SEM where this exceeds the dimensions of the symbols.

soleus fibers reflected general differences between fast- and slow-twitch fibers. Experiments were therefore performed in sternomastoid muscles which contain macroscopically identifiable bundles of fast-red and fast-white fibers (Dulhunty & Dlutowski, 1979). A 15 msec test pulse was used with discrete depolarizations to each conditioning potential (Protocol 3, Materials and Methods). The results (Fig. 5*A*) suggest that, within this limited survey, the holding potential at which *Vt* is minimal, and the range of potentials over which *Vt* increases, are the same in EDL and in red and white sternomastoid fibers and are therefore specific characteristics of fast-twitch fibers (either red or white).

Effects of Denervation on the Relationship Between *Vt* and Conditioning Potential

Denervated fibers were tested with 50 msec test pulses and progressive +5 mV conditioning depolarizations (Protocol 2, Materials and Methods). As expected from the negative shift in activation curves for K^+ contractures in denervated EDL and soleus fibers (Dulhunty & Gage, 1985), *Vt* was also more negative at conditioning potentials between −60 and −40 mV (Fig. 5*B* and *C*).

The conditioning potential at which *Vt* was minimal, and the slope of the curve through the average *Vt* data between −30 and −20 mV, (i) remained the same as normal in the denervated EDL fibers (Fig. 5*B*) and (ii) was shifted to more positive conditioning potentials in denervated soleus fibers (Fig. 5*C*). As a result of this shift in the average *Vt* after denervation of soleus fibers, contraction threshold occurred at very similar test potentials in denervated soleus and normal (dotted line, Fig. 5*C*) and denervated EDL fibers at conditioning potentials between −30 and −10 mV. These results provide another example in which changes in *Vt* did not reflect changes in the inactivation curve for 200 mM K^+ contracture tension (*see* Introduction).

Can Changes in *Vt* during Conditioning Depolarization Be Attributed to Inactivation of Excitation-Contraction Coupling?

Two observations raised the question of whether inactivation of excitation-contraction coupling had any influence on *Vt*. The first was the mismatch between the changes in *Vt* with depolarization and the inactivation curves for 200 mM K^+ contracture tension in EDL and soleus fibers. The second observation was that *Vt*, at conditioning potentials between −30 and 0 mV, was often only 0.5 to 1.0 mV positive to the conditioning potential and appeared to "track" changes in the conditioning potential (i.e., a +5 mV increase conditioning potential produced a +5 mV increase in *Vt*). To describe this effect in a quantitative way, we measured the slope of a line between *Vt* values at conditioning potential positive to −30 mV (Fig. 6*A*). A slope of 1 indicated a one to one relationship between increments in holding potential and *Vt*.

Figure 6*A* compares data obtained from two soleus fibers in which *Vt* was obtained with 10 and 100 msec test pulses with a line having a slope of 1.0 and arbitrarily intersecting the conditioning potential axis at −40 mV. The slope of the line through the *Vt* data at conditioning potentials positive to −40 mV was close to 1.0 for the 100 msec test pulse (circles), but greater than 1.0 for the 10 msec test pulse (squares). Average slopes for *Vt*, obtained with a 10 msec test pulse were 2.25 for soleus fibers, or 1.75 for EDL (Fig. 6*B*). The

slope decreased with increasing pulse duration and was not significantly different from 1.0 for 100 and 500 msec test pulses in EDL or for 500 msec test pulses in soleus.

Fibers became inactive, and therefore achieved their steepest increase in *Vt* at holding potentials positive to −35 mV in soleus, or −15 mV in EDL (Fig. 4 above). Taken together, the results suggest that the effect of inactivation on *Vt* (i) depends on the duration of the test pulse and is greatest for test pulses with durations less than 100 msec and (ii) is stronger in soleus fibers than in EDL fibers at most pulse durations.

Discussion

The effect of conditioning depolarization on the membrane potential (*Vt*) at which movement is first elicited has been examined. Inactivation of maximum tension that occurs with conditioning depolarizations to potentials between −60 and −40 mV did not inhibit *Vt:* fibers were, in fact, easier to activate (*Vt* became more negative) within this potential range. Further conditioning depolarization to potentials between −40 and 0 mV produced expected positive shifts in *Vt* which were greater when brief test pulses were used (<100 msec). That *Vt* increased, and fibers became inactivatable, with less conditioning depolarization in soleus fibers than in EDL was surprising because maximum tension is depressed with less conditioning depolarization in the EDL fibers (Chua & Dulhunty, 1988). Finally, rheobase was achieved at shorter test pulse durations in EDL than in soleus. This last observation is considered first since it is relevant to later sections of the Discussion.

Rheobase in EDL and Soleus Fibers

The strength-duration curve for contraction threshold does not achieve a true rheobase in skeletal muscle (Costantin, 1974; Chua & Dulhunty, 1988). However, rheobase is *approached* between 20 and 50 msec in EDL fibers (Fig. 7) or between 1,000 and 2,000 msec in soleus (Chua & Dulhunty, 1988). Since the separation between the terminal cisternae and the center of the myofilaments is similar in EDL and soleus fibers, the strength-duration curves suggest that the rate of calcium release with small depolarizations is about 10 times faster in EDL fibers. This is consistent with aequorin responses to strong test depolarizations from −80 to 0 mV, which show that plateau calcium concentrations are achieved in 50 msec in EDL and 300 msec in soleus (Fig. 5 in Eusebi et al., 1985).

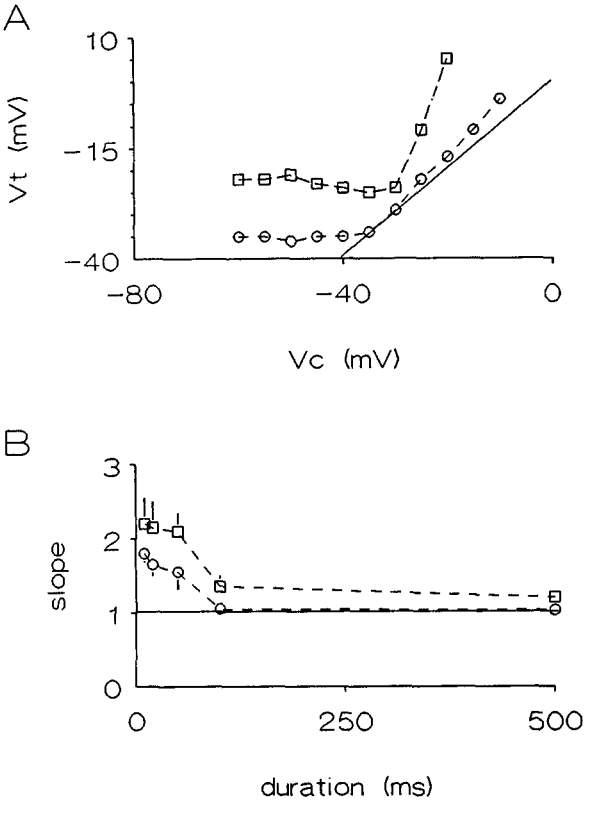

Fig. 6. The slope of the positive component of the relationship between *Vt* and conditioning depolarization. (*A*) *Vt* (in mV) is plotted against conditioning depolarization (*Vc* in mV) in one soleus fiber tested with a 10 msec pulse (squares) and a second soleus fiber tested with a 100 msec pulse (circles). (*B*) Average slope of the positive limb of the relationship between *Vt* and conditioning potential is plotted against pulse duration for EDL fibers (circles) and soleus fibers (squares). The vertical bars show ±1 SEM.

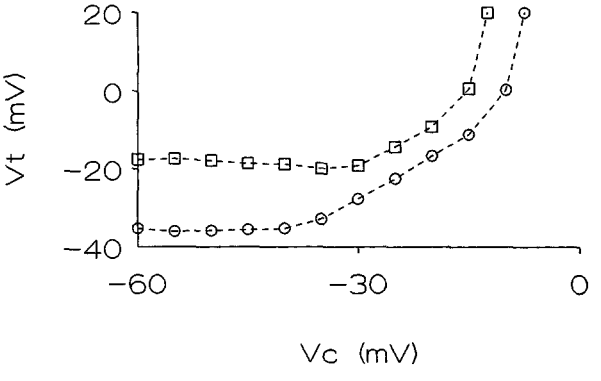

Fig. 7. *Vt* (in mV) is plotted against conditioning potential (*Vc* in mV). Average *Vt* for 9 EDL fibers tested with a 10 msec pulse (squares) is compared with average *Vt* for 6 soleus fibers tested with a 500 msec pulse (circles).

Effect of Test Pulse Duration on Comparisons between *Vt* in EDL and Soleus Fibers

All comparisons between *Vt* in different preparations have thus far been made using *Vt* obtained with test pulses of the same duration. This type of comparison could have led to incorrect conclusions if, for example, the test pulse duration was at rheobase for EDL but not for soleus. To test this possibility, comparisons of *Vt* were made using data obtained with test pulses of 10 msec in EDL and 500 msec in soleus, i.e., test pulses that were comparable with respect to the duration at which rheobase was approached (i.e., at 20 msec in EDL or 1,000 msec in soleus). In contrast to previous comparisons, the increase in *Vt* occurred at conditioning potentials that were 5 mV more negative in EDL than in soleus.

Therefore, taking pulse duration into account reveals differences between EDL and soleus fibers in the *Vt*/conditioning potential relationship that are consistent with the differences in the effect of conditioning depolarization on K^+ contracture tension in the two types of fiber. However, the effects of pulse duration and rheobase cannot explain that inactivation did not influence *Vt* at conditioning potentials between -60 and -30 mV.

Factors that Could Contribute to the Effect of Conditioning Depolarization on *Vt*

The insensitivity of *Vt* to inactivation at conditioning potentials between -60 and -30 mV was not influenced by external calcium ions or by the protocol used to establish the conditioning depolarization. Two additional factors that may have contributed to changes in *Vt* were:

1. Background or Pedestal Calcium Concentrations

K^+ contractures decay either to the baseline or to a small pedestal level within 1 to 2 min if depolarization is maintained (Chua & Dulhunty, 1989). The pedestal tension is attributed to a background concentration of active voltage sensor which induces a small efflux of calcium from the sarcoplasmic reticulum and maintains myoplasmic calcium at concentrations that are close to threshold for contraction (Chua & Dulhunty, 1989; Dulhunty, 1992).

Background calcium concentration can account for the initiation of movement with less than normal test depolarization at conditioning potentials between -60 and -40 mV. The total concentration of calcium at the end of the test pulse is the sum of the background concentration and the concentration induced by the test pulse. Although the concentration due to the test pulse alone may be reduced by inactivation, the total concentration may exceed that achieved by the test pulse in the absence of inactivation (and *Vt* become more negative) if the steady-state concentration is sufficiently high.

The "tracking" effect of *Vt* with changes in conditioning potential can be explained by background calcium concentrations that were equal to, or exceeded, contraction threshold at conditioning potentials between -40 and -10 mV. Since the fibers maintained a steady-state contracture, only a small extra depolarization was required to release additional calcium and generate movement.

2. The Effect of Saturation of the Tension Activation Curve on Vt

The ability of an increase in test pulse amplitude to produce increments in myoplasmic calcium concentration will be limited by the potential at which the tension activation curve saturates, i.e., at potentials between -10 and 0 mV in soleus fibers and between $+10$ and $+20$ mV in EDL fibers (Chua & Dulhunty, 1989). Test pulses to more positive potentials will be no more effective than test pulses to the potentials at which saturation occurs. Indeed, maximum values of *Vt* were around -3 to -5 mV in soleus and $+3$ to $+6$ mV in EDL when long test pulses were used. More positive values of *Vt* were recorded with briefer test pulses, suggesting that the rate of activation may increase with depolarization to potentials that are more positive than the potentials at which the tension response saturates. Therefore, depolarization beyond the saturation potential would increase the amount of calcium released at short times.

Effects of Denervation on the Relationship between *Vt* and Conditioning Potential

The effect of denervation on *Vt* in soleus fibers can be attributed to changes in the strength-duration curve. The positive limb of curve relating *Vt* (for a 50 msec test pulse) to conditioning potential was shifted to more positive conditioning potentials by denervation in soleus fibers and the curve became similar to that obtained in normal and denervated EDL fibers. Denervation alters the profile of strength-duration curve in soleus fibers, but not in EDL, so that rheobase is approached between 20 and 50 msec in soleus (M.F. Patterson and A.F. Dulhunty, *unpublished observations*). This effect of denervation on the strength-duration curve in soleus was equivalent to the shift seen in normal soleus fibers with an increase in test pulse duration from 50 to 500 msec (Fig. 4).

Other Factors that May Contribute to Differences between Results Obtained with K^+ Contracture and Voltage Clamp Techniques

It is likely that the degree of depolarization of the T-tubule membrane differs in K^+ contracture and voltage clamp experiments. The difficulty of controlling radial membrane potential in the entire T-tubule system using voltage clamp techniques is well recognized (Hille & Campbell, 1976). Thus, better control of the total T-tubule membrane potential is obtained during K^+ contracture experiments. However, the determination of *Vt* depends only on the activation of surface myofibrils (Adrian et al., 1969; Costantin, 1974) and areas of T-tubule membrane are likely to be under reasonable voltage control.

A consequence of poorly controlled T-tubule membrane potential in the voltage clamp experiments would be an apparent shift in the voltage dependence of activation to more positive membrane potentials. Although this is a possible explanation for some of the results, it is unlikely to have been a significant factor. *Vt* for long pulses at −80 mV was about −25 mV in EDL fibers and −35 mV in soleus fibers. These potentials are 5 to 6 mV more positive than contraction thresholds measured with K^+ contracture techniques (Dulhunty & Gage, 1985; Chua & Dulhunty, 1989) and this represents the extent of the error that may be introduced by poor voltage control of the T-tubule membrane. The 20 mV difference between the potentials at which inactivation becomes apparent with the two types of experiment cannot be explained by poor voltage clamp control of the T-tubule membrane close to the surface of the fiber.

In conclusion, a comparison of the effects of conditioning depolarization on the membrane potential at which movement is first elicited by a test pulse with the effects of conditioning depolarization on maximum tension is complicated because (i) the *Vt*/holding potential relationship varies with test pulse duration when the durations are less than rheobase, and (ii) rheobase is achieved at very different times in EDL and soleus fibers and is altered by denervation and (iii) *Vt* is very sensitive to small changes in steady-state concentrations of calcium ions, which have little effect on maximal 200 mM K^+ contractures. *Vt* and 200 mM K^+ contracture studies provide very different, but complementary, information about the state of excitation-contraction coupling in skeletal muscle.

The authors are indebted to Professor P.W. Gage for encouragement and discussion and for comments on the manuscript. We are grateful to Suzanne Curtis for assistance throughout the project.

References

Adrian, R.H., Chandler, W.K., Hodgkin, A.L. 1969. The kinetics of mechanical activation in frog muscle. *J. Physiol.* **204:**207–230

Brum, G., Rios, E., Stefani, E. 1988. Effects of extracellular calcium on calcium movements of excitation-contraction coupling in frog skeletal muscle fibres. *J. Physiol.* **398:**441–473

Chandler, W.K., Rakowski, R.F., Schneider, M.F. 1976. Effects of glycerol treatment and maintained depolarisation on charge movement in skeletal muscle. *J. Physiol.* **254:**285–316

Chua, M.F., Dulhunty, A.F. 1988. Inactivation of excitation-contraction coupling in rat extensor digitorum longus and soleus muscles. *J. Gen. Physiol.* **91:**737–757

Chua, M.F., Dulhunty, A.F. 1989. Noninactivating tension in rat skeletal muscle. *J. Gen. Physiol.* **94:**183–203

Costantin, L.L. 1974. Contractile activation in frog skeletal muscle. *J. Gen. Physiol.* **63:**657–674

Dulhunty, A.F. 1979. A refractory period after brief activation of mammalian skeletal muscle fibres. *Neurosci. Lett.* **14:**223–228

Dulhunty, A.F. 1991. Activation and inactivation of excitation-contraction coupling in rat soleus muscle. *J. Physiol.* **439:**605–626

Dulhunty, A.F. 1992. The voltage-activation of contraction in skeletal muscle. *Prog. Biophys. Mol. Biol.* **57:**181–223

Dulhunty, A., Carter, G., Hinrichsen, C. 1984. The membrane capacity of mammalian skeletal muscle fibres. *J. Mus. Res. Cell Motil.* **5:**315–332

Dulhunty, A.F., Dlutowski, M. 1979. Fiber types in red and white segments of rat sternomastoid muscle. *Am. J. Anat.* **156:**51–62

Dulhunty, A.F., Gage, P.W. 1985. Excitation-contraction coupling and charge movement in denervated rat extensor digitorum longus and soleus muscles. *J. Physiol.* **358:**75–89

Dulhunty, A.F., Gage, P.W. 1988. Effects of extracellular calcium concentration and dihydropyridines on contraction in mammalian skeletal muscle. *J. Physiol* **399:**63–80

Dulhunty, A.F., Gage, P.W. 1989. Effect of cobalt, magnesium, and cadmium on contraction of rat soleus muscle. *Biophys. J.* **56:**1–14

Eisenberg, R.S., Johnson, E.A. 1970. Three-dimensional electrical field problems, in physiology. *Prog. Biophys. Mol. Biol.* **20:**1–65

Eusebi, F., Miledi, R., Takahashi, T. 1985. Aequorin-calcium transients in mammalian fast and slow muscle fibers. *Biomed. Res.* **6:**129–138

Freygang, W.H., Goldstein, D.A., Hellam, D.C., Peachey, L.D. 1964. The relation between the late after-potential and the size of the transverse tubular system of frog muscle. *J. Gen. Physiol.* **48:**235–263

Gage, P.W., Eisenberg, R.S. 1969. Action potentials, afterpotentials, and excitation-contraction coupling in frog sartorius fibers without transverse tubules. *J. Gen. Physiol.* **53:**298–310

Hille, B., Campbell, D.T. 1976. An improved vaseline gap voltage clamp for skeletal muscle fibres. *J. Gen. Physiol.* **67:**265–293

Hodgkin, A.L., Horowicz, P. 1960. Potassium contractures in single muscle fibres. *J. Physiol.* **153:**386–403

Kovacs, L., Rios, E., Schneider, M.F. 1979. Calcium transients and intramembrane charge movement in skeletal muscle fibres. *Nature* **279:**391–396

Lannergren, J., Westerblad, H. 1986. Force and membrane potential during and after fatiguing, continuous high-frequency stimulation of single *Xenopus* muscle fibres. *Acta Physiol. Scand.* **128:** 359–368

Luttgau, H.C., Gottschalk, G., Berwe, D. 1987. The effect of calcium and Ca antagonists upon excitation-contraction coupling. *Can. J. Physiol. Pharmacol.* **65:**717–723

Patterson, M.F., Dulhunty, A.F. 1991. Effects of denervation on the activation and inactivation of excitation-contraction coupling in rat soleus muscle. *Proc. Aust. Physiol. Pharmacol. Soc.* **22(2):**79*p*

Sanchez, J.A., Stefani, E. 1978. Inward calcium current in twitch muscle fibres of the frog. *J. Physiol.* **283:**197–209