ACTIVATION AND INACTIVATION PROPERTIES OF NORMAL AND DENERVATED MAMMALIAN SKELETAL MUSCLE

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To Andrew and Hannah
This thesis contains no published or written material of other persons, except where reference has been made in the text.

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ABSTRACT

The effect of denervation on the activation and inactivation of excitation-contraction coupling in fast- and slow-twitch rat skeletal muscle was assessed using potassium (K-) contractures and the two-microelectrode point voltage clamp technique with visual endpoint determination of contraction threshold. Solutions containing sulphate as the impermeant anion were used to obtain better control of depolarization in K-contracture and voltage clamp experiments.

Denervation shifted the voltage dependence of the half maximal activation of K-contractures by -12 mV in fast-twitch extensor digitorum longus (EDL) compared to only a -1.3 mV in slow-twitch soleus fibres. The overall effect of denervation was to make the activation curves similar for the two types of muscle.

The point of initial tension generation in activation curves has been used to predict contraction thresholds for the muscle fibres. Following denervation, changes in the type of the activation curves suggested that the contraction thresholds for soleus and EDL became similar. To test this observation, the two-microelectrode point voltage clamp technique was used to accurately determine contraction thresholds for a variety of test pulse durations (5-500 msec) in normal and denervated soleus and EDL muscle fibres. Results demonstrated that contraction thresholds were significantly more negative in soleus compared to EDL (by about 13 mV). A shift in contraction thresholds at all pulse durations was observed following denervation. Contraction thresholds for soleus became more positive (4 mV) while those for EDL became more negative (6 mV), thereby making contraction thresholds in the two muscles similar.

The effect of conditioning depolarizations on the peak height for test contractures induced by exposure to solutions containing 200 mM K+ (200K) was examined in normal and denervated preparations. Only small negative shifts (1-2 mV) in the voltages for half maximal inactivation were observed between normal and denervated muscle (both soleus and EDL), and the slope factors were hardly altered.

An extensive study was carried out to examine the membrane potential, \( V_t \), at which threshold contractions were elicited by brief test pulses in polarized and depolarized EDL, soleus and sternomastoid muscle fibres. The effects of test pulse duration, inactivation of E-C coupling and denervation were studied. The \( V_t \)-holding potential curve for test pulses with durations of 10 msec to 50 msec demonstrated (a) a shallow negative slope component with a minimum \( V_t \) at a holding potential of -40 mV in soleus fibres, or -35 mV in EDL and sternomastoid fibres and (b) a steep positive slope component at more positive holding potentials.
Characteristics of both components of the \( V_f \)-holding potential curve were examined. It was concluded that the effects of depolarization on threshold contractions elicited by brief test pulses were very different from, and could not be predicted by, the effects of depolarization on maximum 200K contracture tension.

The full time course of maximal contractures in 200 mM K\(^+\) was examined in normal and denervated EDL and soleus. Of particular interest was the decay of contracture tension brought about by the inactivation of E-C coupling. It was found that 200K contractures in normal soleus and EDL decayed more rapidly if preceded by a 5, 10 or 30 minute conditioning depolarization in 30K. The decay of contractures in denervated muscle were slower than normal and five minute conditioning depolarizations had a lesser effect on the decay of 200K contracture tension in denervated muscle. The results are described by a five state model for activation of the voltage sensor for E-C coupling which includes two inactivated states. Denervation of EDL resulted in a distinctive biphasic decay of 200K contracture tension which was interpreted in terms of the unmasking of a second inactive state. The results show that denervation has a strong effect on the time course and voltage dependence of contraction. Most of these effects can be explained by an effect of denervation on the voltage sensor for E-C coupling.

In the above experiments, the osmolarity and ionic strength of the high potassium solutions were elevated and the same as that of the 200K solution. To examine the effect the high ionic strength had on K-contractures, sulphate was replaced by methanesulphonate in some experiments. Results indicated a -11 mV shift in the voltage for half maximal activation in methanesulphonate compared to results obtained with sulphate containing solutions. A hyperpolarization of the membrane potential in most elevated potassium solutions was also observed. The results did not show whether sulphate or methanesulphonate produced anomalous effects on the voltage activation of contraction. Time did not permit a further investigation of this point.

Finally, a series of voltage clamp experiments were carried out to examine the effects of the perchlorate anion on the contraction threshold for soleus, for a variety of test pulse durations (5-500 msec). Results demonstrated a progressive and parallel negative shift in the contraction threshold upon exposure to solutions containing 0 mM, 2 mM and 10 mM perchlorate.
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CHAPTER 1

GENERAL INTRODUCTION

1.1 Structure of skeletal muscle

The overall aim of this study is to investigate the voltage dependence of contraction in mammalian skeletal muscle. Much of the work has been directed towards the inactivation of excitation-contraction coupling and the effects of denervation on the activation and inactivation of contraction.

Skeletal muscle is constructed from individual muscle fibres which are multinucleated (syncitial) cells. The structure of the cells is shown in Figure 1.1. The muscle fibre is surrounded by a sarcolemma: the cell membrane. The sarcolemma is a lipid bilayer containing numerous proteins which form transport systems, pumps and ion channels. Invaginations at regular intervals along the sarcolemma form the transverse tubule (T-) system which extends inwardly to the most axial myofibrils.

Beneath the sarcolemma lies an internal membraneous structure which encapsulates the myofilament lattice of each myofibril. Known as the sarcoplasmic reticulum (SR), this structure sequesters, stores and releases calcium. The SR consists of longitudinal compartments which terminate as blind endings. These endings, the terminal cisternae (TC), are situated adjacent to the T-system, with two TC located on opposite sides of a transverse tubule forming the functionally important triad.

The muscle fibre is composed of myofibrils which contain contractile proteins (myofilaments). The contractile proteins lie in close association with the SR and are arranged in an ordered fashion to form sarcomeres - the fundamental contractile units of skeletal muscle.

1.2 The contractile mechanism

The signal for a muscle fibre (in vivo) to contract is initiated at the level of the spinal cord or higher centres. An action potential travelling along a motor neuron terminates at the presynaptic side of the neuromuscular junction. The action potential causes the release of vesicles, containing the neurotransmitter acetylcholine (ACh), from the terminal axon into the cleft region. ACh interacts with nicotinic receptors located on the postsynaptic muscle fibre membrane, the motor end plate, causing a local depolarization, known as an end plate potential. A postsynaptic
Figure 1.1: Schematic diagram of a muscle fibre (from Eisenberg, Kuda & Peter, 1974).
action potential is generated when the end plate potential reaches a threshold membrane potential. Voltage sensitive ion channels in the sarcolemma of the muscle fibre (section 1.5) ensure that the action potential is propagated in all directions over the entire surface of the muscle fibre in the same way as impulses are conducted along nerve fibres.

The T-system, formed from invaginations in the sarcolemmal membrane, is responsible for the propagation of action potentials to the innermost myofibrils. Again, this is achieved by the activation of voltage sensitive ion channels in the T-tubule membrane (section 1.5).

In mechanisms that are yet to be fully elucidated, excitation-contraction (E-C) coupling encompasses the many processes that allow depolarization of the T-tubule membrane to activate contraction (section 1.9). A voltage sensitive molecule in the T-tubule membrane is responsible for transmitting changes in membrane potential to calcium release channels in the adjacent sarcoplasmic reticulum.

The massive increase in myoplasmic calcium, which occurs when stored calcium in the sarcoplasmic reticulum diffuses down its concentration gradient, allows interaction between contractile proteins to occur. Threshold levels of calcium in the confined region of the myofilament lattice uncouples the inhibitory effects of troponin over tropomyosin. A sideways shift in tropomyosin reveals sites on actin thin filaments suitable for myosin cross-bridge attachment. Cross-bridge cycling leads to force production when energy is released by the hydrolysis of ATP to ADP.

Relaxation occurs when myoplasmic calcium levels fall below threshold and allow inhibition of further cross-bridge cycling. The ATP-dependent SR Ca\(^{2+}\) pump (ie: Ca\(^{2+}\)-ATPase) sequesters Ca\(^{2+}\) into the lumen of the longitudinal SR for as long as the Ca\(^{2+}\) levels in the myoplasm are elevated. Other Ca\(^{2+}\) binding proteins in the myoplasm, such as calmodulin and parvalbumin, also have a regulatory role in the maintenance of myoplasmic calcium levels.

1.3 Skeletal muscle sarcolemma

The sarcolemmal properties of skeletal muscle differ somewhat from those of cardiac muscle. ie: during a skeletal muscle action potential, calcium entering a fibre via voltage sensitive ion channels in the sarcolemma is insignificant in terms of the overall calcium requirement for tension generation (Endo, 1977; Gonzalez-Serratos, Valle-Aguilera, Lathrop & Garcia, 1982) and is not required for contraction (section 1.9.5). This is in contrast to cardiac muscle where extracellular calcium influx is required to trigger calcium release from the SR and, in frog, where some of the calcium required for contraction is derived from the
extracellular medium via the sarcolemma (Eisenberg, Dix, Lin & Wenderoth, 1987; Ruegg, 1988). Nevertheless, the sarcolemma in skeletal muscle is essential for the maintenance of the membrane potential, propagation of the action potential and, most importantly, contains the voltage sensor for E-C coupling (For review see Stefani & Chiarandini, 1982).

1.4 The transverse (T-) tubule

Hill (1948) demonstrated that diffusion of an "activator" into the muscle fibre was too slow to activate the contractile mechanism. Since then, numerous studies have verified the importance of the transverse tubule (T-) system in conducting the action potential to the interior of the fibre to activate contraction (see section 1.6).

The membrane of individual T-tubules is continuous with the sarcolemma. The T-tubules are usually situated at the level of the Z-disc in amphibian muscle or in the region of the I-band in crustaceans, fish and muscles of higher vertebrates (Ruegg, 1988). The section of T-tubule near the opening in the sarcolemma follows a tortuous path with a varying diameter. Values quoted for the diameter of the T-tubule near the periphery of the fibre vary from 17-80 nm (Page, 1964; Huxley, 1964; Franzini-Armstrong & Porter; 1964; Franzini-Armstrong, Landmesser & Pilar, 1975; Luttgau & Stephenson, 1986).

1.5 Distribution of ion channels in the sarcolemma and T-system

Voltage sensitive ion channels are distributed throughout the sarcolemmal and T-system membranes. It is important to note that the actual density of channels within those membranes, together with their pharmacological properties, vary considerably (Caille, Ildefonse & Rougier, 1985). Variation in channel distribution is also apparent between amphibian and mammalian skeletal muscle.

The results of studies looking at the distribution of sodium channels between surface and T-tubular membranes differs considerably although it would appear that there is a lower Na channel density in the T-tubule. In amphibia, estimates of the ratio of surface to T-tubular Na channel density varies between 20:1 (Adrian & Peachey, 1973) down to 4:1 (Jaimovich, Venosa, Shrager & Horowicz, 1976). Le Treut, Boudier, Jover & Cau (1990) suggest that sodium channel density is lower in the T-system of mammals.

Eisenberg & Gage (1969) demonstrated that the delayed rectifier (a voltage dependent potassium channel), as well as resting potassium conductance are distributed between the surface and T-tubule membranes, while resting chloride
Conductance is located almost exclusively on the surface membrane. However, it has been proposed that chloride conductance contributes to the repolarization of the T-tubule action potential (see e.g., Heiny, Valle & Bryant, 1990). Reasons for reported differences were not clear. Bretag (1987) has compiled a comprehensive review of chloride channels in muscle.

The distribution of ionic conductances is different in mammals. While resting potassium conductance is distributed across the surface and T-tubular membranes (Dulhunty, 1979), chloride conductance has been localized mainly to the T-system (Palade & Barchi, 1977; Dulhunty, 1979; Chua & Betz, 1991). Palade & Barchi (1977) give a quantitative estimate of up to 80% of chloride conductance associated with the T-system.

Two major types of voltage-dependent calcium channel are present in mammalian skeletal muscle (Cognard, Traore, Potreau & Raymond, 1986; Hosey & Lazdunski, 1988). The T-type channel is found primarily in the sarcolemma while the L-type channel is situated in the T-system (Romey, Garcia, Dimitriadou, Pincon-Raymond, Rieger & Lazdunski, 1989). It has been shown that the majority of Ca$^{2+}$ channels in skeletal muscle are located in the T-system (Nicola Siri, Sanchez & Stefani, 1980; Potreau & Raymond, 1980). Since it is also recognised that the L-type Ca$^{2+}$ channel protein plays a key role in E-C coupling (Rios & Brum, 1987; Adams, Tanabe, Mikami, Numa & Beam, 1990), a more detailed description of calcium channel properties will be given in Section 1.8.

1.6 The skeletal muscle action potential

The propagation of the skeletal muscle action potential into and along the T-system is the first stage in the sequence of events that we know as excitation-contraction (E-C) coupling. Since the distribution of ion channels over sarcolemmal and T-system membranes is not uniform, some consideration must be given to changes in the time course of the action potential as it passes from the surface to the T-tubule membrane, together with possible physiological effects.

The changes in ionic conductances that occur during an action potential are similar in nerve and muscle (Adrian, Chandler & Hodgkin, 1970; reviewed by Luttgau & Stephenson, 1986). The upstroke in the action potential is due to an increase in the conductance of voltage-sensitive Na channels in the sarcolemma since the maximum amplitude of the action potential can be reduced by lowering extracellular sodium (Nastuk & Hodgkin, 1950) and abolished by tetrodotoxin (Adrian et al. 1970). Repolarization of the membrane potential occurs following inactivation of the Na channels (Hodgkin & Huxley, 1952; Simoncini & Stuhmer,
together with an increase in the conductance of the delayed rectifier potassium channel (Adrian et al., 1970).

The "local activation" experiments of Huxley and Taylor (1958) demonstrated that depolarization of the surface membrane at specific points (i.e., over openings to the T-system) could lead to a graded contraction within the fibre. Since the degree of contraction depended on the strength of the depolarizing current, an electrotonic, non-regenerative spread of excitation within the T-tubule was proposed. These experiments provided the first evidence that the site of activation of contraction was at the level of the T-system. Gage & Eisenberg (1969) performed the definitive experiment when they demonstrated that disruption of the T-system by glycerol treatment abolished twitches, while action potentials could still be recorded.

Despite the importance of localizing contraction to the T-system, Huxley & Taylor (1958) were incorrect in their assumption that the nature of the spread of excitation within the T-system was not regenerative. Many studies, beginning with Gonzalez-Serratos (1966), demonstrated the existence of a regenerative tubular action potential (Gonzalez-Serratos, 1966, 1971; Adrian, Costantin & Peachey, 1969; Costantin, 1970; Bastian & Nakajima, 1974; reviewed by Costantin, 1975; Caille et al. 1985). A description of the T-tubule action potential is important since it is responsible for the activation of contraction in the in vivo system.

Action potentials recorded from frog muscle fibres display a depolarization following the spike which is called an "early afterpotential." The afterpotential is caused by the slow recharging of membrane capacitance and has been attributed to an effect of action potentials in the T-system since it is abolished following glycerol treatment (Gage & Eisenberg, 1969).

The dimensions of the T-tubule (Section 1.4) make measurement of membrane potential and action potentials across the T-tubule membrane impossible with conventional electrophysiological techniques. Measurement of the T-tubule depolarization with potential sensitive dyes has provided a measure of the action potential in the T-system (Nakajima & Gilai, 1980a, 1980b, 1981; Heiny & Vergara, 1982, 1984; Delay, Ribalet & Vergara, 1986; Heiny et al. 1990). These optical studies prove conclusively that the T-tubule membrane contains activatable Na channels responsible for a regenerative action potential (Vergara & Bezanilla, 1981; Heiny & Vergara, 1982). Unfortunately, all studies of T-tubule action potentials have been on frog skeletal muscle which, as has already been mentioned, is discretely different from mammalian skeletal muscle. A complete analysis of the ionic currents underlying the T-tubule action potential in any species has yet to be performed.
1.7 The triad

A large body of information suggests that mediation between depolarization of the T-tubule membrane and calcium release from the SR occurs at a structure known as the triad. The triad, so named because it is composed of three anatomical units (Porter & Palade, 1957), is formed by the close apposition of two terminal cisternae of the sarcoplasmic reticulum to either side of a T-tubule. Mobley & Eisenberg (1975) have reported that, in frog, over 67% of the T-tubule membrane is surrounded by SR. This is significant since it means that a large area of the T-system is in intimate contact with the calcium containing SR. There is strong evidence to suggest that there is no direct electrical continuity between the SR and the T-system. In particular, membrane capacitance is too low to support continuity (Sabbadini & Dahms, 1989), and the SR and extracellular fluid have different ion distributions (Oetliker, 1982). Despite the fact that morphological studies also rule out direct continuity (see eg Somlyo, 1979), it has been proposed that an ion channel could exist through the foot structure (see below) and establish a link between the SR and the T-tubule (section 1.10).

The anatomy of the triad is complex. There is a 12-14nm gap (Franzini-Armstrong, 1970) between the membranes of the T-tubule and the terminal cisternae of the SR. Morphological evidence shows structures such as feet (Franzini-Armstrong, 1970), bridges (Somlyo, 1979), pillars (Eisenberg & Eisenberg, 1982) and rods (Dulhunty, 1988) in the triad junction.

1.8 Protein structure of the triad

In an attempt to elucidate a mechanism for E-C coupling, attention has focussed on proteins which reside in the T-tubule and sarcoplasmic reticulum membranes. It is thought that some of these proteins span the junctional gap and provide communication between the two membranes (see reviews by Ashley, Mulligan & Lea, 1991; Caswell & Brandt, 1989; Lai & Meissner, 1989).

1.8.1 The dihydropyridine (DHP) receptor of the T-tubule

A review of the biochemical and structural composition of the T-system membrane has been compiled by Sabbadini & Dahms (1989). One of the most important developments in this area has been the identification of receptors with a high affinity for dihydropyridine (DHP) compounds in the T-tubule membrane. It
has been shown that these receptors play a key role in E-C coupling (Rios & Brum, 1987; Tanabe, Beam, Powell & Numa, 1988; Adams et al. 1990) (Section 1.9.5).

In the late 1970's electrophysiological studies on skeletal muscle revealed a slow, voltage-dependent calcium current (Sanchez & Stefani, 1978) which was later shown in binding experiments to be preferentially localized in the T-system (Fosset, Jaimovich, Delpont & Lazdunski, 1983; Glossman, Ferry & Boschek, 1983). This current, known as an L-type calcium current, can be pharmacologically blocked by a variety of calcium channel antagonists including dihydropyridines (eg: nifedipine, nitrendipine) (Fosset et al. 1983; Lamb & Walsh, 1987), phenylalkylamines (eg: verapamil) and benzothiazepines (eg: diltiazem) (Galizzi, Borsotto, Barhanin, Fosset & Lazdunski, 1986) (reviewed by Catterall, Seagar & Takahashi, 1988; Tuana & Murphy, 1990). Ligand binding studies indicate that the three different classes of antagonists bind to separate, allosterically coupled sites on the channel protein.

Skeletal muscle T-tubule membranes possess DHP receptor sites that are 30-fold higher in density than in sarcolemmal membranes (Fosset et al. 1983). Consequently, T-tubule membrane preparations were used in a number of studies for the purification of the DHP receptor (Borsotto, Barhanin, Norman & Lazdunski, 1984; Borsotto, Barhanin, Fosset & Lazdunski, 1985; Curtis & Catterall, 1986). Recent research suggests that the DHP receptor consists of five subunits:

The α1 subunit is a 165 kDa protein which possesses the channel forming properties of the receptor together with the binding sites for agonist and antagonist drugs. The amino acid sequence of the α1 subunits of calcium and sodium channels show extensive homology (Trimmer & Agnew, 1989), with a structure consisting of four domains (I-IV) each with six transmembrane spanning peptides (S1-S6)(Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa & Kojima, 1987; Montal, 1990). The S3 segment of each domain is thought to contribute to a heterotetramer forming the pore of the DHP-receptor channel with ion conducting capabilities (Grove, Tomich & Montal, 1991). This has been proposed since the amino acid sequence of the S3 segment is largely conserved between skeletal muscle and isoforms of cardiac muscle, brain, and aorta (Tanabe et al. 1987; Mikami, Imoto, Niidome, Mori, Takashima, Narumiya & Numa, 1989; Snutch, Leonard, Gilbert, Lester & Davidson, 1990; Grove et al, 1991). The cDNA for the α1 subunit of skeletal muscle expressed in murine cells (with no known Ca-currents) yields DHP-sensitive, voltage-gated calcium channels (Perez-Reyes, Kim, Lacerda, Horne et al. 1989).
In rabbit skeletal muscle the $\alpha_1$ subunit coexpresses with $\alpha_2$, $\beta$, $\tau$- and $\delta$-subunits in a 1:1:1:1:1 stoichiometry (Chang & Hosey, 1990). De Jongh, Warner & Catterall (1990) have shown that the $\alpha_2$ subunit, a 143 kDa peptide, is disulphide linked to the 24-27 kDa $\delta$-subunit. The two subunits form a glycoprotein and are encoded by the same gene (De Jongh et al. 1990). Significantly, DHP-sensitivity and Ca channel activity can be generated in the absence of these subunits suggesting that they function in a modulatory role (Perez-Reyes et al. 1989; Kim, Wei, Ruth, Perez-Reyes, Flockerzi, Hofmann & Birnbaumer, 1990).

The remaining subunits, $\beta$ and $\tau$, have been shown to associate specifically with the $\alpha_1$ subunit (Catterall, 1991). The $\beta$ subunit is a 54 kDa protein which, like the $\alpha_1$ subunit, can be phosphorylated. Importantly, it has been shown that coexpression of the $\beta$- and $\alpha_1$ subunits in murine cells reveals Ca currents which have time courses 100 times faster than when $\alpha_1$ alone is expressed in the same cells (Lacerda, Kim, Ruth, Perez-Reyes et al. 1991). Therefore, it appears likely that the $\beta$-subunit is responsible for modifying the activation kinetics of the DHP-sensitive Ca channel. While the primary structure of the 25 kDa $\tau$-subunit has been described (Jay, Ellis, McCue, Williams, Vedvick Harpold, 1990), its role is not fully understood. Co-expression of the $\tau$-subunit with the $\alpha_1$ subunit of the DHP receptor has been shown to significantly increase the rate of inactivation of Ca$^{2+}$ currents in oocytes (Singer, Biel, Lotan, Flockerzi, Hoffman & Dascal, 1991).

\subsection*{1.8.2 The ryanodine receptor of the terminal cisternae}

Studies with the plant alkaloid ryanodine, have been instrumental in analysing the major junctional protein which spans the gap separating T-system and SR membranes and forms the calcium release channel in the SR (the physiological actions of ryanodine have been reviewed by Jenden & Fairhurst (1969)). The direct binding of low concentrations of ryanodine to receptors on the terminal cisternae ("locking" calcium release channels in an open state with a lower than normal conductance) was first demonstrated by Fleischer, Ogunbunmi, Dixon & Fleer (1985).

Preliminary studies leading to the identification of junctional gap proteins showed that the "foot" structure, originally described by Franzini-Armstrong (1970), was a high molecular weight doublet (Cadwell & Caswell, 1982).
Kawamoto, Brunschwig, Kim & Caswell (1986) raised monoclonal antibodies against this protein and suggested that it consisted of a 300 kDa dimer subunit, with four such subunits forming the intact spanning protein. A major breakthrough came in 1987 with the revelation that the ryanodine receptor was in fact the junctional foot protein (JFP) (Inui, Saito & Fleischer, 1987). More importantly however, were electrophysiological studies which demonstrated that the purified ryanodine receptor formed Ca\(^{2+}\) - activated Ca\(^{2+}\) channels in lipid bilayers (Imagawa, Smith Coronado & Campbell, 1987; Hymel, Inui, Fleischer & Schindler, 1988; Lai, Erickson, Rousseau, Liu & Meissner, 1988; Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988; Liu, Lai, Rousseau, Jones & Meissner, 1989). ie: the ryanodine receptor, the Ca\(^{2+}\) release channel of the terminal cisternae and the junctional foot protein are one and the same structure.

Block, Imagawa, Campbell & Franzini-Armstrong (1988) have described the architecture of the molecular units which make up the triad junction. The ryanodine receptor/foot protein appears in electron micrographs as a tetrameric structure which extends 12 nm from the face of the terminal cisternae membrane. There appears to be a hole (2nm diameter) in the middle of this structure with four others located around its' periphery (Wagenknecht, Grassucci, Frank, Saito, Inui & Fleischer, 1989). On the apposing T-tubule membrane diamond shaped clusters of particles form tetrads. Morphological and binding studies suggest that it is likely that the tetrads are four DHP receptors lined up opposite the foot proteins (Block et al. 1988; Anderson, Grunwald, El-Hashem, Sealock & Meissner, 1990; reviewed by Ashley et al. 1991).

Other proteins also contribute to the anatomy of the junctional gap. Kim et al. (1990) have described a 95 kDa protein, triadin, which interacts with both the DHP receptor and the JFP. In addition, a second bridging protein, glyceraldehyde-3-phosphate dehydrogenase (GAPD), has been described by Brandt, Caswell, Wen & Talvenheimo (1990). GAPD is a 36 kDa protein, and has previously been shown to promote junctional formation between isolated T-tubule and terminal cisternae membranes (Corbett, Caswell, Brandt & Brunschwig, 1985). Kim et al. (1990) also demonstrated the presence of a third, 170 kDa protein, in the junctional region. It was proposed that this protein interacted with triadin and served as an anchor to calsequestrin in the terminal cisternae of the SR.
1.9 EXCITATION-CONTRACTION COUPLING

1.9.1 Voltage dependence of activation

Kuffler (1946) was one of the first workers to use elevated potassium in bathing solutions to induce contractures (K-contractures) in muscle. From these experiments, and the classic contracture studies of Hodgkin & Horowicz (1960a) it was demonstrated that the degree of activation of contraction was graded and not dependent on action potentials alone, but rather, on the extent of membrane potential depolarisation.

In addition to high potassium concentrations, depolarization by voltage clamp (Heistracher & Hunt, 1969; Caputo & De Bolanos, 1979; Caputo, Bezanilla & Horowicz, 1984) and skinned fibre techniques (Donaldson, 1985; Stephenson, 1985; Lamb & Stephenson, 1990a, b), have been used to illustrate the voltage dependence of contraction.

When considering the process of E-C coupling, four key factors are widely accepted:
(a) The site where depolarization initiates contraction is in the T-tubule.
(b) There is a voltage sensor in the T-tubule membrane.
(c) There is a calcium release channel in the SR membrane.
(d) The contractile process is activated when myoplasmic calcium concentrations reach a threshold level.

Our current understanding of the mechanism of E-C coupling is based on the following observations of the response of the voltage sensor to depolarization and on the calcium release channel in the SR.

1.9.2 Asymmetric Charge Movement

In 1973, Schneider & Chandler used the three microelectrode voltage clamp technique (pioneered by Adrian et al. 1970) to describe a phenomenon which they termed "asymmetric charge movement". The charge movement is thought to reflect the reorientation of charge in the muscle fibre membrane following a change in the membrane electrical field. Charge movement can be pharmacologically and electrophysiologically separated into component parts (see eg: reviews by Huang, 1988; Rios & Pizarro, 1991). Charge I is sigmoidally related to membrane potential (Chandler, Rakowski & Schneider, 1976a, b; Dulhunty & Gage, 1983, 1985;
Lamb, 1986a, b; Melzer, Schneider, Simon & Szucs, 1986) and, importantly, is located in the T-tubule (Chandler et al. 1976b, Dulhunty, Gage & Barry, 1981). In addition, it moves in the voltage range for contraction threshold (Schneider & Chandler, 1973; Horowicz & Schneider, 1981a, b; Adrian & Huang, 1984) and it closely follows the activation and inactivation of tension (Huang, 1982; Hui, 1983)(see section 1.12).

Significantly, charge I always appears to precede calcium release (Kovacs, Rios and Schneider, 1979, Melzer et al. 1986) although it would seem that calcium release is responsible for a "hump" component (Q₉) in the current record (Csernoch, Pizzaro, Uribe, Rodriguez & Rios, 1991). The amount of charge moved corresponds closely to the rate of Ca²⁺ release (Rakowski, Best & James-Kracke, 1985; Melzer et al. 1986), which lends credence to a role of asymmetric charge movement in E-C coupling.

1.9.3 Components of Asymmetric Charge Movement

Adrian & Almers (1976a,b) and Schneider & Chandler (1976) independently demonstrated the existence of a second form of charge, distinct from charge I. Charge 2, also sigmoidally related to membrane potential, is seen as a change in fibre capacitance at depolarized membrane potentials. This charge has characteristics that differ from Charge 1 (Chandler et al, 1976a,b). Charge 2 is seen in depolarized fibres following inactivation of Charge I. Charge 2 exhibits a lower sensitivity to changes in membrane potential and is of lesser magnitude compared to Charge 1. Adrian & Almers (1976b) describe both Charge 1 and Charge 2 as representing "redistribution of charges or dipoles within the membrane, resulting from changes in the configuration of charges on proteins or other molecules which are influenced by the electric field in the membrane."

Adrian & Peres (1977) proposed that one physiological role of charge movement was to provide a gating signal for the delayed rectifier potassium channel. However, a later paper (Adrian & Peres, 1979) questioned this theory on the grounds of physical and kinetic anomalies. In addition, this paper described fundamental properties of charge movement which have provided the basis for further study into mechanical inactivation. Adrian & Peres (1979) described charge movement as consisting of three component charges - a rapid component termed Qᵦ, a slower component, Q₉, and third charge (Qₐ) which appears following prolonged depolarization and is the same as charge 2 (described by Adrian and Almers, 1976a; Adrian, Chandler & Rakowski, 1976). Charge 1 (Schneider &
Chandler, 1973) was shown to consist of the sum of $Q_B$ and $Q_r$. Subsequent studies have provided additional information on the kinetic properties and pharmacology of these component charges (reviewed by Huang, 1988; Rios & Pizarro, 1991). A model proposed by Csemoch et al. (1991) infers that $Q_B$ is responsible for opening calcium channels in the SR. The released calcium binds to sites on the T-tubule membrane thereby negating surface potential and inducing an additional charge movement ($Q_r$).

The situation in mammalian preparations is quite different. A species of charge with the characteristics of $Q_r$ is virtually absent in rabbit (Lamb, 1986b) and has not been demonstrated in rat (Hollingworth & Marshall, 1981; Dulhunty & Gage, 1983; Hollingworth, Marshall & Robson, 1990). Interestingly, it has been shown that tetracaine reduces charge only when the bathing solution is hypertonic. Isotonic solutions containing tetracaine have no effect on charge (Hollingworth et al. 1990). Therefore it seems plausible that tetracaine is acting primarily at another step in the E-C coupling mechanism.

1.9.4 Charge movement and the DHP-sensitive $Ca^{2+}$ channel

One of the most important discoveries in E-C coupling was that charge movement and the release of Ca from the SR could be simultaneously immobilized by the presence of low concentrations of dihydropyridines (Rios & Brum, 1987). Since slow $Ca^{2+}$ currents in the T-tubule membrane and Charge I can be blocked with dihydropyridines in a voltage dependent manner (Lamb & Walsh, 1987; Cognard, Rivet & Raymond, 1990), this result provided the first evidence that the DHP receptor might generate the asymmetric charge movement and be a critical link in E-C coupling.

1.9.5 The role of the DHP receptor in E-C coupling

The reason why the T-system is so rich in DHP receptors is unclear since less than a few percent form functional $Ca^{2+}$ channels (Schwartz, McCleskey & Almers, 1985) (although this proposal has been questioned (Lamb, 1991)). Nevertheless, the calcium current generated by these channels is too slow to be involved in the generation of action potentials (Sanchez & Stefani, 1978) and external calcium itself is not essential for the generation of twitches, tetani or K-contractures (Armstrong, Bezanilla Horowicz, 1972; Luttgau & Spiecker, 1979; Cota & Stefani, 1981; Miledi, Parker & Zhu, 1984; Dulhunty & Gage, 1988). The role of external calcium is most likely one of modulation of the E-C coupling

Confirmation of the role of the DHP receptor in E-C coupling came with the elegant experiments of Numa's group. Muscular dysgenesis is a fatal autosomal recessive mutation (Gluecksohn-Waelsch, 1963) in which the slow Ca$^{2+}$ current and charge movement are absent and there is a failure of E-C coupling (Beam, Knudsen & Powell, 1986; Rieger, Bournaud, Shimahara, Garcia, Pincon-Raymond & Romey, 1987; Adams et al. 1990). Tanabe et al. (1988) demonstrated that E-C coupling could be restored in myotubes from embryonic dysgenic mice by microinjection of the cDNA for the DHP receptor. This is accompanied by a coincident restoration of charge movement (Adams et al. 1990). Furthermore, Tanabe et al. (1990) injected cDNA for the cardiac DHP receptor into dysgenic skeletal muscle myotubes and induced cardiac-like E-C coupling, ie: a slow Ca$^{2+}$ current and a dependency on extracellular Ca$^{2+}$ concentration.

In contrast to cardiac muscle, extracellular Ca$^{2+}$ is not required for skeletal muscle E-C coupling (Armstrong et al. 1972; Dulhunty & Gage, 1988; Dulhunty, 1992). This would suggest that subtle differences exist in the DHP receptors for skeletal and cardiac muscle. Subsequently in another series of experiments, Tanabe, Beam, Adams, Niidome & Numa (1990) demonstrated that the cytoplasmic regions linking domains I & II, and II & III are critical in the determination of skeletal-type E-C coupling.

1.10 MODELS FOR EXCITATION-CONTRACTION COUPLING

While there is now a great deal of information about E-C coupling, the question still remains: what is the mechanism that opens Ca$^{2+}$ release channels on the surface of the terminal cisternae? Several mechanisms (listed below) have been postulated and evaluated in recent reviews by Rios, Ma & Gonzalez (1991), Rios & Pizarro (1991) and Ashley et al. (1991).

Direct coupling of T-system and SR membranes.

Mathias, Levis & Eisenberg (1981) proposed a model for E-C coupling where a transient ionic current couples the T-system with the SR. In this way an action potential is propagated across the junction separating the two membranes and initiates Ca$^{2+}$ release by depolarizing the SR. This mechanism has been questioned because it cannot account for conductance and capacitance properties of
the surface membrane (Oetliker, 1982; Ashley et al. 1991) and because of the lack of evidence demonstrating the presence of a T-SR channel (Gilly, 1981). Also, there is no evidence for a potential across the SR membrane (Somlyo, Gonzalez-Serratos, Shuman, McClennan & Somlyo, 1981). However, it would appear that SR Ca^{2+} channels do in fact demonstrate a strong voltage dependence in both native form, and when incorporated into lipid bilayers (Smith, Coronado & Meissner, 1985, 1986), and "sarcoballs" (Stein & Palade, 1988). Interpretation and comparison of these results needs to be done with caution since there may be more than one type of Ca^{2+} channel in the SR (Dulhunty, 1991).

**Calcium-induced calcium release (CICR)**

It is well known that an influx of calcium across the sarcolemma is essential for E-C coupling in cardiac muscle because it provides a calcium source for the induction of CICR. It is also clear that external calcium is not required for E-C coupling in skeletal muscle (section 1.9.5 above). However, various studies have shown that CICR could possibly be initiated by calcium released from internal calcium stores. Supporting a role for CICR in E-C coupling, it has been demonstrated that Ca^{2+} ions can augment the release of Ca^{2+} from the SR in skinned fibres under certain conditions (Stephenson, 1981) and increase the probability of opening of SR Ca^{2+} channels incorporated into lipid bilayers (Smith et al. 1986). In addition, the effects of CICR are enhanced in MH swine (Fill, Coronado, Mickelson, Vilven, Ma, Jacobson & Louis, 1990). Klein, Simon & Schneider (1990) have also shown that, in the presence of caffeine, CICR contributes significantly to the activation of SR calcium release. They could not rule out a contribution of CICR during depolarization in the absence of caffeine. These studies demonstrate that CICR is important in the E-C coupling mechanism.

**Inositol trisphosphate (IP_3).**

IP_3 as a chemical link in E-C coupling was proposed by Vergara, Tsien & Delay (1985) when they observed increased levels of IP_3 in electrically stimulated fibres. Numerous studies have also reported contractures in skinned skeletal muscle fibres exposed to IP_3 (Vergara et al. 1985; Volpe, Salvati, Di Virgilio & Pozzan, 1985; Donaldson, Goldberg, Walseth & Huetteman, 1987; Rojas & Jaimovich, 1990). In addition, IP_3 precursors have been isolated from T-tubule membranes (Hidalgo & Jaimovich, 1989). It is interesting to note that some studies have reported that IP_3 activates ryanodine-sensitive Ca^{2+} channels in SR vesicles.

There is a body of evidence which suggests that IP$_3$ is not directly involved in E-C coupling (reviewed by Hidalgo & Jaimovich, 1989; Ashley et al. 1991). This includes (a) the time course of contractures due to IP$_3$-induced Ca$^{2+}$ release from the TC being too slow to be of physiological importance and (b) the degradation of IP$_3$ by enzymes being too slow to account for the speed of termination of Ca$^{2+}$ release during a twitch.

The general consensus of opinions now favour a modulatory role for IP$_3$ in E-C coupling.

**The mechanical hypothesis for E-C coupling**

This putative mechanism for E-C coupling remains speculative but is the most popular at present. First proposed by Chandler *et al.* (1976b), the theory predicts that charged particles in the T-tubule membrane are mechanically linked to the SR membrane via a rigid rod connected to a plug. The calcium release channel in the SR is blocked by the plug in the resting position. In the activated position the charged particles move across the T-tubule membrane causing the plug to be removed from the Ca$^{2+}$ release channel in the SR.

Present day variations of this model are consistent with much of the molecular structure of the triad junction (reviewed by Rios *et al.* 1991). The three dimensional structure of the SR calcium release channel proposed by Wagenknecht *et al.* (1989) shows that the junctional proteins could act as the "plungers" in Chandler *et al.*'s (1976b) original hypothesis. Etter (1990) proposed that arginine residues in the S$_4$ segment of the DHP receptor protein could move the plungers. The movement of a large number of these charged residues could displace a protein with a high molecular mass over a very small distance and thus generate charge movement (Rios *et al.* 1991).

Experimental evidence favouring this mechanism for E-C coupling is scarce. The DHP receptor and the ryanodine receptor/calcium release channel of the SR do not appear to have a high affinity for each other. In addition, other complicating factors need to be addressed. ie: do all Ca$^{2+}$ release channels reside in the junctional region or do the extra-junctional ryanodine receptors described by Dulhunty, Junankar & Stanhope (1991) also play a crucial role? The calcium concentration gradient across the terminal cisternae would be abolished if extrajunctional Ca$^{2+}$ were not present. ie: if all channels were in the junctional
face, the enormous efflux of calcium into the gap region would negate the calcium concentration gradient and prevent calcium release (Dulhunty, Junankar & Stanhope, 1991; Dulhunty, 1992). It would be difficult to explain the release of calcium from extra-junctional regions with the "plunger" hypothesis.

1.11 HISTORICAL PERSPECTIVES ON ACTIVATION AND INACTIVATION OF EXCITATION-CONTRACTION COUPLING

In order to initiate E-C coupling, the voltage sensor in the T-system membrane must be able to undergo conformational changes. A variety of conformational states have been attributed to the voltage sensitive molecule.

1.11.1 Contractile Inactivation

Contractile inactivation is a phenomenon that (a) causes the slow decay of voltage activated tension if depolarization is maintained and (b) reduces maximum tension with further depolarization (Hodgkin & Horowicz, 1960a). Upon application of a 190 mM potassium-containing solution to a single muscle fibre, or small bundle of fibres, tension rapidly increases and then undergoes a slowly declining plateau followed by a rapid exponential decay. This phenomenon can be explained in terms of a model describing the conformational state of the voltage sensor:

\[ P \Leftrightarrow A \Leftrightarrow I \]

Depolarization rapidly converts the voltage sensor from the precursor state (P) to the active form (A). The conversion is faster with greater depolarization. Contraction occurs in the presence of a threshold concentration of A. This accounts for the rising phase of the contracture, the steepness of which is related to the amount of depolarization (see below). If the membrane potential is depolarized for a prolonged period of time, a percentage of activator is converted to an inactive end product (I). The tension generated is linearly dependent on the concentration of activator, whose availability is dependent on the level of precursor, some of which may already have been converted by depolarization to the inactive state.
1.11.2 Force - membrane potential relationship

The relationship between peak force and membrane potential in skeletal muscle is examined under different conditions in several sections of this thesis. Hodgkin & Horowicz (1960a) were first to describe this relationship. They induced contractures in single muscle fibres of the frog and demonstrated an S-shaped "activation" curve relating force to the logarithm of the external potassium concentration ([K]_0). Initial tension generation, or contraction threshold, occurred in the presence of low (20mM) [K]_0. Thereafter tension was graded in a manner which was steeply membrane potential dependent (as determined by [K]_0) until a maximal plateau level reached around -25mV ([K]_0=100mM).

Alternatively, maximum test contracture tension can be plotted as a function of membrane potential achieved after a period of time in a conditioning solution. The conditioning solution depolarizes the membrane potential and results in the steady-state conversion of A to I (above). With large conditioning depolarizations the maximum height of a test contracture decreases as less P is available for conversion to A. A plot of peak test contracture tension against conditioning membrane potential is known as an "inactivation" curve.

1.11.3 Effects of external calcium concentration on force production and inactivation

The role of external calcium in modulating skeletal muscle contraction has been recognized for many years (see eg: Denton, 1948; Bianchi & Shanes, 1959; Frank, 1960). Although external calcium is not necessary for E-C coupling, it has been shown to alter the kinetics of activation and inactivation (Lüttgau, 1963; Caputo & Giminez, 1967; Lüttgau & Spiecker, 1979). Lüttgau (1963) was first to describe the effect of external calcium on the time course of the potassium contracture in single frog muscle fibres. He observed a progressive shortening of the plateau phase of the contracture when external calcium was lowered to less than 4 mM. In addition, a negative shift in the curve relating tension (i.e. the height of a test contracture following a conditioning depolarization for a set period of time - section 1.11.2) to membrane potential was described. The term "contractile inactivation" was coined by Lüttgau (1963) to describe the decay in tension which accompanies prolonged depolarization.

Lüttgau (1963) proposed that an inward flux of calcium was responsible for the "... shift of an activator of contraction from the outside to the inside of the membrane..." Caputo & Giminez (1967) also examined the effect of external
calcium on E-C coupling and suggested that since contractile processes still took place in the absence of extracellular calcium, the translocation of calcium rather than an influx was responsible for mediating the contractile response.

Further studies by Frankenhaeuser & Larnergren (1967) confirmed many of the findings of previous workers (Hodgkin & Horowicz, 1960a; Lüttgau, 1963; Foulks & Perry, 1966; Caputo & Giminez, 1967). They also demonstrated changes in the voltage sensitivity of peak tension generation when the extracellular calcium concentration was altered. This effect was attributed to membrane potential depolarization in the absence of external calcium. Importantly, these authors postulated that the action of calcium was to provide a "stabilizing" effect at a specific site on the cell membrane.

1.11.4 Caffeine and contractile inactivation

Caffeine was shown by Axelsson & Thesleff (1958) to activate the contractile mechanism in a persistent manner without surface membrane depolarization. In isolated skeletal muscle fibres of the frog, Lutgau & Oetliker (1968) induced maximal tension with caffeine in fibres that were mechanically inactive when tested with a depolarization.

In theory, the contracture was induced by bypassing the inactivated voltage sensor. Importantly, the voltage sensor was considered to occupy a site within the T-system. We now know that caffeine activates the ryanodine receptor calcium release channel (see reviews by Fill & Coronado, 1988; Lai & Meissner, 1989).

1.11.5 Contractile inactivation measured under voltage clamp conditions

The development of the voltage clamp technique allowed further study of mechanical inactivation. Heistracher & Hunt (1969) used short,twitch-type fibres located between adjacent scales in the skin of snakes. Force was measured in response to voltage steps applied using a two-microelectrode voltage clamp technique. Mechanical inactivation similar to that observed by Hodgkin & Horowicz (1960a) was seen. ie: a decay in tension following a prolonged depolarization. In addition, a novel depolarizing pulse protocol was used to study the effect of instantaneous changes in membrane potential on the time-course of inactivation. The time-course of contractile inactivation was shown to be membrane potential dependent. While the slow rise and fall of tension in low potassium solutions had been previously observed (Hodgkin & Horowicz, 1960), the rate of inactivation was not entirely dependent on the rate of activation. ie: a fibre which is
rapidly depolarized well in excess of contraction threshold would, under normal circumstances, rapidly inactivate. In the study by Heistracher & Hunt (1969) membrane potential was quickly changed from a very depolarized level to a less depolarized level (still more positive than contraction threshold) after the peak of the contracture. Immediately, the time-course of inactivation slowed to a level determined by the new membrane potential, demonstrating that the rate of contractile inactivation is dependent on the membrane potential of the fibre.

Caputo & De Bolanos (1979) also studied the dependency on membrane potential of tension relaxation during prolonged depolarization. In voltage clamp experiments, the relaxation rate of tension following short (< 200ms) depolarizing pulses depended on whether the membrane potential was either partially or fully repolarized. If the repolarized membrane potential was still more positive than the contraction threshold, additional activator was released and the time course of relaxation was prolonged by an amount that was dependent on the new membrane potential. If the repolarization was to a membrane potential more negative than contraction threshold, relaxation was independent of the new membrane potential.

1.11.6 Effects of temperature on inactivation and repriming

The pioneering work of Hodgkin & Horowicz (1960a) demonstrated "repriming," ie: the ability of a fibre to restore its contractile response following inactivation with prolonged depolarization. Caputo (1972) carried out an extensive examination of the temperature dependence of the repriming process in single muscle fibres of the frog.

In particular, Caputo (1972) showed that the time course of a contracture was significantly prolonged when the temperature was reduced from 20°C to 3°C. The slower time course of the 3°C potassium contracture was attributed to a slower release and more efficient usage of calcium from the SR. In contrast to events occurring at 20°C, Caputo (1972) demonstrated that the repriming process was severely slowed when the muscle fibre was maintained at 3°C.

1.11.7 Further effects of low [Ca^{2+}]_o and other divalent cations on activation and inactivation of E-C coupling

Armstrong et al. (1972) used EGTA to study the role of external calcium in E-C coupling. In the presence of < 10^{-5}M calcium, twitches could be elicited 15-20 mins after immersion of single fibres in the EGTA ringer. It was proposed that the slow abolition of twitches was a result of progressive membrane depolarization
induced by 1mM EGTA. The authors suggested that an influx of calcium played no role in triggering contraction.

Initial work by Lüttgau (1963) demonstrated a specific "stabilising" role of external calcium on the time course of potassium contractures. Stefani & Chiarandini (1973) also showed smaller and shorter contractures in low external Ca$^{2+}$, while Armstrong et al. (1972) believed that an influx of Ca$^{2+}$ was not a prerequisite for a twitch. However, in contrast to these findings, Barrett & Barrett's (1978) results using high concentrations of EGTA in voltage clamped fibres suggested an important role for external Ca$^{2+}$ in E-C coupling.

These conflicting reports on the role of external calcium in E-C coupling prompted Lüttgau and Spiecker (1979) to re-investigate the area. Using standard K-contracture techniques these authors measured tension in single muscle fibres of the frog. The solutions they used contained different concentrations of Ca$^{2+}$; from above physiological (10^{-3}M) to well below this level (10^{-9}M). Effects shown were a reduction in the duration of the plateau of maximum force (30-50%) and an increase in the half-time of relaxation (0-200%). Maximum force decreased by < = 15%. A later study by Lüttgau, Gottschalk & Berwe (1987) also demonstrated a slowed recovery of maximal tension following prolonged depolarization and a negative shift in the steady-state potential dependence of force inactivation in low calcium solutions. Similar results on the effects of low calcium solutions on the steady-state inactivation curve have been reported by Cota & Stefani (1981).

Barrett & Barrett (1978) suggested that very high concentrations of EGTA were required to lower Ca$^{2+}$ concentrations in the vicinity of the T-tubules. In a second series of experiments, Lüttgau & Spiecker (1979) used high concentrations of EGTA (80mM) together with 5mM MgCl$_2$ added to the solution (low Ca$^{2+}$ concentrations in EGTA depolarize the membrane, but this effect is lessened by the addition of Mg$^{2+}$). Results showed that twitches and tetani could still be elicitted more than an hour after the preparation was placed in the high EGTA solution. Barrett & Barrett's results could therefore be explained by depolarization since they did not use Mg$^{2+}$ to maintain membrane potential.

Lüttgau (1963) showed that Mg$^{2+}$ has similar effects to Ca$^{2+}$ in maintaining the resting membrane potential and retarding inactivation. The more powerful effect of Ca$^{2+}$ on slowing the time course of inactivation could be due to the inflow of Ca$^{2+}$ through voltage sensitive channels upon depolarisation (Stefani & Chiarandini, 1973). Lüttgau & Spiecker (1979) used the Ca$^{2+}$ channel blocker D600 to test this theory. The effect of D600 was similar to that seen in low Ca$^{2+}$ solutions and therefore in agreement with Stefani & Chiarandini (1973).
Activation and inactivation curves for contraction showed that neither the threshold potential, maximum tension, nor the shape of the activation curve was significantly altered by the replacement of Ca^{2+} with Mg^{2+}. However, the steady-state inactivation curve was shifted by 20-30mV towards more negative potentials (Lüttgau & Spiecker, 1979).

Finally, Lüttgau & Spiecker (1979) showed that the effects of high EGTA on twitch force provided results which supported Armstrong et al. (1972) by showing that the initiation of contraction is not dependent on the influx of Ca^{2+}. However, one effect of Ca^{2+} is to contribute to the coupling process by delaying the onset of inactivation.

1.11.8 Characterization of E-C coupling processes by modification of the response of the voltage sensor

The effects of numerous cations, anions and drugs on E-C coupling have revealed much about the activation and inactivation processes. A brief summary is given below:

1) Cations

In addition to information already presented on the effects of low external calcium concentrations (sections 1.11.3 & 1.11.7), Dulhunty (1991) reported that solutions containing 20 mM EGTA and 10 mM Mg^{2+} have a minimal effect on contractures in mammalian preparations. An exciting observation by Caputo (1981) showed that nickel (Ni^{2+}), substituted for calcium in the bathing solution, reversed all the effects on K-contractures in frog muscle caused by low external calcium. Nickel does not carry current through calcium channels (Almers & Palade, 1981). It was postulated that Ni^{2+} acted at a site near the T-tubule and SR junction which regulates E-C coupling, including contractile inactivation. Similar results have been reported by Lorkovic & Rudel (1983).

Several other cations have also been shown to influence contraction. Ba^{2+} (1.8mM) is an ineffective substitute for Ca^{2+} in the external bathing solution, producing similar effects on the inactivation of tension as low Ca^{2+} (Bolanos, Caputo & Velaz, 1986). Lanthanum (< 1mM) has a similar effect to elevated calcium in that it also prolongs the time course of potassium contractures in frog muscle by an action at the level of the voltage sensor (Andersson & Edman, 1974; Bolanos et al. 1986).
It is generally agreed that the action of cations on inactivation in frog muscle is by their screening effect on fixed negative charge located on the extracellular (T-system) membrane. Dulhunty & Gage (1989) reported that many effects of divalent cations on both activation and inactivation of contraction could not be attributed to a general screening of surface charge in mammalian skeletal muscle. Cadmium, cobalt and magnesium depressed tension in a manner that suggested binding of cations to specific sites rather than screening, since there was an order of effectiveness (Cd\(^{2+}\) > Co\(^{2+}\) > Mg\(^{2+}\)) which was the same as for the depression of calcium currents.

2) Anions

The effect of anions on contractile activation has been well documented (Hodgkin & Horowicz, 1960b; Foulks, Miller & Perry, 1973a, b; Gomolla, Gottschalk & Lüttgau, 1983; Lüttgau, Gottschalk, Kovacs & Fuxreiter, 1983; Dulhunty, 1988; Dulhunty, Zhu, Patterson & Ahern, 1992). Numerous anions, in particular perchlorate, (ClO\(_4^-\)) have been shown to produce a negative shift in the activation curve relating peak tension to membrane potential in both frog and mammalian skeletal muscle (Foulks et al. 1973; Dulhunty et al. 1992). Perchlorate has little effect on the the steady-state curve for inactivation in frog (Foulks et al. 1973; Gomolla et al. 1983). However, in mammals it has been shown to produce a negative shift in the steady-state inactivation curve and to expose a "slow" component in the decay of K-contracture tension (Dulhunty et al. 1992).

The anions NO\(_3^-\) and SCN\(^-\) potentiate twitches and K-contracture tension (Hodgkin & Horowicz, 1960b; Sandow, 1965). This has been attributed to an effect of the anions on the T-system membrane since the potentiation is seen soon after addition of the ions to the bathing solution (see eg: Nagai, Takauji, Kosaka & Tsutsu-Ura. 1979). However, the potentiating effect on K-contractures and more recent evidence showing inhibition of inactivation (Nagai et al. 1979), could be due to a direct action on the SR since caffeine contractures are potentiated (Matsushima, Fujino & Nagai, 1962; Nagai, Oota & Nagai, 1978) and calcium uptake by isolated SR is inhibited in the presence of the anions (Nagai, 1959-cited in Nagai et al. 1979; Ebashi, 1965).

3) Calcium channel blockers

Calcium channel blockers (dihydropyridines, phenylalkylamines and benzothiazepines - see section 1.8.1) have provided an additional tool for the
analysis of events associated with the inactivation of tension. In addition to effects on calcium currents (Eisenberg, McCarthy & Milton, 1983; Gottschalk & Luttgau, 1985; Ildefonse et al. 1985; Avila-Sakar, Kota, Gamboa-Aldeco, Huerta, Muniz & Stefani, 1986; Rios, Brum & Stefani, 1986) and charge movement (Hui, Milton & Eisenberg, 1984; Lamb, 1986a, b; Rios & Brum, 1987), calcium channel blockers also have a profound effect on the kinetics of isometric twitch and tetanic contractions and K-contractures (Luttgau & Spiecker, 1979; Eisenberg et al. 1983; Gallant & Goettl, 1985; Berwe, Gottschalk & Luttgau, 1987; Caputo & Bolanos, 1987; Luttgau et al. 1987; Rakowski, Olszewski & Paxson, 1987; Dulhunty & Gage, 1988; Gamboa-Aldeco, Huerta & Stefani, 1988; Erdmann & Luttgau, 1989; Neuhaus, Rosenthal & Luttgau, 1990).

The mechanism by which calcium channel blockers act is complex. Luttgau et al. (1987) proposed that diltiazem (a benzothiazepine) and D600 (a phenylalkylamine) acted at a calcium receptor site located on the voltage sensor for force activation. Inactivation of the voltage sensor is dependent on calcium dissociation from this site. By accelerating the release of calcium from this site, the drugs increase the rate of inactivation of the voltage sensor and send the muscle fibre into a paralysed state. It has been shown that D600 acts in a temperature and voltage dependent manner (Eisenberg et al. 1983; Berwe et al. 1987; Caputo & Bolanos, 1987).

Reports on the actions of the dihydropyridine class of calcium channel blockers are conflicting. Under appropriate conditions, DHP's have been shown to depress contraction (Ildefonse et al. 1985; Avila-Sakar et al. 1986; Rios et al. 1986), to potentiate it (Gallant & Goettl, 1985) or to have no effect (Lamb, 1986b; Neuhaus, 1986; Luttgau et al. 1987; Rakowski et al. 1987). The results can be explained in terms of the effects of (a) the extracellular calcium concentration and (b) the extent of depolarization (Dulhunty & Gage, 1988). Briefly, Dulhunty & Gage (1988) identified the following characteristics of calcium and DHP interactions with contractions in mammalian muscle:

(a) Contractions were initially potentiated, then depressed with time in low calcium solutions.
(b) Nifedipine potentiated contractions in 2.5 mM calcium solutions, but depressed contractions in low calcium solutions.
(c) Potassium contracture amplitude was relatively unaffected by nifedipine in presence of 2.5mM calcium. However nifedipine depressed contracture amplitude in bathing solutions containing low calcium.
(d) Nifedipine accelerated the rate of decay of potassium contractures at all calcium concentrations.
In an extension of previous models for activation and inactivation of E-C coupling (see eg. Hodgkin & Horowicz, 1960a; Lüttgau et al. 1987; Dulhunty & Gage, 1988), Dulhunty (1991) proposed that the voltage sensor for force production could reside in five possible states:

$$P \rightleftharpoons Q \rightleftharpoons A + Ca^{2+} \rightleftharpoons I \rightleftharpoons Y + Ca^{2+} \downarrow C \rightleftharpoons O.$$ 

P, A and I have the same meaning as described in section 1.11.1. The conversion of P to Q is a voltage dependent step which generates charge (Q). Y is a second inactive state (see below). The number of SR calcium channels in the open configuration is proportional to the concentration of activator (A). To account for enhanced activation in low external calcium (eg: the potentiation of twitch height) (Graf & Schatzman, 1984; Lüttgau et al. 1987; Brum et al. 1988), the dissociation of calcium is required in the conversion of Q to A. The more rapid conversion of I to Y in the presence of low external calcium (Lüttgau et al. 1987; Dulhunty & Gage, 1988) requires that this step also involves the dissociation of calcium. In the presence of low calcium, DHP's accentuate the rate of conversion of A to I and retard the repriming of the molecule back to the precursor form. The second inactive state, Y, is required to explain biphasic decay seen in some high potassium contractures. This state is also achieved more readily in the presence of low calcium solutions (Luttgau et al. 1987).

1.12.1 ACTIVATION AND INACTIVATION OF ASYMMETRIC CHARGE MOVEMENT

Many of the characteristics of charge movement, including the inactivation or immobilization of charge, were described by Chandler et al. (1976a,b). Prolonged depolarization resulted in a decline in measureable charge movement. The time course of appearance and disappearance of charge as a function of membrane potential was similar to the activation, inactivation and repriming of the depolarization-induced contractures first described by Hodgkin & Horowicz (1960a). A similar correlation between force development, contractile repriming and charge movement kinetics was noted by Caputo, Gottschalk & Lüttgau (1981).
The model proposed by Chandler et al. (1976a,b) requires 3 configurations of charge (Q) in order to describe the observed results - resting, activating and refractory.

The model predicts that an amount of charge (Q) will be rapidly converted from resting (R) to activating (A) upon depolarization. The amount of Q converted is dependent on the extent of depolarization. The rate constant for this process is rapid (milliseconds). If the duration of depolarization is sufficiently long, conversion of charge to the refractory state will proceed with a much slower rate constant (in the order of seconds). Adrian et al. (1976) demonstrated that the reappearance of charge movement following prolonged depolarization (repriming) coincided with the restoration of contractile performance.

The effect of temperature on the inactivation parameters of tension has already been described (section 1.11.6 - see Caputo, 1972). Significantly, temperature has similar effects on charge movement (Chandler et al. 1976b; Rakowski, 1981). As for tension, the time course of decay in charge movement with depolarization is highly temperature dependent. Depolarizations to -20mV give time constants for decay of charge of 1.5 s at 5°C (Chandler et al. 1976) compared with 13 s to 24 s at 2°C (Rakowski, 1981).

Another similarity between charge movement and tension is the repriming process and the effect of temperature on the repriming process. Repriming of charge movement and tension are similarly retarded by low temperature (Rakowski, 1981). These results suggest that both activation and inactivation of tension depend on changes in the voltage sensor.

1.12.2 Effects of low external calcium and calcium channel blockers on charge movement

Brum et al. (1988) demonstrated that low external calcium depressed the levels of both SR calcium release and Charge 1, while increasing the movement of Charge 2. The effect of low external calcium was to shift the inactivation curve for Charge 1 to more negative potentials. It was proposed that this shift was due to a
more rapid disappearance (inactivation) of charge in the presence of low external calcium.

The inhibitory effects of calcium channel antagonists on charge movement have also been well documented. D600 has been shown to suppress recovery of charge movement following prolonged depolarization (Feldmeyer, Melzer & Pohl & Zollner, 1990). Also, as with nifedipine, Charge 1 is decreased (Hui et al. 1984; Lamb, 1986b; Rios & Brum, 1987) while Charge 2 is increased (Rios & Brum, 1987; Melzer & Pohl, 1987; Caputo & Bolanos, 1989). The overall effect of the calcium channel antagonists is to lower the affinity of a receptor on the voltage sensor to calcium. A decrease in Charge 1 suggests that a higher percentage of voltage sensors are in the inactivated state. This analogy is very similar to the effect of calcium channel antagonists on the inactivation of tension (section 1.11.8).

1.12.3 Effects of surgical and drug treatment on asymmetric charge movement.

The use of animals which have undergone surgical or drug treatment has provided further information on the characteristics of charge movement, and the way in which charge movement reflects changes in the conformational state of the voltage sensor for E-C coupling. Alterations to the contractile properties of muscle, which were brought about by denervation, paraplegia and thyrotoxicosis, coincided with parallel changes in asymmetric charge movement (Dulhunty & Gage, 1983, 1985; Dulhunty, Gage & Lamb, 1987). In all cases, shifts in the voltage sensitivity of contraction for either soleus or EDL were coincident with similar shifts in the voltage sensitivity of asymmetric charge movement.

In summary, while there is still confusion over specific properties of charge movement (Rios & Pizarro, 1991) evidence for its role as the voltage sensor for E-C coupling is compelling, since charge movement and tension are so closely related. For example,

(a) Charge movement and tension are both strongly voltage dependent.
(b) The immobilization of charge is affected by temperature in much the same way as the decay in the tension response for maximal contractures.
(c) The repriming characteristics of tension and charge movement are similar.
(d) They are both similarly affected by low calcium concentrations in bathing solutions and by calcium channel antagonists.
(e) Parallel changes in the voltage dependence of tension and charge movement are apparent after denervation, paraplegia and thyrotoxicosis.
1.13 EFFECTS OF DENERVATION ON PROPERTIES OF SKELETAL MUSCLE

Fast- and slow-twitch muscle fibres have specific differences in terms of their metabolic enzyme profiles, myosin isoforms and myosin ATPase (mATPase) activities (Barany, Barany, Reckard & Volpe, 1965; Barnard, Edgerton, Furukawa & Peter, 1971; Peter, Barnard, Edgerton, Gillespie & Stempel, 1972; reviewed by Pette & Staron, 1990) which closely parallel differences in contractile properties (Close, 1967; Barnard et al. 1971; Edgerton & Simpson, 1971). Other studies have shown a correlation between the number of indentations in the terminal cisternae of the SR and the speed of twitch contraction (soleus having fewer indentations) (Dulhunty & Valois, 1983) and that the voltage sensitivity of charge movement, like contractile activation, occurs at more negative potentials in slow-twitch (soleus) muscle fibres than in fast-twitch (EDL) fibres (Dulhunty & Gage, 1985).

Skeletal muscle fibres are generally classified according to diverse contractile, morphological and histochemical properties. Delineation and subsequent classification of muscle fibres into specific subgroups is therefore made difficult owing to the enormous heterogeneity that can, and does, exist. However, it is the heterogeneity that provides functional specialization and allows plasticity to occur within and between species.

A review by Pette & Staron (1990) summarized the cellular and molecular diversities that exist between mammalian skeletal muscle fibres and procedures used to classify them. At present, the most favoured classification protocols are based on the studies of Brooke & Kaiser (1970a, b) and Barnard et al. (1971). The original distinction between slow-twitch (Type I) and fast-twitch (Type II) fibres, based on mATPase activity (Engel, 1962), was considered an oversimplification of the situation.

A more detailed examination of mATPase activity by Brooke & Kaiser (1970a, b) delineated two major subgroups of fast-twitch fibres (IIA and IIB). It has now been shown that EDL preparations (which have been used extensively in the experiments described in this thesis) consist of a mixture of type IIA and IIB fibres, while soleus muscle (also used in this thesis) contains predominantly slow-twitch (type I) muscle fibres (Ariano, Armstrong & Edgerton, 1973).

The major effects of denervation on twitch characteristics occur over a period of about three weeks (Dulhunty, 1985; Dulhunty & Gage, 1985). The potentiation of the twitch following denervation, together with increases in the time for contraction and relaxation have been described in detail (Lewis, 1972; Drachman & Johnson, 1975; Finol, Lewis & Owens, 1981; Dulhunty, 1985). As a
consequence of twitch potentiation, the twitch to tetanus ratio also increases (Eccles, Eccles & Kovak, 1962; Lewis, 1972; Finol et al. 1981; Dulhunty, 1985). Another effect of denervation is to alter the contractile properties of fast- and slow-twitch muscle such that they become similar (Karpati & Engel, 1968; Jolesz & Sreter, 1982; Baldwin, Roy, Edgerton & Herrick, 1984; Hoffman, Roy, Blanco & Edgerton, 1990; Jiang, Roy & Edgerton, 1990; Graham, Roy, Navarro, Jiang, Pierotti, Bodine-Fowler & Edgerton, 1992). Importantly, the effects of denervation on the twitch correlate well with changes to the voltage sensitivity of contractile activation, the amount of charge moved, and fewer indentations in the terminal cisternae of the SR (for EDL) (Dulhunty, Gage & Valois, 1984; Dulhunty, 1985; Dulhunty & Gage, 1985; Gage, Dulhunty, Lamb & Wakefield, 1989).

1.14 Aims of the experiments.

The main objective of this thesis was to investigate the E-C coupling process, and the way in which the voltage sensitivity of contractile activation and inactivation was altered under varied conditions. These conditions included differences in fibre-type, or modifications due to denervation.

In Chapter 3, potassium contractures were used to measure contractile activation and inactivation in normal and denervated, fast- and slow-twitch rat muscle. Contraction thresholds were also measured under voltage clamp conditions to confirm the results obtained with potassium contractures.

The aim of Chapter 4 was to study the effects of conditioning depolarizations on the membrane potential for threshold contractions in fast- and slow-twitch muscles. The effects of denervation were also considered.

An examination of the time course of maximal potassium contractures was the major objective of Chapter 5. Again, the effects of denervation on parameters measured in normal muscles was studied.

Chapter 6 consists of two parts. In Part I, potassium contractures were used to examine the voltage sensitivity of contractile activation, and the effect of substitution of impermeant anions in bathing solutions. Part II forms a section of published work dealing with the effect of perchlorate anions on the membrane potential for contraction threshold.
CHAPTER 2

GENERAL METHODS

2.1 Biological preparations

Approval for experiments presented in this thesis was granted by the Animal Experimentation Ethics Committee of the Australian National University.

Adult male Wistar rats (Rattus norvegicus) weighing 250-400g were used in all experiments. Three muscles were used: soleus and extensor digitorum longus (EDL), hindlimb muscles containing a predominance of slow- and fast-twitch fibres respectively, and the sternomastoid which is a superficial neck muscle containing fast-twitch fibres.

Animals were killed by asphyxiation with carbon dioxide (CO₂) and then pinned to a cork dissecting board. Hair and skin were removed from the area overlying the required muscle, and the muscle quickly excised.

2.2 Macro-dissection

Soleus: The calcaneal (Achilles) tendon was severed and external connective tissue cut both medially and laterally to the knee, thereby progressively exposing the deep surface of the soleus muscle. The calcaneal tendon was pinned medially away from the hindlimb allowing the tendon of origin, connecting the superior end of the soleus to the posterior head of the fibula, to be severed. The soleus and gastrocnemius muscles were completely separated by raising the tendon of origin with forceps and carefully cutting the connective tissue between the dorsal surface of the soleus and the anterior surface of the gastrocnemius.

EDL: The distal tendon of the tibialis anterior was severed and external connective was removed both medially and laterally so that the muscle could be removed to expose the underlying EDL. The distal tendon of the EDL was then severed and raised with forceps. Light connective tissue attaching the EDL to the rest of the hindlimb was cut and the muscle was easily removed by cutting the proximal tendon attached to the femur.

Sternomastoid: Overlying connective tissue, submandibular glands, etc, which cover the sternomastoid muscle were removed first. A piece of bone from the
mastoid process together with the tendon was removed from the skull. The muscle was freed from connective tissue along its length to the point of insertion onto the sternum. Extreme care was taken to avoid damaging the many blood vessels which supply this area to prevent bleeding over the muscle. The same dissection procedure was repeated on the contralateral side. Section of the lower portion of the sternum allowed two sternomastoid muscles to be freed from the animal.

Following isolation, preparations were pinned out in a Petri dish lined with Sylgard 184 (Dow Corning) and bathed in a low chloride Krebs solution in which sulphate was the major anion (section 2.5).

2.3 Microdissection

Extraneous connective tissue was removed from the muscle. This, and all microdissecting procedures, were carried out using Dumont N° 5 microdissecting tweezers and Teufel 8 cm microdissecting scissors with curved blades.

In this study, two different experimental techniques were employed, and different muscle dissections were required:

(a) preparation for two-microelectrode voltage clamp - the purpose of this dissection was to provide a thin sheet of muscle fibres, intact from tendon to tendon, to facilitate light transmission and allow identification of individual muscle fibres for electrode impalement. Bundles of muscle fibres were teased laterally from the underside of the preparation under low power of the microscope. Connective tissue between bundles of fibres was cut along the longitudinal axis of the muscle from tendon to tendon. This procedure was repeated across the width of the underside of the muscle. Initially large bundles were removed, followed by progressively smaller bundles until an almost transparent sheet of muscle, approximately five fibres thick, remained.

(b) preparations for potassium contractures - the purpose of the dissection was to obtain a small bundle of fibres intact from tendon to tendon. Isolation of single fibres from EDL and soleus muscle has proven unsuccessful primarily due to the tight association between fibres and connective tissue.

A small piece of tendon which contained a number of terminating muscle fibres was cut away from the rest of the preparation by cutting connective tissue between fibre bundles. This procedure was continued along the longitudinal axis of
the muscle to the opposite tendon. Any damaged or unwanted fibres were removed to leave a small bundle of 10-20 intact muscle fibres. The bundles were flat and usually only 2 fibres or 150 μm thick. The small cross-sectional dimensions avoid the major diffusion limitations of whole muscles (Goldberg, Martel & Kushmerik, 1975).

2.4 Denervation surgery

Denervated soleus and EDL muscles were obtained by section of the sciatic nerve. Animals to be denervated were anaesthetized with ether and placed dorsal side up on an operating table. The animal's condition was constantly monitored by eye: the operation proceeded when a toe pinch stimulus failed to elicit a hindlimb reflex.

Hair was removed from the lower left-hand lumbar region. A small incision (~1.5cm) was made in the skin revealing the underlying musculature. A blunt dissection was performed, where closed scissors were pushed through the musculature located just distal to the sciatic notch and then opened. The sciatic nerve could be clearly seen beneath the musculature. A small section (4-5mm) of the nerve was removed causing a violent twitch of the hindlimb. Wounds were sutured and the animals allowed to recover for 3 to 6 weeks prior to experimentation.

2.5 Solutions

All solutions used in experiments are shown in Table 2.1. The Control solution was a low chloride Krebs solution in which the major impermeant anion was sulphate. This was the standard solution used to bathe preparations during dissection, and in rest/recovery periods of experiments. Unless otherwise stated it was the only solution used in voltage clamp experiments (see section 2.7).

All solutions contained a constant low chloride concentration (16 mM). The use of low chloride solutions reduced the effects of high T-tubule chloride conductance on the rate of T-tubule depolarization (Dulhunty, 1979). Substitution of chloride ions with the impermeant sulphate anion gives a more accurate control of membrane potential in the T-system by increasing membrane resistance (and hence the T-tubule length constant) and eliminating the effects of chloride ion redistribution upon changes to membrane potential. This is not especially important in voltage clamp experiments where contractions of surface myofibrils only are measured (section 2.7.3) although the increased length constant of the T-tubule allows for a more accurate voltage clamp.
Table 2.1: Composition of bathing solutions used in experiments. Ion concentrations are given in mM.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Cl⁻</th>
<th>SO₄²⁻</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.5</td>
<td>3.5</td>
<td>7.6</td>
<td>16</td>
<td>42.6</td>
<td>170</td>
</tr>
<tr>
<td>10K</td>
<td>190</td>
<td>10</td>
<td>7.6</td>
<td>16</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>20K</td>
<td>180</td>
<td>20</td>
<td>7.6</td>
<td>16</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>30K</td>
<td>170</td>
<td>30</td>
<td>7.6</td>
<td>16</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>40K</td>
<td>160</td>
<td>40</td>
<td>7.6</td>
<td>16</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>60K</td>
<td>140</td>
<td>60</td>
<td>7.6</td>
<td>16</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>80K</td>
<td>120</td>
<td>80</td>
<td>7.6</td>
<td>16</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>120K</td>
<td>80</td>
<td>120</td>
<td>7.6</td>
<td>16</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>200K</td>
<td>-</td>
<td>200</td>
<td>7.3</td>
<td>16</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

In addition, all solutions contained: 1 mM Mg²⁺; 11 mM glucose; 2 mM TES (N-tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid) pH buffer. pH=7.40±0.05, adjusted with NaOH. (*temp = 22 ± 1°C*)
In the case of potassium contractures, a rapid and synchronous depolarization of the entire cross section of the bundle of muscle fibres is essential. Standard Krebs solutions containing normal and elevated potassium (constant [K] X [Cl] product) failed to produce contractures in mammalian muscle which were equal in height to contractures induced in low chloride solutions (Dulhunty & Gage, 1985). It is thought that cable properties of the T-system are responsible for this effect. One explanation put forward by Chua & Dulhunty (1988) is that peak tension is achieved by the passive spread of depolarization throughout the T-system rather than direct depolarization brought about by the diffusion of high potassium solutions, since time to peak tension is much faster than the time required for potassium to fully diffuse into the T-system (~ 40 sec; Dulhunty, 1979). The passive spread of depolarization could be accelerated by the use of low-Cl solutions which increase the T-tubule length constant (Dulhunty, Carter & Hinrichsen, 1984) resulting in synchronous contractile activation and increased contracture amplitude. Consequently low-chloride sulphate solutions have been used in numerous studies on mammalian muscle (Dulhunty & Gage, 1985, 1988; Chua & Dulhunty, 1988, 1989; Dulhunty, 1991; Dulhunty, Zhu et al. 1992)

In solutions 10K-120K (10-120mM K⁺), elevated potassium was achieved by substituting potassium sulphate for sodium sulphate while maintaining a constant ionic strength. The ionic strength of the low chloride control solution was similar to that of normal Krebs. Sucrose was added to increase the osmolarity to that of a normal Krebs solution. The two conflicting considerations in using the low chloride control solution were (a) that the lower than normal sodium concentration concentration depresses peak tetanic force slightly (Cairns, 1991), and (b) 70 mM Na₂SO₄ (140 mM Na⁺ as in normal Krebs) would increase ionic strength and produce faster rundown of force (A.F. Dulhunty - personal communication). A compromise was to use the low Na⁺, normal ionic strength control solution and a high ionic strength test (high K⁺) solution. An additional complication is that sodium sulphate and potassium sulphate do not fully dissociate, making calculations of exact ionic strength difficult. The concentration of ionised calcium in all solutions was ~2.3mM (Dani, Sanchez & Hille, 1983). Calcium sulphate has a low dissociation constant and so its concentration in each solution was raised to achieve the desired free calcium level. Further consideration will be given to some of these problems in Chapter 6, Part I.

It was necessary to use a 200 mM K⁺ solution to achieve depolarization to -5 mV and to obtain a maximal tension response. The ionic strength of all high potassium solutions was increased to that of the 200K solution, meaning that all high potassium solutions had the same ionic strength which was elevated compared
with the control solution. The anion, methanesulphonate, was used in some experiments (Chapter 6, Part I) to test the effects of (a) continuous exposure to a divalent anion \((SO_4^{2-})\) and (b) effects of changes in ionic strength and tonicity between the control and high \(K^+\) solutions.

All solutions were pH buffered with TES (N-tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid). pH was set to \(7.40\pm0.05\) pH units by addition of sodium hydroxide (1M).

2.6 Potassium contracture technique

Bundles of fibres were set up in a bath (0.5ml) suitable for rapid changeover of solutions (2ml/s)(Figure 2.1). A small stainless steel hook was placed through the tendon at one end of the preparation and attached to a semiconductor force transducer (Akers, model AE875, SensoNor a.s. Horten, Norway). The other tendon was attached to forceps held perpendicular to the preparation by Narashige micromanipulators, allowing for easy length adjustment. Tension could be induced either by the application of high potassium solutions or by direct electrical stimulation to produce twitches and tetani.

The small volume bath (0.5 ml) was mounted on a thermostatically controlled water jacket which maintained temperatures of bathing solutions at \(22\pm1\) °C throughout the experiments. The changeover of solutions was achieved by an electronic switching solenoid designed and built at the ANU. Solutions containing normal or elevated potassium were flowed into the bath (max. rate = 2 ml/s) via an inlet located close to the forceps. The equilibration time for solutions was less than 0.5 sec. A motor driven pump (Neuberger, miniport pump) removed solutions through an outlet located close to the force transducer. Large platinum electrodes were fitted to the long walls of the bath and extended over the entire length of the preparation.

Experiments were controlled by an interface between an Osborne 386 personal computer and an isolated stimulator (designed and built at the ANU). A software program ("MUSCON") written within the department enabled parameters and data for each experiment to be saved in digital form for later analysis.

At the beginning of each experiment the length of the preparation was adjusted to achieve maximal twitch height. During recovery phases of experiments, fibres were stimulated continuously at a frequency of 0.1 Hz. Pulse durations were usually less than 1.0 msec and pulse amplitudes around 70V. Fused tetani could be achieved by trains of pulses (50-70 Hz) for a period usually less than 1.5 s. This produced maximal contractions with a plateau phase.
Figure 2.1: Diagram illustrating the main features involved in the stimulation, recording and analysis of force in contraction studies.
Specific contractures, tetani and twitches were saved digitally and a continuous record of each experiment was displayed on a digitizing oscilloscope (Tektronix 5223) and plotted on a two channel chart recorder (7402A, Hewlett-Packard Co., Palo Alto, CA, USA). The chart recorder had a frequency response of 55 Hz for full-scale deflections and 125 Hz for 10mm (twitch height) deflections.

2.7.1 Two-microelectrode voltage clamp

Intracellular glass microelectrodes of 4-8 MΩ were filled with 2.5 M KCl and used to point voltage clamp single muscle fibres. The design layout of circuit is shown in Figure (2.2). Operational amplifiers (TL074* op-amps) 1 and 2 are unity gain voltage followers (buffers), providing a low output resistance from a high resistance voltage source (ie. a 10kΩ potentiometer). The outputs of these buffers, together with the input signals of the voltage electrode (e_m) and reference (bath) electrode (e_b) respectively were fed into low-pass filters 3 and 4, also constructed from the TL074 chip. The purpose of the low-pass filters was to eliminate high frequency noise.

The output of op-amp 3 was fed into a unity gain op-amp (5) constructed from an OP-07C chip**. This allowed the membrane potential, V_m, to be monitored.

In addition, the output of op-amp 3 was also fed, together with the output of op-amp 4, into a unity gain differential amplifier, 6. The membrane potential, V_m, was then displayed on an oscilloscope and also fed back into a summing operational amplifier, 7. A command potential, -V_c, and the membrane potential, +V_m, were compared at the summing point. The command potential is the required membrane potential; and a combination of a holding potential (-V_h) and a brief depolarising test pulse (-V_c).

The feedback system ensured that the actual membrane potential (V_m) was the same as the required membrane potential (V_h or V_c). If the two signals were unequal (ie: non-zero signal following subtraction) then the difference was fed back through a variable gain voltage amplifier and applied to a current passing microelectrode inserted in the muscle fibre. The gain of the clamp could be changed so that depolarisation of the muscle fibre membrane closely resembles the applied rectangular pulses. The 10% to 90% response time of the voltage clamp was 100 μs.

The inputs to the differential amplifier had switches which could isolate either part of the circuit (e_m or e_b). A 10kΩ 10-turn potentiometer on the non-
Figure 2.2 The two microelectrode voltage clamp set-up. A full description is given in the text.
inverting inputs of the buffers was used to "zero" the potential on the tip of the microelectrodes before insertion of \( e_m \) into the fibre.

The bath ground electrode for the system was a chloride coated, tightly coiled, silver wire attached to a virtual earth current to voltage convertor. Voltage, current and command signals were continually displayed on an oscilloscope.

(*) The TL074 is a low noise operational amplifier with low input bias and offset currents and fast slew rate. The low harmonic distortion and low noise make this amplifier ideally suited for high fidelity and audio pre-amp applications. Each amplifier on the chip has JFET inputs for high input impedance.

(**) The OP07C is an operational amplifier with ultra-low input offset voltage. The differential inputs with wide input voltage range and good CMRR provide accurate results in high-noise environments.

2.7.2 Capacitance Neutralization

Each voltage microelectrode \((e_m \text{ or } e_b)\) input was connected to a capacitance neutralisation circuit, so that the response time of a biological amplifier system to microelectrode input signals was not limited by stray capacitance arising from various sources. eg; between the drain and gate of FET input transistors, long connecting wires, capacitance across the microelectrode wall to the bath, etc.

With capacitance neutralization the current through the stray capacity is compensated for with current generated by a variable amplified output \((1 - 10X)\) applied through a fixed capacitor to the input. Problems occur in adjusting the compensation correctly so as not to distort the input signal by inducing oscillations due to overcompensation, and also from the injection of current noise with the compensating signal. Optimal performance of the capacitance neutralization gives a maximum bandwidth response with minimal overshoot.

2.7.3 Contraction Threshold measurement

Thin sheets of intact muscle fibres (section 2.3) bathed in the low chloride control solution (Table 2.1) were pinned out on a Sylgard 184 (Dow Corning) lined bath (volume 3 ml) prior to microelectrode insertion.

A high resolution (800 lines/inch) video camera (National WV-1800/B) attached to a phototube on a compound microscope (Zeiss) allowed the preparation to be observed on a television monitor (National WV-5410) connected to the video
camera. The microscope was fitted with a 16 X air objective (Zeiss) and a 16 X 12.5mm working distance eyepiece (Zeiss). Calibration of the optical system with a stage micrometer indicated overall magnification was 500. The microscope, stage and objective were earthed to a central point and positioned on a vibration-free air table.

Micromanipulators (Narashige) placed on either side of the microscope objective were used to position the voltage recording and current passing microelectrodes. These electrodes were inserted into opposite edges of a muscle fibre at a separation of 50-100 μm (Figure 2.2). This configuration was the most appropriate in order to avoid nonuniformities in membrane potential around the current microelectrode (Adrian, Costantin & Peachey, 1969; Eisenberg & Johnson, 1970; Dulhunty, 1982). A third microelectrode was placed in the bathing solution in close proximity to the voltage recording microelectrode.

When the clamp gain was increased, the muscle fibre membrane was point voltage clamped in the region between the voltage recording and current passing electrodes. Fibres were usually clamped at a holding potential of -80mV (unless otherwise stated). Rectangular depolarizing (test) pulses of varying duration and amplitude could be applied to the muscle fibre via the voltage clamp circuitry. The
CHAPTER 3

ACTIVATION AND INACTIVATION OF CONTRACTION IN NORMAL AND DENERVATED RAT SOLEUS AND EDL.

3.1 INTRODUCTION

It has been well established that denervation of skeletal muscle produces many changes in the properties of muscle fibres. These include atrophy, potentiation of the twitch, proliferation of acetylcholine receptors and TTX-insensitive sodium channels, and a variety of contractile, morphological and histochemical alterations (see section 1.13.).

A consistent observation in many studies is that denervation makes the properties of fast- and slow-twitch fibre types similar (section 1.13). A number of studies in this laboratory have examined contractile properties, asymmetric charge movement and morphology in the denervated muscle fibre (Dulhunty, 1985; Dulhunty & Gage, 1985). In particular, it has been shown that denervation of both fast-twitch (EDL) and slow-twitch (soleus) muscle produces changes in the voltage sensitivity of potassium contracture tension and charge movement that make the two fibre types appear similar (Dulhunty & Gage, 1985). An interesting observation from that study was that the slope of the curve relating tension generation to membrane potential (activation curve) becomes steeper following denervation. As a result, it would appear that (a) although in denervated soleus fibres the voltage for half-maximal activation shifts to negative potentials, the threshold potential (contraction threshold) becomes more positive and (b) the points of initial tension generation in denervated soleus and EDL are shifted to similar values on the ordinate (membrane potential) scale.

The inactivation curves for denervated preparations have not been previously studied. Consideration of inactivation in denervated mammalian muscle will provide an important insight into the effects of denervation on the voltage sensor for E-C coupling.

The aims of these experiments were twofold. Firstly, to use potassium contractures to examine the effect of denervation on the inactivation curve relating peak test contracture tension to conditioning membrane potential for fast- and slow-twitch mammalian skeletal muscle. At the same time data was generated for the voltage dependence of activation in normal and denervated muscle. A second objective was to use the two micro-electrode point voltage clamp technique to test
the hypothesis that the contraction thresholds for control preparations are altered by
denervation such that threshold is either not changed, or shifted to positive
potentials in denervated soleus fibres so that contraction thresholds in fast- and
slow-twitch fibres become similar.

3.2 METHODS

Procedures for the handling of animals, macro- and microdissection of
muscle preparations and denervation surgery are described in detail in Chapter 2,
sections 2.1-2.4. Denervated muscles were used in experiments if they satisfied
two criteria: (a) fibrillations were present upon exposure of the denervated muscle
to the low chloride control solution, and (b) muscles in the leg on the denervated
side of the animal exhibited obvious signs of atrophy.

The composition of solutions for use in the following experiments have been
described in section 2.8.

3.2.1 Potassium contracture experiments

A description of the K-contracture technique can be found in Chapter 2,
section 2.5. The K-contracture experiments presented in this chapter were based on
the two-pulse protocol described in detail by Dulhunty (1991). Figure 3.1 outlines
this protocol. The sequence of events is as follows:

* A tetanus (P1) was generated in fibres equilibrated in a control 3.5mM
potassium (3.5K) solution. This was immediately followed by a test 200K
contracture (tK-1) which produced maximal tension. The high potassium solution
was washed out shortly after the peak of the contracture had been reached.

* A second tetanus (P2) was generated following a period of recovery in the
control solution, usually around 20 minutes. Subsequently, a five minute
conditioning period in elevated potassium (10K-120K) was immediately followed
by a test 200K contracture. The height of the contracture in the conditioning
solution, tA, and the height of the test contracture following the conditioning
depolarization, tJ, were measured and used to obtain activation and inactivation
parameters respectively, curves fitted to the data (see below).

* Following recovery from conditioning and test contractures, a final
tetanus (P3) and 200K contracture (tK-3) were measured.

In order to analyse data, all contractures were normalized to their preceeding
tetanus. This procedure avoided assumptions about the time course of "rundown"
Figure 3.1: Records obtained using the two-pulse protocol to measure inactivation of K-contracture tension. Shown are chart records of tension as a function of time for EDL (A) and soleus (B & C) muscle. Each experiment was begun by generating a control tetanus (P1) and 200K (t\textsubscript{c1}) contracture. The small vertical deflections are twitches. Examples of submaximal contractures (t\textsubscript{a}) upon exposure to conditioning high potassium solutions (indicated) are shown in each panel. These contractures were preceded by a second tetanus (P2). Test 200K contractures (t\textsubscript{t}) were generated after 5 minutes of conditioning in the indicated high potassium solutions. A further tetanus (P3) and 200K contracture (t\textsubscript{c3}) were generated at the end of the series. Vertical calibration bar: 4 mN. Horizontal calibration bar 2 minutes.
of preparations (Dulhunty, 1991). Data was only accepted from preparations in which the ratio of contracture to tetanic tension remained constant. The mean amplitude of control (ie: pre- and post-conditioning) 200K contractures, expressed relative to peak tetanic tension, was taken as maximal tension, $T_{\text{max}}$, in soleus fibres since the activation curve saturates between 120K and 180K in slow-twitch fibres (Dulhunty & Gage, 1985). Maximum tension in EDL fibres was not always achieved with 200K solutions. Therefore peak tetanic tension was assigned the value of $T_{\text{max}}$ in these preparations. The normalization procedure was as follows:

$$T_{\text{max}} = \frac{(t_{\text{c1}}/P1 + t_{\text{c3}}/P3)}{2} \quad (1)$$

The amplitude of normalized tension ($T_a$) generated by exposure to the conditioning solution and expressed as a function of $T_{\text{max}}$ is given by:

$$T_a = \frac{(t_{\text{a}}/P2)}{T_{\text{max}}} \quad (2)$$

Inactivation curves were obtained from the normalized height of the test contracture, $T_i$, following a conditioning depolarization. The amplitude of the test contracture, $t_i$, was expressed as a fraction of $T_{\text{max}}$ as follows:

$$T_i = \frac{(t_i-t_p)}{T_{\text{max}}} \quad (3)$$

where $t_p$ represents pedestal tension (a depolarization dependent, non-inactivating tension). (see Figure 3.2)

Activation and inactivation curves were obtained by plotting average values of $T_a$ and $T_i$ respectively, against the membrane potential ($V_m$) achieved by the conditioning solution. Comparisons of the voltage sensitivities of activation and inactivation for control and experimental (eg: denervated) preparations were obtained by fitting Boltzmann-type equations to the above data (Dulhunty, 1991; Dulhunty & Gage, 1983, 1985; Chua & Dulhunty, 1988, 1989; Dulhunty et al. 1992). The activation curve was fitted by a Boltzmann equation of the form:

$$T_a = T_{\text{max}} / [1 + \exp(V_a - V_m)/k_a] \quad (4)$$

where $V_a$ is the $V_m$ at which $T_a$ equals $0.5T_{\text{max}}$ and $k_a$ is a slope factor.
Similarly, the inactivation curve, at time t (i.e. 5 minutes), was fitted by a corresponding Boltzmann equation of the form:

\[ T_i(t) = \frac{T_{\text{max}}}{1 + \exp(V_m - V_i(t))/k_i(t)} \] (5)

where \( V_i(t) \) is the \( V_m \) at which \( T_i(t) \) equals 0.5\( T_{\text{max}} \) and \( k_i(t) \) is a slope factor.

3.2.2 Contraction threshold determination

The visual determination of contraction threshold using the two-microelectrode point voltage clamp technique has been described in section 2.7.3. Strength-duration curves were generated by determining the membrane potential for contraction threshold at a variety of pulse durations from a holding potential of -80 mV.
3.3 RESULTS

3.3.1 Potassium contractures in normal and denervated soleus and EDL muscle.

The traces shown in Figure 3.2 A-C show typical digitally recorded tension responses for control and denervated soleus and EDL muscle fibres obtained with the two-pulse protocol (methods, section 3.2.1). Each trace shows tension in response to conditioning depolarizations in elevated potassium solutions, followed immediately by a test 200K contracture. In many examples, particularly those in which elevated potassium is 30 mM or less, a small initial tension generation was often due, in part, to action potential activity (see Figure 3.2). Twitches brought about by action potentials looked very different to K-contractures. The action potential activity occurred briefly at the commencement of depolarization in elevated potassium solutions, and then ceased. The cessation of activity was presumably due to the inactivation of sodium channels. However, with small depolarizations in 10K to 30K, action potential activity may have been maintained. Therefore, in order to extract threshold contractures which were not contaminated by action potential activity, separate experiments were carried out in elevated potassium solutions (10-30K) together with tetrodotoxin (TTX) which abolishes sodium conductance (see Ritchie & Rogart, 1977). These experiments showed that contractures could not be generated by solutions containing less than 30 mM potassium. Therefore, any tension response measured in 10K or 20K solutions was induced by action potential activity.

3.3.2 Activation curves for normal and denervated soleus and EDL muscle.

Activation curves express the relative peak tension generated by a bundle of muscle fibres as a function of their membrane potential in solutions containing elevated potassium. Separate experiments were carried out to measure membrane potentials in fibres bathed in the high potassium solutions used to invoke contractures. As shown by Dulhunty & Gage (1985), the average membrane potential for fibres bathed in low-chloride solutions was not significantly different between normal and denervated fibres. Nevertheless, average membrane potentials in fibres conditioned in solutions containing more than 30 mM potassium are hyperpolarized in EDL compared to soleus (Table 3.1).

Activation curves for control and denervated soleus muscle are shown in Figure 3.3. These curves have been plotted from average data presented in Table 3.2A. It can be seen that the effect of denervation on the voltage sensitivity of peak
Figure 3.2: Examples of tension responses obtained from normal soleus (A), denervated soleus (B), normal EDL upon exposure to high potassium solutions (concentration of K⁺ indicated) (ta) immediately followed by exposure to a 200K solution (ti). Asterisks indicate tension considered to be generated by action potentials. Vertical calibration bar equals 5 mN and horizontal bar represents 1 minute.
Table 3.1: Membrane potentials ($V_m$) recorded from soleus and EDL fibres exposed to various external potassium concentrations.

<table>
<thead>
<tr>
<th>[K+] (mM)</th>
<th>Soleus $V_m$ (mV)</th>
<th>EDL $V_m$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>-85.9±0.3 (115)</td>
<td>-83.0±0.2 (125)</td>
</tr>
<tr>
<td>10</td>
<td>-69.5±0.6 (22)</td>
<td>-67.6±0.9 (24)</td>
</tr>
<tr>
<td>20</td>
<td>-54.9±0.7 (22)</td>
<td>-54.9±0.9 (22)</td>
</tr>
<tr>
<td>30</td>
<td>-45.9±0.7 (16)</td>
<td>-46.6±0.5 (16)</td>
</tr>
<tr>
<td>*</td>
<td>-45.9±0.8 (16)</td>
<td>* -46.5±0.9 (15)</td>
</tr>
<tr>
<td>40</td>
<td>-40.3±0.6 (20)</td>
<td>-37.9±0.8 (22)</td>
</tr>
<tr>
<td>60</td>
<td>-30.0±0.3 (50)</td>
<td>-30.2±0.7 (12)</td>
</tr>
<tr>
<td>*</td>
<td>-29.4±0.4 (36)</td>
<td>* -30.5±0.6 (12)</td>
</tr>
<tr>
<td>80</td>
<td>-24.0±0.6 (20)</td>
<td>-24.8±0.5 (22)</td>
</tr>
<tr>
<td>*</td>
<td>-23.4±0.4 (20)</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>-16.0±0.5 (29)</td>
<td>-17.4±0.5 (22)</td>
</tr>
<tr>
<td>200</td>
<td>-1.6±0.4 (21)</td>
<td>-2.7±0.6 (23)</td>
</tr>
</tbody>
</table>

* Denervated preparations
Figure 3.3: The effect of denervation on the activation of contraction in soleus muscle. Relative K-contracture tension, $T_a$ (see methods), is plotted against membrane potential measured in high potassium solutions. The symbols show average results obtained in normal soleus (open diamonds) and denervated soleus (filled diamonds). The curves through the data were generated using equation 4 (methods, section 3.2). For normal soleus (dashed line), $V_a$ and $k_a$ are -26.5 mV and 2.8 mV respectively, while for denervated soleus (continuous line) the values are -27.8 mV and 2 mV.
Table 3.2: Relative activation (A) and inactivation (B) results (mean±SEM) obtained from normal and denervated soleus and EDL fibres which were exposed to various external potassium concentrations. Data was analysed as outlined in section 3.2.1 (methods). Numbers in parentheses indicate sample numbers.

### A

<table>
<thead>
<tr>
<th>[K⁺] (mM)</th>
<th>Soleus (normal)</th>
<th>Soleus (Denervated)</th>
<th>EDL (normal)</th>
<th>EDL (Denervated)</th>
</tr>
</thead>
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<tr>
<td>3.5</td>
<td>0</td>
<td>0</td>
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<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>0 (5)</td>
<td>0 (5)</td>
</tr>
<tr>
<td>20</td>
<td>0 (7)</td>
<td>0 (9)</td>
<td>0 (5)</td>
<td>0 (5)</td>
</tr>
<tr>
<td>30</td>
<td>0.03±0.01 (15)</td>
<td>0 (11) †</td>
<td>0 (9)</td>
<td>0.01±0.00 (11)</td>
</tr>
<tr>
<td>40</td>
<td>0.03±0.01 (9)</td>
<td>0.01±0.01 (9)</td>
<td>0 (8)</td>
<td>0.12±0.04 (5)</td>
</tr>
<tr>
<td>60</td>
<td>0.19±0.05 (9)</td>
<td>0.39±0.05 (12) *</td>
<td>0.06±0.03 (8)</td>
<td>0.42±0.04 (5)</td>
</tr>
<tr>
<td>80</td>
<td>0.77±0.05 (10)</td>
<td>0.76±0.04 (7)</td>
<td>0.21±0.03 (6)</td>
<td>0.88±0.03 (7)</td>
</tr>
<tr>
<td>120</td>
<td>0.97±0.03 (8)</td>
<td>1 (7)</td>
<td>0.58±0.04 (5)</td>
<td>1 (5) *</td>
</tr>
<tr>
<td>200</td>
<td>1</td>
<td>1</td>
<td>0.93</td>
<td>0.962</td>
</tr>
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</table>

### B

<table>
<thead>
<tr>
<th>[K⁺] (mM)</th>
<th>Soleus (normal)</th>
<th>Soleus (Denervated)</th>
<th>EDL (normal)</th>
<th>EDL (Denervated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.92±0.04 (5)</td>
</tr>
<tr>
<td>20</td>
<td>0.89±0.04 (7)</td>
<td>0.79±0.04 (9) ††</td>
<td>0.78±0.07 (5)</td>
<td>0.72±0.02 (5)</td>
</tr>
<tr>
<td>30</td>
<td>0.76±0.04 (15)</td>
<td>0.61±0.06 (11)</td>
<td>0.64±0.07 (9)</td>
<td>0.54±0.04 (11)</td>
</tr>
<tr>
<td>40</td>
<td>0.39±0.08 (9)</td>
<td>0.11±0.03 (9) ††</td>
<td>0.31±0.03 (8)</td>
<td>0.22±0.03 (5)</td>
</tr>
<tr>
<td>60</td>
<td>0.03±0.01 (9)</td>
<td>0.05±0.01 (12) ††</td>
<td>0.03±0.01 (8)</td>
<td>0.06±0.03 (5)</td>
</tr>
<tr>
<td>80</td>
<td>0 (10)</td>
<td>0 (7)</td>
<td>0 (6)</td>
<td>0 (7)</td>
</tr>
<tr>
<td>120</td>
<td>0 (8)</td>
<td>0 (7)</td>
<td>0 (5)</td>
<td>0</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant at p<0.01 level.
† Significant at p<0.05 level.
++ Significant at p<0.10 level.
tension is minimal. Similarly, the voltage dependence of activation of charge movement in soleus is barely altered with denervation (Dulhunty & Gage, 1985). The Boltzmann parameters, fitted to average data are very similar in control and denervated preparations (Table 3.3). In denervated preparations the voltage for half-maximal tension is shifted to more negative membrane potentials by just 1.3 mV, while the voltage sensitivity of the slope is only slightly increased (k = 2 mV for denervated compared to 2.8 mV in normal soleus). However, a Student t-test demonstrated that average 60K contracture tension was significantly augmented (P < 0.01) in denervated soleus muscle compared to normal. This result had little effect on the overall shape of the Boltzmann curve because the voltage sensitivity of tension generation at the membrane potential achieved by a 60K solution is extremely steep. The increase in submaximal contracture tension shows that denervation alters the voltage dependence of tension such that the point of initial tension generation is more positive than that for control (see section 3.3.5).

In contrast to soleus, denervation of EDL produced a strong shift in the activation curve to more negative membrane potentials (Figure 3.4). Denervation moved the voltage for half-maximal activation (V_a) by 12 mV in the negative direction. This shift in V_a is almost identical to a shift in V_a for the activation of charge movement observed following denervation of EDL (Dulhunty & Gage, 1985). Figure 3.4 also shows that the point of initial tension generation (contraction threshold) in denervated EDL is probably more negative than that of control EDL. This observation confirms that of Dulhunty & Gage (1985) and will also be considered in more detail in section 3.3.5. In addition to changes in half-maximal activation of tension, the slope factor (k) for denervated EDL decreased from 4.5 mV to 3 mV, making the relationship between contracture tension and membrane potential steeper (Table 3.3A).

The end effect of denervation on the activation curves for EDL and soleus is to make the curves appear similar. Differences in the activation curves for normal EDL and soleus fibres can be seen in Figure 3.5A. The voltage for half-maximal activation is 8.7 mV more positive in EDL (open triangles) compared to soleus (open diamonds), while the soleus curve is considerably steeper (see Table 3.3A). Figure 3.5B clearly shows that denervation changes the voltage sensitivity of the activation curves for EDL and soleus such that they almost become superimposed. In fact, denervation has such an effect on EDL that the voltage for half-maximal activation becomes more negative than the corresponding parameter for denervated soleus. Consequently, differences between EDL and soleus were not only reduced by denervation, but actually reversed. Also, the voltage sensitivity of tension generation becomes much steeper in denervated EDL compared to control. As a
**Table 3.3A:** Effects of denervation on $V_a$ and $k$ obtained from the best, least squares fit of equation 4 (methods) to the averaged normalized K-contracture data.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$T_{max}$</th>
<th>$V_a$ (mV)</th>
<th>$k$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.00</td>
<td>-26.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Denervated</td>
<td>1.00</td>
<td>-27.8</td>
<td>2</td>
</tr>
<tr>
<td>EDL:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.00</td>
<td>-17.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Denervated</td>
<td>1.00</td>
<td>-30</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 3.3B:** Effects of denervation on $V_i$ and $k$ obtained from the best, least squares fit of equation 5 (methods) to the averaged normalized K-contracture data.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$T_{max}$</th>
<th>$V_i$ (mV)</th>
<th>$k$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.00</td>
<td>-41.5</td>
<td>4</td>
</tr>
<tr>
<td>Denervated</td>
<td>1.00</td>
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<td>4.5</td>
</tr>
<tr>
<td>EDL:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
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<td>7</td>
</tr>
<tr>
<td>Denervated</td>
<td>1.00</td>
<td>-47</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 3.4: Relationship between average relative K-contracture amplitude and membrane potential in bundles of fibres from normal EDL (open triangles) and denervated EDL (filled triangles). The curves through the data were generated using equation 4 (methods, section 3.2). For normal EDL (dashed line), $V_a$ and $k_a$ are -17.8 mV and 4.5 mV respectively, while for denervated EDL (continuous line) the values are -30 mV and 3 mV.
Figure 3.5: (A) Activation curves showing differences in the voltage sensitivity of relative K-contracture amplitude for normal EDL (open triangles) and normal soleus (open diamonds). Curves fitted to average data show that $V_a$ equals -26.5 mV and -17.8 mV for soleus (continuous line) and EDL (dashed line) respectively, while $k_a$ is slightly less, at 2.8 mV, for soleus compared to 4.5 for EDL. (B) Activation curves showing differences between denervated soleus (filled diamonds) and denervated EDL (filled triangles). For denervated EDL (dashed line), $V_a$ and $k_a$ are -30 mV and 3 mV respectively, while for denervated soleus (continuous line) the values are -27.8 mV and 2 mV.
result, it can be seen that average contracture tension is less for soleus upon exposure to all potassium concentrations below 80K, suggesting that denervated EDL muscle is more easily activated for a given depolarization (Table 3.2A).

3.3.3 Inactivation curves for normal and denervated soleus and EDL.

Conditioning depolarizations were applied for a period of 5 minutes and the degree of inactivation assessed by the height of a test 200K contracture. Inactivation in conditioning solutions containing less than 60 mM K⁺ does not reach steady-state within five minutes, although most of the changes to measured inactivation are almost complete within this time (Dulhunty, 1991). Figure 3.6 shows average values of Tᵢ for normal and denervated soleus, plotted against membrane potential in each conditioning solution (see Table 3.1). Average values for Tᵢ are given in Table 3.2B.

Denervation of soleus muscle produces a negative shift (4.5 mV) in the voltage for half-maximal inactivation (V₁/₂) (Table 3.3B). The slope of the steady-state inactivation curve declined slightly after denervation, with k increasing from 4 mV to 4.5 mV (Table 3.3B). There were significant differences between average test contracture tension in normal and denervated muscle. Inactivation reduced the average value for Tᵢ following a 40K conditioning depolarization to 0.39 of T_max in normal soleus, and to 0.11 of T_max in denervated soleus (Student t-test, p<0.05). Following a 60K conditioning depolarization, Tᵢ was close to zero in both control and denervated muscle, although significantly greater in the denervated muscle (p<0.1).

Denervation of EDL did not cause large changes to the voltage sensitivity of steady-state inactivation compared to control values. The steady-state inactivation curves for control and denervated EDL (Figure 3.7) show that denervation induced a -3 mV parallel shift in the voltage for half-maximal inactivation (V₁/₂) (Table 3.3B). However, student t-tests on average normalized test contracture tension demonstrated that there were no significant differences between control and denervated data at any point.

The inactivation curves for control and denervated soleus and EDL are shown in Figure 3.8A & B. A comparison of Boltzmann parameters for these curves demonstrates that the curves undergo a shift in V₁/₂ to more negative potentials and a tendency towards similar voltage sensitivities following denervation (Table 3.3B). The small negative shift in denervated soleus together with the larger negative shift in denervated EDL brings V₁/₂ in denervated EDL and soleus closer together.
Figure 3.6: The effect of denervation on the inactivation of contraction in soleus muscle. Relative peak amplitude of test K-contracture tension, T_i (see methods), is plotted against membrane potential measured in high potassium solutions following a 5 minute conditioning depolarization. The symbols show average results obtained in normal soleus (open diamonds) and denervated soleus (filled diamonds). The curves through the data were generated using equation 5 (methods, section 3.2). For normal soleus (dashed line), V_i and k_i are -41.5 mV and 4 mV respectively, while for denervated soleus (continuous line) the values are -46 mV and 4.5 mV.
Figure 3.7: Inactivation of average normalized test 200K contracture tension in normal (open triangles) and denervated (filled triangles) EDL. The curves through the data were generated using equation 5 (methods). $V_i$ and $k_i$ are -44 mV and 7 mV respectively for normal fibres (dashed line) and -47 mV and 7 mV for denervated preparations (continuous line).
Figure 3.8: Comparison of the differences in inactivation curves between normal soleus (open diamonds) and EDL (open triangles) in A, and denervated soleus (filled diamonds) and EDL (filled triangles) in B. $V_i$ is 2.5 mV more positive in normal soleus (dashed line) than in normal EDL (broken line), and $k_i$ is 4.5 mV for soleus and 7 mV for EDL (A). Denervation shifts $V_i$ to more negative potentials in both soleus (continuous line) and EDL (dashed line) (soleus 1 mV more positive than EDL) and the curves become less steep. ie: $k_i$ in denervated soleus is 4.6 mV compared to 7 mV in denervated EDL (B).
The changes described above demonstrate a number of important findings. Firstly, the -5 mV shift in the inactivation curve for denervated soleus suggests that this preparation becomes more susceptible to the effects of prolonged depolarization than control soleus. Hence, denervated soleus displays inactivation characteristics which are similar to those of EDL. In contrast, the negative shift in the inactivation curve for denervated EDL suggests that this fibre-type is even more susceptible to the effects of prolonged depolarization than its' control counterpart.

3.3.4 Comparison of activation and inactivation curves in normal and denervated skeletal muscle.

A combined plot of activation curve and inactivation curve reveals a small overlap between the two curves. This overlap, or "voltage window", has been used to predict the amount of noninactivating tension which is often seen in conditioned fibres (Chua & Dulhunty, 1989). Figure 3.9 A-D shows combined activation and inactivation curves for control and denervated soleus (A & C) and EDL (B & D). It can be clearly seen that denervation alters the amount of overlap that occurs, together with the voltage dependence of peak overlap. The amount of overlap appears to increase in EDL, and decrease in soleus following denervation. Examples of preparations displaying noninactivating tension are shown in Figure 3.2. While the area of the region beneath the activation and inactivation curves is indicative of the existence of pedestal or noninactivating tension, it cannot be used to predict the exact value of the measured tension (Dulhunty & Zhu, personal communication). Hence, significant pedestal tensions were seen frequently in denervated EDL only. The observations in Figure 3.2 were made following conditioning depolarizations in 60K, where overlap of the activation and inactivation curves was maximal.

3.3.5 Contraction threshold in normal and denervated soleus and EDL muscle

The results shown in Figure 3.4 above (normal and denervated EDL activation curves) suggest that the point of initial tension generation (contraction threshold) in denervated EDL is shifted to more negative membrane potentials. This result has previously been shown by Dulhunty & Gage (1985). The results presented by these authors also suggested that the contraction threshold for denervated soleus was shifted, in this case to more positive membrane potentials. This observation was discussed in section 3.3.2 (Figure 3.3). However, since the K-contracture technique would be considered "crude" for the measurement of
Figure 3.9: Comparison of steady-state activation and inactivation curves in normal soleus (A) and EDL (B), and denervated soleus (C) and denervated EDL (D). The graphs illustrate the effect of denervation on the area of overlap between activation and inactivation parameters.
contraction thresholds, the observation regarding denervated soleus was not convincing. Therefore, to examine the effects of denervation on small contractions, the two-microelectrode point voltage clamp technique was used to accurately determine contraction thresholds in normal and denervated soleus and EDL muscle fibres.

Strength-duration curves, which show the average membrane potential for contraction threshold expressed as a function of pulse duration (5-500 msec), have been plotted in Figure 3.10 for normal (n=19) and denervated (n=22) soleus in (A), and normal (n=19) and denervated (n=8) EDL in (B). These strength-duration curves are of a similar shape to those described previously (Adrian et al, 1969; Costantin, 1974; Dulhunty & Gage, 1983; Chua & Dulhunty, 1988). The results clearly show that denervation induces a positive shift in the contraction threshold for soleus, and a negative shift in the contraction thresholds for EDL, at all pulse durations. The shifts in contraction threshold following denervation were significantly different from control values at the P<0.001 level. Significance was based on F values obtained from a two-way analysis of variance (ANOVA) for repeated measures.

Another feature of the strength-duration curves is that a rheobase potential was not achieved with a 500 msec pulse duration, although the change in threshold between 100 and 500 msec is greater in soleus than EDL. The failure to reach rheobase also establishes results from previous studies. In fact, Chua & Dulhunty (1988) were not able to reach rheobase in mammalian soleus and EDL with pulse durations of almost 2 seconds.

Strength-duration curves have been replotted in Figure 3.11A & B which compare contraction thresholds obtained with normal soleus and EDL in (A), and with denervated soleus and EDL in (B). It can be seen that denervation alters the contraction thresholds of soleus and EDL, making them appear similar. The average difference between contraction thresholds in control EDL and soleus was 13.5 mV compared to 3.3 mV in denervated EDL and soleus.

The data obtained with the 2 micro-electrode voltage clamp confirms the changes in contraction threshold with denervation that were indicated by K-contracture data.
Figure 3.10: Strength-duration curves showing differences in contraction thresholds for normal (open squares) and denervated soleus (filled squares) in A, and normal (open triangles) and denervated EDL (filled triangles) in B. The vertical bars denote ±1 SEM.
Figure 3.11: Strength-duration curves for normal soleus (open squares) and EDL (open triangles) in A, and denervated soleus (filled squares) and EDL (filled triangles) in B. Note the shift towards similar contraction thresholds for soleus and EDL in the denervated preparations.
3.4 DISCUSSION

The activation curves obtained in this study were similar to those previously shown by Dulhunty and Gage (1985) and Chua & Dulhunty (1988). Differences in the voltage for half maximal inactivation shown in inactivation curves for normal soleus and EDL were not as large as the 22 mV difference reported by Chua & Dulhunty (1988). I have no explanation for this difference except that different colonies of animals were used and it is possible that the voltage for half maximal inactivation varied between the colonies.

3.4.1 The effects of denervation on E-C coupling.

The results obtained in this study demonstrated that denervation made the voltage activation properties of fast-twitch (EDL) and slow-twitch (soleus) muscle similar. This observation is not unique. Many early studies which altered or eliminated nervous input to skeletal muscle such as spinal cord transection (paraplegia), denervation, and pharmacological inhibition of nervous stimuli have reported the shift in physiological parameters, usually a shift from fast- to slow-twitch characteristics (section 1.13). As a result of these studies debate has concentrated on whether the effects of denervation are due to lack of nervous activity generating movement of the limb, or the absence of a trophic factor supplied by the nerve. In this study, denervation was induced, not so much to determine why denial of nervous activity causes specific alterations to contractile properties in skeletal muscle, but rather to examine the plasticity of the E-C coupling mechanism.

3.4.2 Activation of mammalian skeletal muscle. Effects of denervation.

The results for the effects of denervation on soleus activation curves require some discussion. Margreth, Salvia, Di Mauro & Turati (1972) have shown that a transformation in the properties of slow muscle SR to those of fast muscle SR occurs with denervation. However, in this study denervation had little effect on the voltage for half maximal activation in soleus, although an examination of average data plotted in the activation curve for normal and denervated soleus, shows that the relative tension generated in a 60K solution is much greater in denervated soleus than in control soleus. In spite of this finding, the voltage sensitivity of contraction is so steep for both preparations, that the overall curve is not much altered. The small negative shift in voltage for the half-maximal activation of
tension following denervation differs from the results of Dulhunty & Gage (1985) who demonstrated a -8 mV negative shift in the same parameter for denervated soleus. This difference may have been due to different periods of denervation used in each study, i.e. 21 to 42 days in this study, 2 to 68 days in Dulhunty & Gage (1985). However, since charge movement in soleus is barely altered, if at all, by denervation (Dulhunty & Gage, 1985) then the result obtained in this study is consistent with charge movement data.

The effect of denervation on activation curves in EDL muscle fibres was much stronger than it was in soleus. Denervation induced a 12 mV shift towards more negative membrane potentials in the voltage for half-maximal tension in EDL. This shift corresponds to a parallel shift in the voltage sensitivity of charge movement in denervated EDL (Dulhunty & Gage, 1985). It is likely that the large shift in charge movement seen in denervated EDL is responsible for changes to the activation curve for tension in denervated EDL. However, other factors may contribute to the changes in the tension response, particularly in soleus.

3.4.3 What point(s) in the E-C coupling mechanism are affected by denervation?

There are three likely sites where denervation could affect E-C coupling. Firstly, the voltage sensitive molecule in the T-membrane and the associated communication with the terminal cisternae of the SR. Secondly, the calcium release channel of the SR and thirdly, the contractile proteins. With reference to these particular sites, a number of findings from previous studies are pertinent.

In considering changes to the activation curve for denervated muscle, Dulhunty & Gage (1985) have shown that the time course of change for contractile activation is not the same as that for charge movement. Therefore additional modifications to one or more sites in the E-C coupling mechanism together with changes to the voltage sensor must occur. A number of studies have reported that the contractile proteins are not altered in the first few weeks after denervation (Syrovy, Gutmann & Melichna, 1971; Margreth et al. 1972; Jaweed, Herbison & Ditunno, 1975; Finol et al. 1981). Alterations to contractile properties could, however, be due to an effect of denervation on the sensitivity of myofilaments to calcium (Dulhunty & Gage, 1985; Kotsias, Paz Muchnik, 1987) thereby causing a change to the force-pCa relationship. The studies by Syrovy et al. (1971), Jaweed et al. (1975) and Finol et al. (1981) looked at changes to myosin ATPase activities, but did not consider alterations in myofilament calcium sensitivity in their assessment of the effect of denervation on contractile proteins. It has been shown that fast- and slow-twitch muscle show different sensitivities in the numbers of
calcium ions required to activate the contractile apparatus. Stephenson & Williams (1981) demonstrated that six calcium ions are required by the regulatory unit for contraction in EDL compared to only two in soleus. Since many of the contractile properties of EDL shift towards those of soleus following denervation, it is possible that a similar shift in the calcium sensitivity of EDL contractile proteins also occurs. Indeed, Trachez, Sudo & Suarez-Kurtz (1990) described significant augmentation of tension in skinned, denervated rabbit EDL upon exposure to levels of calcium (pCa=6.0) which gave submaximal tension responses. Minimal changes to the calcium sensitivity of contractile proteins in denervated soleus could account for the apparent (see 60K data point) change to the voltage sensitivity of tension generation.

An increase in resting intracellular calcium concentrations has been reported for denervated rat EDL (Kirby & Lindley, 1981). Therefore, a second possibility to describe augmentation of submaximal contractures is that the amount of calcium released per unit of asymmetric charge moved is altered, leading to an increased level of myoplasmic calcium and altered force production for a given depolarization. In addition to the findings of Kirby & Lindley (1981), there are several pieces of evidence which favour an increase in resting myoplasmic calcium following denervation. Engel & Stonnington (1974) showed that an increased volume fraction of the sarcotubular system developed in the first 20 days after denervation. If the releasing properties of the SR increased following denervation (ie: altered calcium release channel in the TC, or increased number of channels if SR area increases) then an increased myoplasmic calcium concentration during activation could be possible. This theory is not unreasonable since an increased sensitivity of denervated mammalian EDL muscle to caffeine has been demonstrated (Gutmann & Sandow, 1965; Kotsias et al. 1987). An interesting experiment would be to study the properties of calcium release channels from SR vesicles, obtained from denervated muscle and incorporated into lipid bilayers. Alternatively, increased myoplasmic calcium could occur via influx of calcium through voltage sensitive calcium channels in the sarcolemma/T-system, since the action potential is prolonged (Redfern & Thesleff, 1971 a,b) and there is a proliferation of T-tubules (Salvatori, Damiani, Zorzato, Volpe, Pierobon, Quaglino, Salviati & Margreth, 1988) and nitrendipine sensitive calcium channels (Schmid, Kazazoglou, Renaud & Lazdunski, 1984) following denervation.
3.4.4 Effects of denervation on inactivation.

Denervation had a more marked effect on the inactivation of tension in soleus than it did on activation parameters. Denervation of soleus and EDL made the parameters of the inactivation curves shift away from control values, such that the Boltzmann parameters for EDL and soleus became more alike in denervated preparations. EDL and soleus muscles have been classified according to their susceptibility, or lack thereof, to fatigue (Burke, Levine, Tsairis & Zajac, 1973; Clamann & Robinson, 1985; Westerblad & Lannergren, 1986), EDL being more susceptible than soleus. In retrospect, this classification bears some similarity to the more negative inactivation curve in EDL. Indeed, inactivation might well be a significant factor in muscle fatigue (see eg Donaldson, 1986).

The results presented for denervated EDL in this study show a small shift in inactivation properties compared to control values. However, denervated EDL generated less relative tension following a given conditioning depolarization than its control counterpart. Activation data shows EDL becoming more like soleus after denervation. If all properties of EDL were transformed to those of soleus in denervated muscle, then denervation would be expected to produce a positive shift in the inactivation curve in EDL. Although the shifts in the inactivation curves were small following denervation, the curves for both EDL and soleus were shifted to slightly more negative membrane potentials, making soleus more like EDL. This is not inconsistent with previous studies showing soleus to EDL shifts in some properties, while EDL to soleus shifts occurred in other aspects of contraction (see eg: Jaweed et al. 1975; Dulhunty & Gage, 1985; Gage et al. 1989; Graham et al. 1992).

While denervation induced only a minimal shift in the inactivation curve for soleus, it is important to note that the degree of inactivation for control soleus was less for all potassium concentrations except 60K where inactivation was virtually complete. This shows that denervated soleus is more vulnerable to the effects of prolonged depolarization than control soleus, which is again consistent with the above observation that denervation makes the properties of soleus similar to those of EDL. Nevertheless, the denervated activation and inactivation curves for EDL and soleus are not identical, suggesting that each muscle-type maintains some of its innervated properties.

The reason why denervation causes a shift in the steady-state inactivation curve for soleus and EDL muscle is not clear. A number of considerations are possible:
(a) Given the effects of denervation on the activation of charge movement in EDL, it is most likely that the inactivation of charge movement is also similarly affected since tension and charge are closely related (Dulhunty & Gage, 1985). The increase in protein turnover following denervation (see eg: Goldspink, 1976; Furuno, Goodman & Goldberg, 1990) may be a major factor underlying changes to the E-C coupling mechanism. The DHP-sensitive calcium channel in the T-system which acts as the voltage sensor for E-C coupling (section 1.9.5) may be altered with denervation. Changes to ion channel kinetics and pharmacology following denervation, including a negative shift in the voltage sensitivity for inactivation of sodium channels, have been reported (see eg: Pappone, 1980; Duval & Leoty, 1985). Interestingly, site-directed mutagenesis has been used to modify regions within the shaker potassium channels from drosophila, resulting in alterations to the voltage dependence of activation and inactivation (Hoshi, Zagotta & Aldrich, 1990; Jan & Jan, 1990; Papazian, Timpe, Jan & Jan, 1991). Since sodium, potassium and calcium channels show a significant degree of conservation of amino acid sequence (Jan & Jan, 1990), it is possible that denervation produces deletions or insertions of amino acids in the channel protein which alters the voltage sensitivity of activation and inactivation of E-C coupling. This is quite feasible, since changes to the voltage dependence of charge movement must reflect changes in the properties of the moving charges (Dulhunty & Gage, 1983).

(b) A number of recent studies have incorporated isolated SR vesicles into lipid bilayer or used skinned fibre techniques to study the characteristics of the calcium release channel of the terminal cisternae. While the general properties of this channel have now been identified (1.8.2), some studies have implicated that the channel can undergo a calcium dependent inactivation (Palade, Mitchell & Fleischer, 1983; Meissner, 1986; Kwok & Best, 1987). This has interesting ramifications for the present study since it could, in theory, provide an alternative mechanism for the decay in tension seen with the inactivation of E-C coupling. However, the time course of the inactivation of calcium release through the calcium channel of the TC is far too rapid (msec) to account for the slow decay time of the voltage sensitive inactivation of tension (seconds). Also, caffeine contracture results in which full activation of depolarized fibres can be achieved, would be unobtainable if the calcium release channels were inactivated.
(c) A third possibility for the effects of denervation on E-C coupling is that the change in potential seen by the voltage sensor is altered by changes to surface charge. Modification of the activation and inactivation kinetics of numerous processes (eg: E-C coupling, ion channel properties etc) has been achieved by the addition of reagents to the bathing solution which alter the surface charge provided by proteins on the surface of the membrane (Agus, Dukes & Morad, 1989; Spires & Begenisich, 1992; McLaughlin, Bruder, Chen & Moser, 1975). For example, the negative ion perchlorate, has been shown to induce positive shifts in the voltage sensitivity of activation of charge movement and tension, together with exposing more than one inactive state of the voltage sensor for E-C coupling (Luttgau et al. 1983; Dulhunty et al. 1992). The actions of perchlorate have been attributed to changes in the surface charge of the sarcolemma. Similarly, modification of protein structure within the membrane by denervation should produce changes to surface charge which could alter the membrane potential perceived by the voltage sensor for E-C coupling.

3.4.5 The two-microelectrode point voltage clamp technique, and interpretation of the strength-duration curve in terms of myoplasmic calcium concentrations.

The two-microelectrode point voltage clamp with visual end point determination of contraction threshold, provided a highly sensitive method of determining the threshold membrane potential for contraction in mammalian muscle. This technique gives a more accurate measure of the voltage dependence of threshold tension for two reasons:-

(a) the voltage clamp procedure does not require uniform depolarization across a bundle of fibres as in K-contracture studies since contraction thresholds are measured in response to movement of surface myofibrils in a single muscle fibre.

(b) the activation of contraction using voltage clamp allows determination of cross-bridge activity at a level which is well below the sensitivity of the the force transducer used in K-contracture studies.

The use of sulphate as the major impermeant anion, changes the cable properties of the muscle fibre to increase the length constant of the T-system and allow for a more uniform clamp, thereby minimising attenuation of depolarizing pulses in axial regions of the muscle fibre. It should be kept in mind that while movement is measured only on surface myofibrils, regions which are not satisfactorily clamped can introduce errors by premature movement in response to
depolarizing pulses. The sulphate solutions serve to increase the region under true voltage clamp.

The general shape of the strength-duration curves presented in section 3.3.5 is consistent with other studies (see eg: Adrian et al, 1969; Costantin, 1974; Chiarandini, Sanchez & Stefani, 1980; Dulhunty, 1982; Miledi et al. 1984; Dulhunty & Gage, 1983; Chua & Dulhunty, 1988, 1989; Dulhunty et al. 1992).

A simple model to explain $V_t$ in terms of myoplasmic calcium can be used to describe the classical shape of the strength-duration relationship. It can be summarized as follows:

1. A depolarizing test pulse of any duration elicits movement when free calcium in the myoplasm exceeds the threshold level required for contraction.
2. The test pulse produces an increase in myoplasmic calcium concentration which reaches a plateau level if the pulse duration is long enough. (NB. the test pulse duration is still short compared with the time for inactivation).
3. At normal resting potentials (around -80 mV) the plateau calcium concentration during a test pulse to contraction threshold is much smaller than the maximum plateau calcium concentration (achieved by a long, large amplitude test pulse).
4. When test pulses that are brief with respect to the time course of $Ca^{2+}$ release are used, calcium concentrations during the pulse do not reach the plateau level. That is, depolarization with long test pulses to a membrane potential $V_t(1)$ that allows the myoplasmic calcium concentration to reach a plateau level which is just threshold for contraction, will be insufficient to achieve threshold concentrations for a short duration test pulse. Therefore a greater depolarization to $V_t(2)$ will increase calcium release so that a threshold calcium level will be achieved within the duration of the test pulse.
5. The briefer the test pulse, the greater will be the depolarization required to generate a threshold level of calcium.

This description suggests that the time required to reach a plateau calcium concentration that is also the threshold for contraction will be indicated by the achievement of a rheobase in the strength-duration relationship.

The difference in contraction thresholds between control soleus and EDL (at all pulse durations) has been shown previously (Dulhunty & Gage, 1983; Chua & Dulhunty, 1988). These results can be primarily explained in terms of differences in the voltage dependence of asymmetric charge movement. Dulhunty & Gage (1985) clearly demonstrated that the voltage for half-maximal charge for EDL was much more positive than that for soleus. The generation of tension will cover a
smaller range of membrane potentials than charge movement, since tension depends on threshold concentrations of activator and may be limited by saturation of the contractile proteins (see Chua & Dulhunty, 1988).

3.4.6 Effects of denervation on strength-duration curves for soleus and EDL.

Potentiation of the 60K contracture in denervated soleus increased the slope of the activation curve relating tension to membrane potential. This result is consistent with a positive shift in the point of initial tension generation observed in K-contractures for denervated soleus (section 3.3.2).

The original hypothesis, that denervation shifts the contraction thresholds for soleus and EDL in opposite directions such that they become similar, was substantiated using the two micro-electrode voltage clamp technique. As for control preparations, the results obtained can be adequately explained in terms of alterations to the voltage sensitivity of charge movement. Denervation produces a negative shift in the voltage for half-maximal charge generation in EDL, and a slight positive shift in the same parameter for denervated soleus (Dulhunty & Gage, 1985). Other effects of denervation which could modulate the contraction thresholds obtained have been outlined in section 3.4.3 above.

Interestingly, Dulhunty & Gage (1983) showed an almost identical effect to denervation on the contraction thresholds for soleus and EDL in spinally transected (paraplegic) rats. The results obtained in this study would suggest that the shifts observed in contraction threshold are primarily due to an effect of the motor nerve on the muscle fibre rather than due to changes caused by higher centres in the CNS.
CHAPTER 4

EFFECTS OF MEMBRANE POTENTIAL ON THE DEPOLARIZATION REQUIRED TO ELICIT CONTRACTION IN RAT SOLEUS AND EDL FIBRES: EFFECTS OF DENERVATION.

4.1 INTRODUCTION

The aim of this section was to investigate the effect of depolarization on contraction threshold in EDL and soleus muscle fibres. Understanding the effects of resting membrane potential on contraction threshold should provide further insight into the properties of the voltage sensor and the state of E-C coupling under conditions of T-tubule depolarization which could occur in damaged muscle fibres, in the hyperkalemic state, or with potassium accumulation during repetitive activity.

To study the effects of depolarization of the resting membrane potential on the membrane potential at which contraction threshold occurs (Vt), the two micro-electrode voltage clamp technique has been employed to measure Vt for contractions elicited by brief (10 msec to 500 msec) test pulses. Since inactivation of E-C coupling occurs during prolonged depolarization, one aim of these experiments was to examine the effect of inactivation on Vt. Maximal test K-contracture tension is reduced as a result of inactivation, and this occurs with less membrane depolarization in EDL compared to soleus (section 3.3.3). Starting hypotheses predicted that (a) Vt should become more positive during steady-state depolarization to membrane potentials producing partial inactivation of maximal K-contracture tension, (b) it would not be possible to elicit threshold contractions during steady-state depolarizations to membrane potentials producing full inactivation of K-contracture tension and (c) both effects of depolarization on Vt should occur at more negative membrane potentials in fast-twitch (EDL) than in slow-twitch (soleus) fibres.

A second objective of this study was to examine the effect of denervation on the relationship between Vt and holding potential. Denervation causes a shift to more negative membrane potentials in the voltage dependence of activation of tension in EDL fibres, and in contraction thresholds in fast-twitch fibres measured at normally polarized potentials (Chapter 3). There is a negative shift in the membrane potential for 50% inactivation of maximal K-contracture tension following denervation of fast-twitch fibres (see section 3.3.3). If these changes in K-contracture activation and inactivation parameters were reflected in the
relationship between \( V_t \) and holding potential then \( V_t \) should become more positive with less depolarization in denervated EDL fibres.

4.2. METHODS

4.2.1 Muscle preparations

Control and denervated rat soleus and EDL preparations were used in these experiments. Removal of whole muscles from animals, micro-dissection procedures and denervation surgery have been described in detail in Chapter 2. The two micro-electrode point voltage clamp technique is also described in Chapter 2.

4.2.2 Solutions

Unless otherwise stated, all experiments were carried out using a low chloride Control solution (Section 2.5) as the bathing solution. For reasons which will be explained in the results section, the solution was modified to also contain 1 mM CoCl\(_2\) or else the CaSO\(_4\) was replaced with 10 mM CaEGTA. Tetrodotoxin was used to block action potential activity to ensure that contraction was in response to applied voltage steps. The concentrations of TTX used were 0.125 \( \mu \)M for control fibres and 5 \( \mu \)M for denervated fibres.

4.2.3 Experimental Protocol

Following insertion of microelectrodes into a muscle fibre using the standard procedures for two microelectrode point voltage clamp, the membrane potential was varied using one or other of the two protocols outlined in Figure 4.1.

**Protocol 1:** A holding potential was established at -80 mV for 2 minutes and the membrane potential for threshold contraction (\( V_t \)) was measured using a rectangular test of varying duration (5-100 msec). The holding potential was then depolarized to -60 mV for two minutes, and contraction threshold determined (Figure 4.1A) between 2 and 3 minutes. The holding potential was then progressively reduced in +5 mV steps. At each level \( V_t \) was measured following a two minute conditioning period. At the end of the experiment the fibre was repolarized to -80 mV. If the fibre was in good condition, less than 100 nA of current was observed and a contraction threshold close to that obtained at the beginning of the experiment could usually be obtained within about 5 minutes at -80 mV.
Figure 4.1: Pulse protocols for depolarization used to measure the effect of holding potential ($V_h$) on the membrane potential at contraction threshold ($V_t$) in rat EDL and soleus muscle fibres. $V_t$ was measured in response to depolarizing test pulses (5-500 msec) applied to fibres voltage clamped at the indicated potentials. (A) Progressive depolarization of $V_h$ in 5 mV steps, with $V_t$ measured after 2-3 minutes at each level. (B) $V_t$ was measured 1-3 minutes after each holding potential was established. The membrane potential was returned to -80 mV for control measurement of $V_t$. 
Protocol 2: \( V_t \) was measured at -80 mV and then the fibre depolarized to a new holding potential (more positive than -60 mV) over a period of 2 minutes. \( V_t \) was measured between 1 and 3 minutes after the depolarized potential had been reached. The holding potential was returned to -80 mV for about 3 minutes (Figure 4.1B) and if the contraction threshold at -80 mV did not vary by more than \( \pm 1 \) mV from the original measurement, then the experiment continued. A new depolarized holding potential was established and the procedure repeated.

In the first set of experiments described below, holding potential was changed from -80 mV to a conditioning potential that was maintained for 10 to 15 minutes and \( V_t \) measured as a function of time at that holding potential.
4.3 RESULTS

4.3.1 Effects of prolonged depolarization on the time course of changes in contraction threshold.

Reductions in the height of test K-contractures after a period of depolarization in a conditioning high potassium solution (see section 3.3.3) follow a biphasic time course, the rate of each phase being dependent on the strength and duration of the conditioning depolarization (Dulhunty, 1991). Figures 4.2A & B show results from a series of experiments on EDL and soleus muscle fibres which examined the time course of changes to $V_t$ during prolonged periods of membrane depolarization. The observed increases in $V_t$ at holding potentials ($V_h$) more positive than -40 mV in soleus, and -30 mV in EDL, followed a biphasic time course. The increases in $V_t$ often followed negative shifts in $V_t$ at holding potentials between -80 mV and -40 mV in soleus, and between -80 mV and -30 mV in EDL. The positive shift in $V_t$ for EDL fibres (Figure 4.2A) at a $V_h$ of -30 mV was always preceded by a negative shift in $V_t$ ($n=5$). For a soleus fibre clamped at a $V_h$ of -30 mV (shown in Figure 4.2B), $V_t$ rose rapidly in the first 3 minutes from -26 mV to -19 mV and then more slowly over the next 7 minutes to -14 mV. Interestingly, the fast and slow phases of the positive increment in $V_t$ occur at roughly the same time as the fast and slow phases of the steady-state inactivation of K$^+$ contractures.

4.3.2 Changes in $V_t$ with changes in $V_h$ for soleus and EDL fibres.

Since the increments in $V_t$ in the above experiments continued for longer than 10 minutes, a steady-state situation had clearly not been reached. Deterioration of muscle fibres usually prevented reliable data collection for very long conditioning durations. Therefore, to enable comparison with (a) K-contracture studies using 3 minute conditioning depolarizations (Chua & Dulhunty, 1988) and (b) the slow phase of steady-state inactivation (Dulhunty, 1991), $V_t$ was routinely measured after depolarizations lasting around three minutes.

The membrane potential was progressively depolarized in 5 mV steps from -60 mV to between -10 mV and 0 mV (Protocol 1, methods, section 4.2, Figure 4.1A above), with $V_t$ measured after a conditioning period at each level. For $V_h$'s between -60 mV and -30 mV, both EDL and soleus became more easily activatable with a test pulse of 50 ms duration (Figures 4.3.A & B). i.e. $V_t$ became more negative by -2 to -5 mV, with a minimum $V_t$ recorded at -30 mV in the
Figure 4.2: Changes in $V_T$ measured as a function of time spent at -20 mV or -30 mV in EDL (A) and -30 mV or -40 mV in soleus fibres (B).
Figure 4.3: $V_t$ was measured during continuous depolarization to various holding potentials using protocol 1 with an EDL (A) and soleus (B) fibre. $V_t$ was measured with a 50 msec test pulse. Note the decrement in $V_t$ for holding potentials between -60 mV and -40 mV in soleus or -30 mV in EDL, and the steep increase in the threshold for contraction as the holding potential becomes more positive.
EDL fibre in Figure 4.3A and at -40 mV in the soleus fibre (Fig 4.3B).

Depolarizations of V_h to levels more positive than -30 mV always resulted in increments in the amplitude of the test pulse required to activate fibres. That is, V_t increased sharply and many fibres could not be activated by test pulses to +20 mV. The positive shift in V_t was usually steeper in soleus fibres.

Test pulses to levels more positive than +20 mV were not employed because the voltage clamp was not reliable in this potential range. Fibres with a V_t < +20 mV were considered activatable, while those in excess of +20 mV were nominated inactivatable. Statistical analysis of results was made difficult for two reasons. Firstly, plotting average values of V_t was not possible since V_t had no real value if more positive than +20 mV. The situation was compounded because fibres become inactivatable over a range of holding potentials. For example, when V_t in 7 soleus fibres was obtained with a 20 ms pulse, all fibres could be activated from a V_h of -25 mV, four fibres at -20 mV, three from -15 mV and only one from -10 mV. Therefore, in order to include all results in the determinations of averages, inactivated fibres were arbitrarily assigned a value of +20 mV.

Curves displaying average V_t are shown for soleus and EDL in Figures 4.4A & B. Significantly, these curves show that fibres are most easily activated (ie minimum V_f) at membrane potentials (-30 to -40 mV) at which inactivation of E-C coupling produces a significant reduction in test 200K contracture (section 3.3.3). Even more unexpected was the fact that EDL fibres remained activatable at holding potentials at which soleus fibres could not be activated. This was surprising since steady-state inactivation of EDL fibres occurs at more negative membrane potentials than soleus (section 3.3.3): if the ability of a voltage clamp test pulse to activate fibres had been strongly influenced by inactivation, then V_t for EDL would have increased beyond +20 mV at more negative holding potentials than in soleus.

4.3.3 Effect of depolarization protocol on the curve relating V_t to holding potential.

Differences observed between the contraction threshold data outlined above and steady-state inactivation data (section 3.3.3) may have arisen from differences in protocol. For the results presented in section 4.3.2, membrane potentials were maintained in the depolarized state for many minutes. In contrast, fibres were depolarized for three minutes only in the K-contracture experiments, followed by about 20 minutes repolarization in 3.5 mM potassium before further depolarizations. Slow changes in ion concentrations within the fibre or T-system with prolonged depolarizations could account for the observed discrepancies. To
Figure 4.4: Effect of pulse duration on average contraction thresholds plotted as a function of holding potential for EDL (A) and soleus fibres (B). Open circles and open squares represent contraction thresholds measured when $V_h$ was stepped progressively from one potential to the next (protocol 1, methods) with test pulse durations of 10 msec and 20 msec respectively. The filled circles represent contraction thresholds measured with discrete depolarization of $V_h$ (protocol 2, methods) and a 15 msec test pulse duration.
test this possibility, a step depolarization protocol (Protocol 2, methods) was introduced which tested $V_t$ at the end of 1-3 minute sojourns at depolarized holding potentials. $V_h$ was then returned to -80 mV. The discrete conditioning depolarizations yielded values for $V_t$ (Fig 4.4, filled circles) which were essentially the same as those obtained with progressive depolarization (Fig 4, open circles). These results suggest that the value for $V_t$ at each holding potential is independent of the previous history of the fibre.

Chua & Dulhunty (1988) demonstrated that $V_t$ increased at more negative potentials in EDL than soleus. While the actual values for $V_t$ for soleus were the same in Chua & Dulhunty (1988) and in the present study, $V_t$ for EDL fibres was considerably more positive at holding potentials between -30 mV and -40 mV in Chua & Dulhunty (1988). The reason for this difference is not clear. It is interesting to note that the difference in the voltage for half maximal inactivation (Chapter 3) was very much less between EDL and soleus than that reported by Chua & Dulhunty (1988). As discussed in Chapter 3, this may have been due to different rat colonies. Since there was less difference in the inactivation curves, we might expect less of a difference in the effect of inactivation on the $V_t$-holding potential curve. In addition, the Chua & Dulhunty (1988) investigation was carried out at a much higher microscope magnification. It was noted in this study that the muscle fibre underwent a spontaneous contraction around the microelectrodes when the membrane potential was depolarized to levels around -30 to -40 mV. For this reason, it was concluded that distortion of the myofibrils around the microelectrodes at holding potentials positive to -40 mV may have obscured sarcomere shortening and produced an artificial increase in $V_t$ at more positive holding potentials when sarcomere shortening at high magnification was used to determine $V_t$.

### 4.3.4 Does external calcium affect the voltage dependence of $V_t$?

The decrease in $V_t$ at membrane potentials between -80 mV and -40 mV was possibly due to an influx of calcium ions, either through voltage sensitive calcium channels or through the sodium/calcium exchange mechanism. If the influx of calcium brought myoplasmic calcium to levels which were just subthreshold for contraction, then $V_t$ could remain the same or become more negative with depolarization. To test this possibility, either 1 mM cobalt was added to the bathing solution to block calcium channels (see eg. Sanchez & Stefani, 1978)(solid lines Fig 4.5A & B), or calcium in bathing solution was replaced by 20 mM CaEGTA (long dashed line Fig 4.5B). $V_t$ was measured in soleus fibres using a 10 msec test pulse (Fig 4.5A) on continually depolarized fibres (Protocol 1, methods) or using discrete
Figure 4.5: Analysis of the effect of external calcium and calcium influx through voltage sensitive calcium channels on contraction threshold versus holding potential curves in soleus (A&B) and EDL (C) fibres. In A & C, contraction thresholds were measured in response to a 10 msec test stimulus with progressive depolarization of Vh (protocol 1, methods) for fibres bathed in the low chloride control solution (open circles, broken line) or in the low chloride control solution in which calcium concentrations were not altered and 1 mM C\(_{\text{Cl}}\) was added (filled circles, solid line). Discrete depolarizations (protocol 2, methods) and a 15 msec test pulse durations was used in B to generate contraction for fibres bathed in the control solution (open circles), or control solution with 1 mM C\(_{\text{Cl}}\) added (filled circles) or solution with 20 mM CaEGTA (filled squares, see text).
conditioning depolarizations (Protocol 2, methods) with a 15 msec test pulse (Figure 4.5B). The minimum value for $V_t$, which occurred at -40 mV in the control solution (Fig 4.5B), was not altered by addition of 1 mM cobalt or the replacement of calcium with 20 mM EGTA. However, the increase in $V_t$ at holding potentials more positive to -40 mV was steeper in fibres bathed in the modified solutions. In contrast, the average $V_t$ for EDL fibres bathed in the low chloride control solution, containing 1 mM Co$^{2+}$, decreased considerably for holding potentials more negative than -35 mV (Figure 4.5C). At holding potentials more positive than -35 mV, $V_t$ in cobalt treated fibres rose in almost identical fashion to $V_t$ obtained for control preparations ($n=8$).

4.3.5 Effect of test pulse duration on contraction threshold.

An analysis of the effect of pulse duration on the voltage dependence of $V_t$ was performed in normal and denervated, soleus and EDL fibres. As would be expected from strength-duration relationships (this study, section 3.3.5; Adrian et al. 1969; Costantin, 1974; Chua & Dulhunty, 1988), $V_t$ becomes more negative for longer test pulse durations (Figures 4.6A-D). This effect was most obvious in soleus fibres (Figures 4.6B & 4.6D) where $V_t$'s for each set of data fell at more negative membrane potentials as the test pulse duration was increased. In EDL, there was considerable overlap of average $V_t$'s for pulse durations in excess of 20 msec (Figure 4.6A & 4.6C).

It should be noted that test pulse duration has no effect on the holding potential for minimum $V_t$. However, the negative slope of the $V_t$-holding potential curve is greatest when $V_t$ was obtained with brief, 10 msec or 20 msec test pulses (Figure 4.6C & 4.6D). The curves for 500 msec test pulses were either flat between -60 and -30 mV for soleus, or had small positive increments in $V_t$ in the range of holding potentials between -60 and -35 mV for EDL. While the positive increases in $V_t$ became steeper as the test pulse became briefer, there was virtually no difference between results obtained with 100 msec (open circles, broken lines) and 500 msec test pulses (filled circles, continuous lines) in either EDL or soleus.

4.3.6 Effect of test pulse duration on the holding potential at which fibres became inactive.

Soleus and EDL fibres were more subject to inactivation when the duration of the test pulse was brief. For example, at a holding potential of -10 mV (Figure 4.7A) no EDL fibres could be activated by a 10 msec test pulse, whereas the
Figure 4.6: The effect of test pulse duration on average contraction threshold plotted as a function of holding potential for EDL (A) and soleus fibres (B). Test pulse durations used were 10 msec (open triangles), 20 msec (open circles), 50 msec (inverted triangles), 100 msec (open diamonds) and 500 msec (filled circles). C and D show an expanded $V_h$ axis for membrane potentials between -60 mV and -30 mV, in EDL and soleus fibres respectively.
Figure 4.7: (A) Comparison between EDL (filled circles, solid line) and soleus (open circles, broken line) of the number of fibres activatable by a test depolarization of set duration from a holding potential of -10 mV. (B) and (C) Percentage of fibres activatable at holding potentials between -30 mV and 0 mV. Results are plotted as a function of the depolarizing test pulse duration used to elicit threshold contractions in EDL (B) and soleus (C) respectively. Test pulse durations used were 10 msec (filled squares), 50 msec (filled circles), 100 msec (open circles) and 500 msec (open squares).
percentage of activatable fibres increased to 88% when the pulse duration was increased to 20 msec, and 100% for 100 and 500 msec test pulses. In soleus, only 14% of fibres could be activated by a 20 msec test pulse at a holding potential of -10 mV and a maximum of 83% could be activated by 100 msec or 500 msec pulses (Figure 4.7A). Inactivation was first observed in EDL at holding potentials of -15 mV (Figure 4.7B), compared to -35 mV in soleus (Figure 4.7C).

4.3.7. Construction of strength-duration curves at different holding potentials.

The data for average V_t-holding potential curves shown in Figure 4.6 has been replotted in Figure 4.8 to show the effect of holding potential on the strength-duration relationship. Figure 4.8A & B show strength-duration relationships with pulse duration displayed on a linear scale. To display data obtained with short pulse durations, Figure 4.8C & D show the same data with pulse duration expressed in logarithmic form. It can be clearly seen that depolarization of the membrane potential from -60 mV to -40 mV has little effect on the strength-duration relation. However, further depolarization resulted in a parallel shifts in the strength-duration relation to more positive values of V_t. At a holding potential of -10 mV, the positive shift in EDL was +20 mV compared to +30 mV in soleus. Parallel shifts in V_t suggest that the time course of threshold calcium equilibration in the myoplasm, and the rate of calcium release from the SR were not altered in depolarized fibres.

Figure 4.8C shows that a rheobase potential was reached in EDL in the range of 20 to 50 msec. The clustering of V_t's for pulse durations in excess of 20 msec shown in Figure 4.6A & C provides further evidence that rheobase has been reached. In contrast, no clear rheobase was reached in soleus fibres (Figure 4.8B & D).

4.3.8 Are effects of holding potential on V_t the same in different types of fast-twitch muscle fibres?

To examine whether the effects of holding potential on V_t in EDL could be reproduced in fast-twitch fibres of other muscles, a 15 msec test pulse was used with discrete conditioning depolarizations (Protocol 2, methods) on red and white sternomastoid muscle fibres (described by Dulhunty & Dlutowski, 1979). Results obtained with both red and white sternomastoid fibres were very similar to those obtained with EDL. The steep increase in V_t seen at a holding potential of -30 mV in EDL was also observed in sternomastoid fibres (Fig 4.9), in contrast to soleus
Figure 4.8: Strength-duration curves for EDL (A) and soleus (B) plotted from average data obtained Figure 5.6. In A, average $V_t$'s, plotted against test pulse duration, were acquired at holding potentials of -15 mV (open squares), -20 mV (open triangles), -25 mV (open diamonds), -40 mV (open circles) and -60 mV (filled circles). In B, the holding potentials were -15 mV (open squares), -20 mV (open triangles), -30 mV (open diamonds), -40 mV (open circles) and -60 mV (filled circles). C and D show the same data as that in A and B, with pulse duration displayed on a logarithmic scale.
Figure 4.9: Average $V_t$ is plotted against holding potential for white sternomastoid (open circles) and red sternomastoid fibres (open squares). For the purpose of comparison, average data for EDL is also plotted (filled circles). All results were obtained with discrete conditioning depolarizations (protocol 2, see methods) using a 15 msec depolarizing test pulse.
where the increase occurs at a holding potential of -40 mV (Figs 4.3 & 4.4 above). Consequently (within this limited survey), the holding potential at which $V_t$ is minimal, and the range of membrane potentials over which $V_t$ increases, appear to be specific characteristics of the "type" of muscle fibre examined.

4.3.9 *Effects of denervation on the $V_t$-holding potential relationship.*

Progressive 5 mV step depolarizations (Protocol 1, methods) were used to examine the effect of a 50 msec test pulse on denervated fast- and slow-twitch muscle. As expected, $V_t$ for holding potentials between -60 mV and -40 mV became more negative in denervated EDL (Fig 4.10).

Contrary to expectations, the holding potential at which $V_t$ increased sharply in EDL was not altered by denervation and the slope of the positive component of the $V_t$-holding potential curve between -30 and -20 mV (a) remained the same as normal in denervated EDL fibres (Fig 4.10A) and (b) was shifted to more positive holding potentials in denervated soleus fibres (Fig 4.10B). The general shape of the $V_t$-holding potential curve in denervated soleus became almost identical to that of normal and denervated EDL, i.e. the positive slope of the curve was similar in all three preparations.

4.3.10. *Can changes to $V_t$ in depolarized fibres be attributed to inactivation of E-C coupling?*

Of some concern in the results was (a) the apparent mismatch between changes in $V_t$ with depolarization compared to K-contracture data, and (b) the tendency for $V_t$ to "track" the holding potential along the positive arm of the $V_t$-holding potential curve (i.e. a +5 mV increase in holding potential produced a +5 mV increase in $V_t$). In order to test the possibility that $V_t$ was tied to the holding potential and was not influenced by steady-state inactivation, the slope of the positive limb of the $V_t$-holding potential was measured. A slope of 1 was indicative that the $V_t$ was tied to the holding potential, whereas a slope greater than 1 suggested that other factors such as inactivation of E-C coupling were likely to be involved in the steeper increment in $V_t$.

Figure 4.11A shows $V_t$-holding potential relationships for two soleus fibres examined with test pulse durations of 10 msec (triangles) and 100 msec (filled circles). The solid line in Fig 4.11A shows a slope of 1 between holding potential and $V_t$. The $V_t$-holding potential curve for the 100 msec test pulse quite clearly
Figure 4.10: Effect of denervation on average $V_t$ plotted against holding potential for EDL (A) and soleus (B). Control curves are plotted with open triangles joined by a broken line; denervated with filled circles and solid line. Denervation of soleus shifts the curve relating average $V_t$ to holding potential to more positive potentials, making it similar to that of EDL (dotted line in (B), provided for comparison). Results were obtained using a continuous depolarization of the membrane potential (protocol 1, methods) with a 50 msec depolarizing test pulse.
Figure 4.11: (A) Curves relating $V_t$ to holding potential for two soleus fibres obtained with 10 msec (open triangles) and 100 msec (filled circles) test pulse durations. The solid line has a gradient of 1 and demonstrates more clearly the tendency of $V_t$, achieved with longer test pulse durations, to "track" the holding potential. (B) Plot of the average slope of the positive limb of the $V_t$ versus holding potential curve as a function of the depolarizing test pulse duration in EDL (open circles) and soleus fibres (filled circles).
follows the solid line, in contrast to the curve for the 10 msec test pulse which has a much steeper slope. The average slope of the positive limb of the \( V_t \)-holding potential curve for a 10 msec test pulse was 2.25 for soleus compared to 1.75 for EDL (Fig 4.11B). The average slope decreased with all increments of the test pulse duration for both soleus and EDL, but was not significantly different from 1 for 100 msec test pulses in EDL or 500 msec in soleus.

Naturally, the slope of the \( V_t \)-holding potential curve was steepest, but could not be measured, at the point at which fibres become inactive. Taken together, the results show that the effect of inactivation on \( V_t \) (a) is dependent on the duration of the test pulse and is greatest for pulse durations less than 100 msec and (b) is more apparent in soleus fibres than EDL at all pulse durations.

4.4 Considerations in the interpretation of changes in \( V_t \). Analysis of the effect of inactivation on \( V_t \).

4.4.1 Hypothesis for the effect of inactivation of E-C coupling on \( V_t \).

A working hypothesis was developed for the effect of inactivation on relative values for \( V_t \) at different holding potentials, based on K-contracture data and the 3 state model given in section 1.11.1. If it is assumed that K-contracture tension reflects the state of the voltage sensor, then the concentration of voltage sensor available for the conversion of P to A at different holding potentials is given by the inactivation curves fitted to the amplitude of test K-contractures in soleus and EDL following a 3 minute conditioning depolarization in elevated potassium (Chua & Dulhunty, 1988). These curves show that at a membrane potential of -40 mV, test K-contracture tension is reduced to 80% of maximum in soleus fibres, or to 30% of maximum in EDL fibres (ie: available \([A]\) is reduced to 0.8 of maximum in soleus or 0.3 in EDL). The hypothesis assumes that:

1. Test depolarizations that are long, and approach times at which rheobase is achieved, result in an increase in A (and calcium concentration) to a plateau level which would be maintained for several seconds if depolarization was maintained, before inactivation reduced the concentration of A.
2. Depolarization to membrane potentials between -60 and -20 mV results in a partial inactivation of E-C coupling such that the maximum plateau concentrations of A and calcium that can be achieved by long depolarizing pulses (which bring about maximal conversion of P to A) are reduced.
3. Partial inactivation causes a proportional reduction in submaximal plateau calcium concentrations achieved during a test pulse to $V_t(1)$ (see section 3.4.5). Therefore, the test pulse amplitude from depolarized holding potentials must be increased in order to reach threshold calcium concentrations. i.e: a test depolarization to $V_t(1)$ from a holding potential of -80 mV will increase the myoplasmic calcium concentration to a level in excess of that which could be generated by a test depolarization to $V_t(1)$ from -60 mV. Consequently, $V_t(2)$ at -60 mV should become more positive.

4. Fibres cannot be activated by test pulses ($V_t > +20$ mV) once the maximum plateau calcium concentration is below the level required for threshold contractions.  

4. When the maximum plateau concentration is reduced to levels that are close to the threshold for contraction, fibres that can be just activated by long test pulses cannot be activated by briefer test pulses. Therefore the strongest effects of inactivation should be seen with the briefest test pulses.

As predicted by the model, the strongest effects of inactivation were, in fact, seen with brief test pulses. However, the predicted increase in $V_t$ at a holding potential of -60 mV (see point 3 in hypothesis above) was not observed. i.e: the inactivation of test contracture amplitude that occurs between -80 mV and -40 mV did not result in a positive shift in $V_t$. Therefore calcium levels in the myoplasm can apparently reach threshold or higher concentrations with a test depolarization to the same membrane potential, regardless of whether the test depolarization was made from -80 mV or from a partially inactive state at potentials between -80 mV and -40 mV. Possible reasons for differences between the expected and observed changes in $V_t$ are discussed below.

4.4.2 Effect of a background tension on measurement and interpretation of $V_t$.

Steady-state depolarizations to membrane potentials more positive than -40 mV lead to an increase in K-contracture tension followed by a slow decay to either the baseline or to a small pedestal level (Chua & Dulhunty, 1989; this study - see section 3.3.2). The pedestal tension is thought to reflect the existence of a low steady-state concentration of activator which maintains the efflux of calcium from the SR such that myoplasmic calcium levels are close to, or in excess of, the threshold for contraction.

The "tracking" effect seen along the positive limb of the $V_t$-holding potential curve is consistent with the presence of a steady-state just threshold concentration of calcium at holding potentials between -40 mV and 0 mV. Within this range of
holding potentials, a test pulse initiates the release of additional calcium which is superimposed on the threshold calcium concentrations. For long test pulses only a small additional depolarization is required to release extra calcium and generate a visible contraction. The extra depolarization should be constant so long as the pedestal tension is maintained and there is sufficient activator concentrations to generate further calcium release. When activator concentrations become close to zero, additional calcium release is not possible and fibres become inactive. Once again, fibres tested with brief depolarizing pulses should be more affected since a reduction in available precursor should ensure that larger depolarizations are required to generate threshold calcium concentrations.

4.4.3 Consideration of activation and inactivation of K-contracture tension in predicting the effect of holding potential on $V_t$ in different fibre types.

That test 200K contracture tension inactivates at more negative membrane potentials in EDL compared to soleus led to an initial hypothesis that test pulses would fail to elicit contractions at more negative membrane potentials in EDL fibres than in soleus fibres. The fact that the opposite result was obtained can be explained if the effects of inactivation on $V_t$ become apparent only at holding potentials at which the concentration of P became very low. Although the potential for 50% inactivation is more negative in EDL fibres, the slope of the inactivation curve (for 3 minute conditioning depolarizations) is much steeper for soleus fibres ($k_i=2.95$ mV in soleus compared with 7 mV in EDL). Consequently, the inactivation curve approaches zero tension at membrane potentials of -15 mV in soleus fibres or 0 mV in EDL fibres. It would therefore be expected that since, full inactivation occurs at more positive membrane potentials in EDL fibres, the sudden increase in $V_t$ should occur at more positive holding potentials in EDL fibres than in soleus.

An additional consideration arises from the activation curves for tension generation. The tension-membrane potential curve saturates at more positive membrane potentials in EDL (+20 mV) than it does in soleus (0 mV)(Chua & Dulhunty, 1989; this study). Therefore complete conversion of P to A occurs with long test pulses to -10 mV in soleus or to +10 mV in EDL. Consequently, soleus fibres which cannot be activated by a test pulse to -10 mV will not be activated by a pulse to +20 mV since all activator has presumably been used by -10 mV. On the other hand, EDL fibres which have not been activated by test pulses to 0 mV could conceivably be activated if the amplitude of the depolarizing pulse was increased to +20 mV. An extension of this argument is that, if conversion to I is not maximal
by 0 mV in EDL, then these fibres are potentially activatable, whereas soleus fibres are not.

Therefore the activation and inactivation parameters at membrane potentials close to 0 mV are consistent with the observation that the steep component of the $V_t$-holding potential curve occurs at more negative membrane potentials in soleus than in EDL.

4.4.4 Comparison of $V_t$/holding potential curves for different fibre types and test pulse durations.

The strength-duration curves shown in Figure 4.8 show that a rheobase is achieved with a 50 msec pulse in EDL, but not with a 500 msec pulse in soleus fibres. These results suggest that the increase in myoplasmic calcium is complete and plateau concentrations approached by 50 msec in EDL, but not by 500 msec in soleus fibres. The stronger effect of inactivation on $V_t$ obtained with test pulse durations that are short compared with rheobase has been discussed in section 4.3.2 above. Therefore when assessing the relative effects of holding potential on $V_t$ in EDL and soleus, results obtained on the rheobase part of the strength-duration curve should be compared. Figure 4.12 demonstrates that, when $V_t$ is obtained with a 50 msec test pulse in EDL, and a 500 msec test pulse in soleus, the steep increase in $V_t$ appears to occur over a similar range of membrane potentials.

4.5 DISCUSSION

This study provides a detailed analysis of the effect of holding potential on the threshold potential at which contraction can be visually detected in different fibre types and over a range of test pulse durations. In contrast to effects on maximum tension, a reduction in the membrane potentials to values between -80 mV and -40 mV had little or no effect on $V_t$. A small shift in $V_t$ to more negative membrane potentials was seen for holding potentials within this range. A further shift in the holding potential to values more positive to -40 mV produced an increase in the value for $V_t$. An analysis of the positive limb of the $V_t$-holding potential curve suggested that increases in $V_t$ were most strongly influenced by the partial inactivation of E-C coupling only when brief test pulses (<100 msec) were used.

Chua & Dulhunty (1989) have previously reported a small negative shift in $V_t$ for membrane potentials between -80 mV and -40 mV, and explained the effect
Figure 4.12: Examples of $V_t$ plotted against holding potential for a 50 msec test pulse duration in EDL (open triangles) and a 500 msec test pulse in soleus (filled circles).
in terms of steady-state changes to subthreshold myoplasmic calcium concentrations. That the negative shift was most apparent with brief test pulses is inconsistent with this hypothesis since a steady-state increase in calcium should boost the total calcium concentration by the same amount following short and long pulses. An alternative explanation is that the relationship between the rate of calcium release from the SR and the amplitude of the test depolarization becomes steeper as the holding potential is depolarized through the range from -80 mV to -40 mV. If this were the case, then calcium concentrations could increase more rapidly during brief test pulses, and the depolarization required to achieve threshold calcium concentrations would be reduced.

The strength-duration curve for contraction, achieved from holding potentials near the resting membrane potential in voltage clamped fibres, does not achieve a true rheobase. Small reductions of 1 to 2 mV are seen in $V_t$ as the test pulse duration is increased from 500 msec to 1000 msec in frog muscle (Costantin, 1974) or 500 msec to 2000 msec in rat EDL (Chua & Dulhunty, 1988). However, the present experiments have demonstrated that in depolarized EDL fibres rheobase is approached with test pulses of 20 to 50 msec. In soleus fibres, $V_t$ fell by 4 mV when test pulse duration was increased from 100 msec to 500 msec (Figure 4.8). A further decrement of 5 mV has been reported for changes to test pulse duration from 500 to 1000 msec, and a smaller fall between 1000 msec and 2000 msec (Chua & Dulhunty, 1988).

The way in which strength-duration curves approach rheobase suggests that, at threshold calcium concentrations, a plateau calcium concentration in the myoplasm is achieved between 20 to 50 msec in EDL fibres, and between 500 msec to 1000 msec in soleus fibres. Since the distance between the site of calcium release from the terminal cisternae to the myofilament proteins is not significantly different in EDL and soleus fibres (Eisenberg & Kuda, 1976), the strength-duration curves suggest that the rate of calcium release with depolarizations to threshold potentials is 10 times faster in EDL fibres than in soleus fibres. Records of aequorin responses obtained by Eusebi, Miledi & Takahashi (1985) with strong test depolarizations from -80 mV to 0 mV, show that calcium release is faster in EDL fibres and that peak calcium concentrations are achieved in 50 msec in EDL and 300 msec in soleus. Since the rate of calcium release is depolarization dependent (Miledi et al, 1984), the times taken for aequorin transients to reach a plateau are consistent with the strength-duration curves presented in this study.

Denervation shifted the positive limb of the $V_t$-holding potential curve to more positive potentials for a 50 msec test pulse in soleus fibres. Thus, the curve for denervated soleus became similar to that for normal and denervated EDL.
Noticeably, denervation did not alter the way in which contraction threshold approached rheobase in EDL, but it did alter the curve for soleus so that rheobase was approached between 20 msec and 50 msec. The effect of denervation on the strength-duration curve can account for the shift in the $V_T$-holding potential curve which is equivalent to the shift that would be seen in normal soleus fibres with an increase in test pulse duration from 50 msec to 500 msec (Figure 4.6).
CHAPTER 5

ANALYSIS OF THE TIME COURSE OF MAXIMAL CONTRACTURES IN NORMAL AND DENERVATED SOLEUS AND EDL MUSCLE. EFFECTS OF CONDITIONING DEPOLARIZATIONS.

5.1 INTRODUCTION

The following experiments were designed to examine the time course of test maximal potassium contractures induced by exposure to 200K in rat soleus and EDL muscle. Because the amplitude of test contracture tension was markedly affected by conditioning depolarizations (Chapter 3), a primary aim of these experiments was to analyse the effect of conditioning depolarizations on the time course of the decay of test contracture tension. Acceleration of the rate of spontaneous decay of 200K contracture tension following a conditioning depolarization could explain the observation that the time course of decay of contractures in skinned frog muscle fibres induced by ionic depolarization of the T-tubule membrane is more rapid than that of K-contractures in intact muscle fibres (personal communication with Lamb & Stephenson). This could be due to possible depolarization of T-system membranes in skinned fibres.

A second objective of this investigation, to study the effects of denervation on the decay of K-contracture tension, stemmed from a number of observations in earlier studies. In an examination of the time course of maximal contractures in single muscle fibres of the frog, Stuesse, Lindley & Kirby (1974) noted that spontaneous inactivation of contracture tension following exposure to high potassium solutions was accelerated in fibres that had been denervated (6-36 days). Dulhunty (1985) demonstrated that the effect of denervation on the spontaneous inactivation of maximal and sub-maximal potassium contractures in bundles of mammalian muscle fibres was complex. In particular, data was difficult to interpret since denervation appeared to induce a biphasic decay of contracture tension in EDL, and the time taken for contractures to decay to 50% of peak amplitude for normal and denervated soleus varied significantly. As a consequence, the second objective of this study was to further investigate the effects of denervation on the time course of maximal contractures, and to examine the way in which the time course was affected by preceding conditioning depolarizations.
5.2 METHODS

Procedures for the potassium contracture technique, including the solutions, dissection of muscle preparations and measurement of tension responses have been described in section 2.5.

A two-pulse protocol was used, in which fibres were exposed to a conditioning solution for 5, 10 or 30 minutes and then to a test 200K solution. In contrast to previous experiments, all 200K contractures were allowed to run their full time course (ie: completely inactivate, so that tension returned to the base line or a pedestal level) rather than washing out the 200K solution with the low chloride control solution at the peak of the contracture.

These experiments were designed to examine the time course of the inactivation of tension and the effects of depolarization on that time course. The 30K solution was used for a conditioning depolarization since it produced a significant amount of inactivation without completely abolishing the tension response. The time courses of three contractures were recorded: t_c1, t_i and t_c3. i.e. the unconditioned test 200K contracture, the conditioned test 200K contracture and the recovered test 200K contracture respectively (see section 3.2.1).

Data was collected as described in section 2.2. Digitally sampled contractures were saved as "ASCII" files and later transferred to Lotus 123 spreadsheets where they were analysed for peak amplitudes and rise and decay times.
5.3 RESULTS

5.3.1 Effects of a conditioning depolarization on the relative rise and decay times of 200K contractures.

The time course of the tension response for a full 200K contracture of a small bundle of intact soleus muscle fibres has a rapid activation phase followed by a slower monotonic decay phase. A typical 200K contracture for soleus is shown in Figure 5.1. The parameters which were measured on this and other 200K contractures are shown in the figure, next to the tension response. The time taken for tension to rise from 10% to 90% of peak amplitude, and also to decay from 90% to 10% of peak amplitude was measured in all preparations. In addition to the 10% and 90% values, 20%-80% rise times and 80-20% decay times were also measured since, in some preparations (see section 5.3.6 on denervated EDL), the decay of the contracture response took an exceptionally long time to decay from 20% of maximal contracture height back to zero amplitude. This was because the observed tension response became biphasic and could not be meaningfully described by the 90-10% decay time.

In Chapter 3 (section 3.3.4) it was shown that the overlap of activation and inactivation curves could be used to predict pedestal tensions. Even in the absence of pedestal tensions, myoplasmic calcium concentrations could be elevated to subthreshold levels in response to moderate depolarizations which are maintained for many minutes. If this is the case, then the time to rise to peak contracture amplitude in conditioned muscle fibres should be slightly faster than that for unconditioned fibres. The data presented in Table 5.1A shows that average rise and decay times of 200K contractures are faster when preceded by a 5 minute conditioning depolarization in a 30K solution. The conditioning depolarizations significantly decrease (in the order of seconds) the rise times (10-90% and 20-80%) of subsequent maximal contractures. Since the rate of rise of contracture tension is voltage dependent (Hodgkin & Horowicz, 1960) it is probable that conditioning depolarizations alter this voltage sensitivity.

To further quantify the rise times of maximal contractures, five experiments were carried out to measure the exact time to peak amplitude in conditioned and unconditioned fibres upon exposure to 200K solutions. Immediately prior to addition of 200K to the bath, a small voltage step was imposed on the tension response. The time for the contracture to reach peak height was measured from the
Figure 5.1: Tension output of a bundle of soleus muscle fibres upon exposure to a 200K solution. Tension on the Y-axis was expressed as a percentage of the peak height for the contracture. The vertical calibration bar is 5 mN. Parameters which were measured on this and all other preparations are shown next to the tension response (inset).
**Table 5.1A:** Average absolute rise and decay times (seconds) for 200K contractures in soleus fibres. \( t_c1, t_j \) and \( t_c3 \) represent maximal 200K contractures obtained under different conditions (see text).

<table>
<thead>
<tr>
<th>(n=5)</th>
<th>10-90%</th>
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<th>90-10%</th>
<th>80-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_c1 )</td>
<td>5.0±0.20</td>
<td>3.0±0.12</td>
<td>21.5±4.6</td>
<td>14.7±3.4</td>
</tr>
<tr>
<td>( t_j ) (after conditioning)</td>
<td>3.9±0.7</td>
<td>2.7±0.6</td>
<td>11.5±2.2*</td>
<td>7.9±1.5*</td>
</tr>
<tr>
<td>( t_c3 )</td>
<td>5.3±3.2</td>
<td>3.2±0.1</td>
<td>18.4±4.0</td>
<td>12.7±2.8</td>
</tr>
</tbody>
</table>

**Table 5.1B:** Average absolute rise and decay times (seconds) of maximal contractures in soleus fibres following 3 consecutive exposures to 200K. A considerable recovery period preceded each exposure to 200K.

<table>
<thead>
<tr>
<th>(n=4)</th>
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<th>90-10%</th>
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<tbody>
<tr>
<td>(1)</td>
<td>4.3±0.8</td>
<td>2.8±0.6</td>
<td>27.1±6.8</td>
<td>19.0±5.0</td>
</tr>
<tr>
<td>(2)</td>
<td>4.7±1.1</td>
<td>3.2±0.9</td>
<td>21.9±5.3</td>
<td>14.5±3.7</td>
</tr>
<tr>
<td>(3)</td>
<td>5.3±1.4</td>
<td>3.7±0.9</td>
<td>19.6±4.2</td>
<td>13.2±2.7</td>
</tr>
</tbody>
</table>

* \( p<0.1 \)
end of the voltage step, which coincided with the onset of exposure to the 200K solution.

Results showed that soleus fibres, conditioned in 30K for 5 minutes, reached peak height on average 15% faster than unconditioned fibres (n=5). That is, the average time to reach peak height following exposure to 200K was 8.05±0.18 seconds (mean ± sem) in unconditioned fibres compared to 6.85±0.39 seconds in conditioned fibres. This difference was significant at the p<0.05 level (student t-test).

Differences in the rate of decay of 200K contracture tension were obvious following a five minute conditioning depolarization. Figure 5.2A shows the same contracture as that in Figure 5.1 (pre-conditioning control 200K, tc1), together with a test 200K contracture (tj) on the same preparation which was preceded by a five minute conditioning in a 30K solution. The test 200K contracture (tj) was scaled and superimposed on the peak height for the tc1 contracture and quite clearly shows an increased rate of inactivation. (ie: faster decay of the tension response). The capacity of the preparation to recover to its pre-conditioned state is shown in Figure 5.2B. The post-conditioned control 200K contracture (tc3) was also scaled and superimposed on the peak height for the tc1 contracture. It can be seen that the rate of decay of the tc3 contracture recovers, such that it becomes closer to the decay rate of the tc1 contracture.

5.3.2 Control experiments studying three consecutive 200K contractures in soleus without a conditioning depolarization.

In each experiment, the tc3 contracture was elicited when any tetanus had recovered to a stable amplitude which was within 10% of height of the tetanus recorded prior to the tc1 contracture (usually about 20 minutes). Table 5.1B shows that decay times, in particular, of tc3 contractures recorded at this time did not always fully recover to the values obtained for tc1 contractures. Therefore, either the effects of the prolonged depolarization were still present, or rundown of preparations occurred such that the optimal tension response could not be maintained. To test this possibility, muscle fibre preparations were subjected to three consecutive exposures to 200K, with a long recovery (approx. 20-30 minutes) between each exposure.

Figure 5.3 shows tension responses for a normal soleus preparation which underwent 3 full 200K contractures as described above. This figure demonstrates the slow increase in the rate of decay as the number of exposures to 200K increases.
Figure 5.2: (A) Maximal (200K) contractures in an *pre*-conditioned (\( tc_1 \), Figure 5.1) and conditioned (\( ti \)) bundle of soleus muscle fibres. The \( ti \) contracture (broken line), which has been scaled and superimposed on the peak height of \( tc_1 \), was obtained after a 5 minute exposure to 30K. (B) In addition to the contractures shown in A, a third contracture (\( tc_3 \) (dotted line) was obtained to test the recovery of the preparation. This contracture has also been scaled and superimposed on the peak height for \( tc_1 \). The vertical calibration bar is 5 mN for the \( tc_1 \) contracture.
Figure 5.3: Three maximal contractures showing the effect of exposures to 200K on the time course of successive contractures. t\textsubscript{c2} and t\textsubscript{c3} have been scaled and superimposed on the peak height for the t\textsubscript{c1} contracture. Vertical calibration bar equals 5 mN for t\textsubscript{c1}.
Table 5.1B confirms this finding, showing that normal soleus preparations undergo a progressive reduction of decay times as the number of exposures to the 200K solution increases. This finding should be viewed with caution since sample numbers are small. It is interesting to note, however, that the recovery of decay times for $t_c{3}$ contractures was, on average, much more complete following 10 and 30 minute conditioning depolarizations, as compared to the 5 minute protocol (Table 5.2A). In general it took 40-50 minutes for tetani to reach stable amplitudes following long conditioning periods (10 and 30 min), compared to 15-25 min recovery periods following 5 minute conditioning depolarizations. As a consequence, it was possible that the long recovery times following 10 and 30 minute conditioning depolarizations allowed the preparations to recover from the effects of inactivation and therefore produce $t_c{3}$ contractures with decay times almost identical to those of $t_c{1}$ contractures. These results suggest that recovery of the tetanus following 5 minute conditioning depolarizations does not coincide with the recovery of the ability of the muscle fibres to produce a 200K contracture of normal duration.

5.3.3 Effect of duration of conditioning depolarization on the time course of decay of a 200K contracture.

To determine whether the changes to the timecourse of a 200K test contracture ($t_t$) had reached a steady-state level after a 5 minute depolarization in 30K, the effects of longer conditioning depolarizations on the parameters describing the tension response for a 200K test contracture were examined. Examples of contractures $t_c{1}$, $t_t$ and $t_c{3}$ respectively, are shown in Figures 5.4A & B for 10 minute and 30 minute conditioning depolarizations in 30K. The data obtained for activation and inactivation times for 5, 10 and 30 minute depolarizations is presented in Table 5.2A.

Since a full recovery of the tension response did not usually occur after 5 minute conditioning depolarizations (see section 5.3.1), the inactivation parameters (i.e. 90-10% and 80-20% decay times) of the test contracture ($t_t$) could not be directly compared to an average of the parameters for the pre- and post-conditioned control 200K contractures ($t_c{1}$ & $t_c{3}$). Consequently, parameters describing the rise and decay of contracture tension for $t_t$ and $t_c{3}$ are given as a fraction of the corresponding parameter for $t_c{1}$ (for the same preparation) in Table 5.2B.

The average relative rise and decay times for $t_c{1}$, $t_t$ (following a 5 min conditioning depolarization) and $t_c{3}$ 200K contractures in soleus have been plotted in histogram form in Figure 5.5. The histograms presented in this section are
Table 5.2A: Effect of duration of conditioning depolarization on average absolute rise and decay times (seconds) for 200K contractures in soleus fibres.

<table>
<thead>
<tr>
<th></th>
<th>10-90%</th>
<th>20-80%</th>
<th>90-10%</th>
<th>80-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>**5 min ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t_c1 (n=8)</td>
<td>4.5±0.4</td>
<td>3.1±0.3</td>
<td>27.9±3.7</td>
<td>18.3±2.3</td>
</tr>
</tbody>
</table>
| t_c2       | 3.2±0.3⁺⁺| 2.2±0.2⁺⁺| 14.1±1.0⁺⁺| 9.4±0.7   
| t_c3       | 4.3±0.5| 3.0±0.4| 18.1±1.2| 11.8±0.9 |
| **10 min *** |        |        |        |        |
| t_c1 (n=6) | 4.4±0.9| 3.0±0.5| 23.8±3.9| 16.0±2.6|
| t_c2       | 2.9±0.3⁺⁺| 2.0±0.2⁺⁺| 10.4±1.1⁺⁺| 7.2±0.8   
| t_c3       | 4.6±0.5| 3.1±0.4| 23.2±5.2| 15.4±3.3 |
| **30 min *** |        |        |        |        |
| t_c1 (n=6) | 5.6±0.9| 3.4±0.4| 36.5±6.2| 25.7±4.5|
| t_c2       | 4.2±0.4| 2.8±0.2| 16.4±2.8⁺⁺| 10.2±2.1   
| t_c3       | 6.2±0.6| 3.8±0.2| 34.7±5.0| 24.3±3.7 |

* Conditioning Duration

** p < 0.01
⁺⁺ p < 0.025
⁺⁺⁺ p < 0.005
θ  p < 0.1
Table 5.2B: Effect of duration of conditioning depolarization on average relative rise and decay times (seconds) for 200K contractures in soleus fibres.

<table>
<thead>
<tr>
<th></th>
<th>10-90%</th>
<th>20-80%</th>
<th>90-10%</th>
<th>80-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>**5 min ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{c1}$ (n=8)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$t_i$</td>
<td>0.72±0.04†</td>
<td>0.74±0.03†</td>
<td>0.56±0.07‡</td>
<td>0.57±0.07‡</td>
</tr>
<tr>
<td>$t_{c3}$</td>
<td>0.96±0.08</td>
<td>0.99±0.09</td>
<td>0.75±0.13</td>
<td>0.79±0.13</td>
</tr>
<tr>
<td>**10 min ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{c1}$ (n=6)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$t_i$</td>
<td>0.68±0.06⊕</td>
<td>0.70±0.08⊕</td>
<td>0.43±0.03‡</td>
<td>0.47±0.04‡</td>
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<tr>
<td>$t_{c3}$</td>
<td>1.12±0.09</td>
<td>1.08±0.09</td>
<td>0.95±0.10</td>
<td>0.95±0.10</td>
</tr>
<tr>
<td>**30 min ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{c1}$ (n=6)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$t_i$</td>
<td>0.85±0.16</td>
<td>0.89±0.16</td>
<td>0.47±0.06†</td>
<td>0.45±0.07‡</td>
</tr>
<tr>
<td>$t_{c3}$</td>
<td>1.21±0.19</td>
<td>1.20±0.18</td>
<td>0.99±0.07</td>
<td>0.99±0.07</td>
</tr>
</tbody>
</table>

* Conditioning Duration

**p < 0.01
† p < 0.025
‡‡ p < 0.005
⊕ p < 0.1
Figure 5.4: Examples of maximal contractures - $t_{c1}$, $t_i$ and $t_{c3}$, where $t_i$ was obtained following a 10 minute conditioning depolarization in 30K (A), or after a 30 minute conditioning depolarization in 30K (B). Vertical calibration bar equals 5 mN for $t_{c1}$ contractures.
Figure 5.5: Histogram of the average relative rise and decay times for $t_c^1$ (filled black), $t_j$ following a 5 minute conditioning depolarization (hatched pattern) and $t_c^3$ (rising diagonal lines) obtained from bundles of normal soleus muscle ($n=8$). Vertical bars show ±s.e.m.

+ $p < 0.025$

* $p < 0.005$
plotted from data summarized in Table 5.2B. It can be seen that the average time to rise from 10-90% and 20-80% of peak contracture height, relative to control (tc1) contractures, is reduced to 0.72 and 0.74 respectively, following a five minute conditioning depolarization (ti)(see section 5.3.1). The faster rise times following a conditioning depolarization of five minutes were seen in all preparations. Following recovery, the average relative rise times for tc3 contractures were almost identical to those of tc1. Similarly, the average relative times for the decay of the tension response from 90-10% and 80-20% with respect to the peak tc1 amplitude were decreased if the muscle fibres had been conditioned by a preceding depolarization. The partial recovery of the decay rate following a conditioning depolarization is evident from the average decay times of the tc3 contractures. The average relative values of the 80-20% decay times increased from 0.57 of control for ti (for a 5 min conditioning depolarization) to 0.79 of control for tc3 (Table 5.2B).

Histograms of the average relative rise and decay times for maximal contractures of soleus muscle are plotted in Figure 5.6 for ti (A) and tc3 (B) for different durations of the conditioning depolarization. It can be seen that the effect of a prolonged conditioning depolarization on the parameters describing the time course of tension decay reaches a steady-state level by 10 minutes of conditioning in 30K, since the effect of a 30 minute depolarization on these parameters was not significantly different from the effect of a 10 minute conditioning depolarization (student t-test). It should be noted however, that while the time course of the decay of tension was not altered by conditioning depolarizations lasting longer than 10 minutes, inactivation of peak tension (as described in Chapter 3) was still changing after conditioning depolarizations of 30 minutes duration. Average inactivation for a 5 minute conditioning depolarization in 30K was 0.80±0.05 (n=8), compared to 0.79±0.05 (n=5) for a 10 minute conditioning in 30K, or 0.42±0.07 (n=7) for a conditioning period lasting 30 minutes. The depressed peak tension after 30 minute depolarizations compared with 10 minute depolarizations can be explained in a number of ways. Either steady-state inactivation is never reached, or only after a very long time. It is unlikely that conditioning depolarization of this duration have a detrimental effect on the ability of a fibre to produce maximal tension since experiments with caffeine have shown that maximal contractures can still be generated many hours after exposure to high potassium solutions (Axelsson & Thesleff, 1958).
Figure 5.6: Histograms showing the average relative rise and decay of contracture tension in normal soleus (n=8) for $t_1$ (A) and $t_{c3}$ (B) contractures with respect to control contractures. $t_1$ and $t_{c3}$ were obtained following conditioning depolarizations of 5 minutes (hatched pattern), 10 minutes (rising diagonal lines) and 30 minutes (spotted pattern) with respect to control ($t_{c1}$, filled black bar) contractures. Vertical bars show ±s.e.m.

** $p<0.01$
+ $p<0.025$
++ $p<0.005$
⊙ $p<0.1$
5.3.4 Effects of denervation of soleus on the time course of relative decay of 200K contracture tension.

To understand the way in which denervation might alter contracture time course, and to compare data with results presented in Chapter 3, I studied denervated soleus muscle using a 5 minute conditioning depolarization. These experiments showed an effect of denervation on the rate at which maximal contractures activate and then undergo spontaneous relaxation of tension. Table 5.3A summarizes the data obtained for rise and decay times of normal and denervated soleus muscle. Comparison of the two muscle types shows that denervated preparations take, on average, longer to rise to peak amplitude compared to normal soleus, and are also much slower to decay from the peak contracture amplitude.

An analysis of the relative rise and decay times for denervated soleus (Table 5.3B) shows that this muscle was less affected by a 5 minute conditioning depolarization than its control counterpart. The histograms shown in Figure 5.7A & B display this result. The relative increase in the rate of decay of contracture tension (80-20%) in \( t_1 \) (A) for denervated muscle was less than in normal muscle \( (p<0.20) \), and \( t_{2-3} \) (B) recovered to a greater extent. Given that very small control and denervated preparations were used in these experiments, the rates of depolarization of fibres must have been similar in both cases. Therefore, these results reflect changes in the properties of E-C coupling following denervation. Interestingly, the results suggest that the relative rates of contractile inactivation is slowed by denervation, suggesting that the rate of conversion of the voltage sensor for E-C coupling between different conformational states, is also slowed with denervation. Denervation also slowed activation. The rate of rise of tension in denervated soleus was less abbreviated by a conditioning depolarization than control.

5.3.5 Effects of a conditioning depolarization on the time course of maximal contractures in bundles of EDL fibres.

EDL fibres did not recover well from 200K contractures. Both tetanic tension and subsequent 200K contractures often failed to recover. In the present experiments, the use of full 200K contractures on EDL made the acquisition of complete sets of reliable data difficult. The tension responses of one preparation which did recover well have been scaled and superimposed and are shown in Figure
Table 5.3A: Comparison of average *absolute* rise and decay times for $t_c$, $t_i$ and $t_c^3$ contractures in normal and denervated soleus.

<table>
<thead>
<tr>
<th></th>
<th>10-90%</th>
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<th>90-10%</th>
<th>80-20%</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=8)</td>
<td>5min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_c^1$</td>
<td>4.5±0.4</td>
<td>3.1±0.3</td>
<td>27.9±3.7</td>
<td>18.3±2.5</td>
</tr>
<tr>
<td>$t_i$</td>
<td>3.2±0.2**</td>
<td>2.2±0.2†</td>
<td>14.1±1.0++</td>
<td>9.4±0.7++</td>
</tr>
<tr>
<td>$t_c^3$</td>
<td>4.3±0.5</td>
<td>3.0±0.4</td>
<td>18.1±1.2</td>
<td>11.8±0.9</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Denervated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>5min</td>
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<tr>
<td>$t_c^1$</td>
<td>8.4±0.9</td>
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<tr>
<td>$t_i$</td>
<td>7.3±1.0</td>
<td>5.5±0.7</td>
<td>28.7±2.9Ø</td>
<td>18.6±1.8Ø</td>
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<tr>
<td>$t_c^3$</td>
<td>8.8±1.1</td>
<td>6.3±0.8</td>
<td>45.1±6.9</td>
<td>28.9±4.5</td>
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</table>

* conditioning duration

** $p<0.01$
† $p<0.025$
++ $p<0.005$
Ø $p<0.05$
Ø' $p<0.1$
Table 5.3B: Comparison of average relative rise and decay times for $t_c1$, $t_i$ and $t_c3$ contractures in normal and denervated soleus.

<table>
<thead>
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<th>90-10%</th>
<th>80-20%</th>
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</tr>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>$5 min^*$</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$t_c1$</td>
<td>0.72±0.04$^{*}$</td>
<td>0.74±0.03$^+$</td>
<td>0.56±0.07$^{**}$</td>
<td>0.57±0.07$^{**}$</td>
</tr>
<tr>
<td>$t_i$</td>
<td>0.96±0.08</td>
<td>0.99±0.09</td>
<td>0.75±0.13</td>
<td>0.79±0.13</td>
</tr>
<tr>
<td>$t_c3$</td>
<td>0.56±0.07</td>
<td>0.57±0.08</td>
<td>0.75±0.13</td>
<td>0.79±0.13</td>
</tr>
<tr>
<td><strong>Denervated</strong></td>
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<tr>
<td>(n=6)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$5 min^*$</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$t_c1$</td>
<td>0.87±0.10</td>
<td>0.96±0.11</td>
<td>0.70±0.06$^*$</td>
<td>0.72±0.08$^*$</td>
</tr>
<tr>
<td>$t_i$</td>
<td>1.05±0.09</td>
<td>1.09±0.10</td>
<td>1.05±0.07</td>
<td>1.07±0.10</td>
</tr>
<tr>
<td>$t_c3$</td>
<td>1.05±0.07</td>
<td>1.05±0.07</td>
<td>1.05±0.07</td>
<td>1.07±0.10</td>
</tr>
</tbody>
</table>

* conditioning duration.

$^{*} p<0.01$

$^{+} p<0.025$

$^{**} p<0.005$

$^{*} p<0.05$

$^{*} p<0.10$
Figure 5.7: Comparison of the average relative rise and decay times for $t_i$ following a 5 minute conditioning depolarization (A) and $t_c3$ (B) in normal (hatched pattern) ($n=8$) and denervated (rising diagonal lines) ($n=6$) soleus muscle as a function of respective control ($t_c1$, filled black) contractures. Vertical bars show ±s.e.m.

** $p<0.01$
$+$ $p<0.025$
$++$ $p<0.005$
$*$ $p<0.05$
5.8. As for soleus, the rate of inactivation of EDL fibres, after a five minute conditioning depolarization in 30K, was also accelerated. Average rise and decay parameters for $t_{c1}$, $t_i$ and $t_{c3}$ are presented in Table 5.4A. The acceleration of decay after conditioning depolarization is further demonstrated by comparison of the rise and decay times expressed relative to control ($t_{c1}$) contractures (Table 5.4A and Figure 5.9). These results also show that the relative rate of rise of test contracture tension in denervated EDL is not significantly affected by preceding conditioning depolarization. Similar experiments to those described in section 5.3.2. showed the trend that successive maximal depolarization in 200K, with 15 to 20 minute recovery periods, resulted in a progressive shortening in relative decay times. However, sample numbers were insufficient for any statistical analysis.

5.3.6 Effects of denervation on the time course for contractile inactivation in EDL.

Denervation of EDL muscles led to changes in the time course of contractile activation and inactivation. As for soleus, the rise and decay times for maximal contractures were slowed following denervation (Table 5.4B). In addition, denervation of EDL produced a resistance to the effects of a 5 minute conditioning depolarizations on the decay of the tension response for a 200K test contracture ($t_i$) compared to its' control ($t_{c1}$). Of particular interest was the very high value of the 90-10% and 80-20% relative decay times, compared to normal EDL, for $t_{c1}$, $t_i$ and $t_{c3}$ shown in Figures 5.10A & B respectively (see also Table 5.4B). This was similar to the result obtained for normal and denervated soleus. Interestingly, in contrast to soleus, the denervation of EDL lead to the appearance of many $t_{c1}$ contractures which had an obvious biphasic decay. The biphasic nature of the decay was not altered by conditioning depolarizations. Examples of the biphasic decay of contracture tension are given in Figures 5.11A &B. The implications of this observation were exciting because, as for perchlorate contractures in mammalian soleus, a biphasic decay of contracture tension supported the hypothesis that a second conformational state of the inactivated voltage sensor could be unmasked, in this case by denervation.

In order to examine the characteristics of the two phases of decay of maximal contractures in denervated EDL, four additional parameters were introduced and used to compare data obtained from normal and denervated EDL. The parameters presented in Table 5.5 are: 90-50%, 50-10%, 80-50% and 50-20% absolute and relative decay times. The times to decay from 90-50% and 80-50% were similar in normal and denervated EDL for the $t_{c1}$ contracture. Following a conditioning depolarization however, the initial phase of the descending contracture
Figure 5.8: Maximal 200K contractures obtained from a bundle of normal EDL which was (a) previously at rest (tc1), (b) immediately following a five minute conditioning depolarization in 30K (ti), and (c) following a recovery period (tc3). The latter contractures were scaled and superimposed on the peak height for the tc1 contracture. Vertical calibration bar equals 5 mN for the tc1 contracture.
Table 5.4A: Comparison of average absolute and relative rise and decay times for $t_{c1}$, $t_i$ and $t_{c3}$ contractures in normal EDL.

<table>
<thead>
<tr>
<th>Normal (n=3)</th>
<th>10-90%</th>
<th>20-80%</th>
<th>90-10%</th>
<th>80-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute times</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{c1}$</td>
<td>5.0±0.2</td>
<td>3.0±0.1</td>
<td>21.5±4.6</td>
<td>14.7±3.4</td>
</tr>
<tr>
<td>$t_i$</td>
<td>3.9±0.7</td>
<td>2.7±0.6</td>
<td>11.5±2.2</td>
<td>7.9±1.5</td>
</tr>
<tr>
<td>$t_{c3}$</td>
<td>5.3±0.2</td>
<td>3.2±0.1</td>
<td>18.4±4.0</td>
<td>12.7±2.8</td>
</tr>
<tr>
<td>Relative times</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{c1}$</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_i$</td>
<td>0.92±0.05</td>
<td>1.08±0.03</td>
<td>0.54±0.01</td>
<td>0.54±0.02</td>
</tr>
<tr>
<td>$t_{c3}$</td>
<td>1.06±0.02</td>
<td>1.05±0.03</td>
<td>0.85±0.04</td>
<td>0.87±0.04</td>
</tr>
</tbody>
</table>

* after 5 minute conditioning depolarization in 30K

** p<0.1
Figure 5.9: Histogram of the average relative rise and decay times for normal EDL (n=3). Times for maximal contractures following a 5 minute conditioning depolarization (t_i, hatched pattern) or following a period of recovery (t_{c3}, rising diagonal lines) are expressed relative to control (t_{c1}, filled black) contractures. Vertical bars show ±s.e.m.

**p < 0.1**
Table 5.4B: Comparison of average *absolute* and *relative* rise and decay times for $t_c1$, $t_i$ and $t_c3$ contractures in denervated EDL.

<table>
<thead>
<tr>
<th>Denervated (n=5)</th>
<th>10-90%</th>
<th>20-80%</th>
<th>90-10%</th>
<th>80-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absolute times</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_c1$</td>
<td>8.0±0.9</td>
<td>5.6±0.6</td>
<td>52.6±15.9</td>
<td>28.8±7.4</td>
</tr>
<tr>
<td>$t_i$ *</td>
<td>7.4±0.8</td>
<td>4.8±0.6</td>
<td>46.0±14.8</td>
<td>25.8±9.0</td>
</tr>
<tr>
<td>$t_c3$</td>
<td>7.6±0.5</td>
<td>5.1±0.4</td>
<td>42.1±15.6</td>
<td>25.7±9.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Relative times</strong></th>
<th>1</th>
<th>1</th>
<th>1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_c1$</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_i$ *</td>
<td>0.93±0.01</td>
<td>0.86±0.02</td>
<td>0.87±0.11</td>
<td>0.86±0.11</td>
</tr>
<tr>
<td>$t_c3$</td>
<td>0.97±0.07</td>
<td>0.93±0.05</td>
<td>0.87±0.2</td>
<td>0.89±0.16</td>
</tr>
</tbody>
</table>

* after 5 minute conditioning depolarization in 30K.
Figure 5.10: Histograms of the average relative rise and decay times for normal (hatched pattern) \( n=3 \) and denervated (rising diagonal line) \( n=5 \) EDL. Times for maximal contractures following a 5 minute conditioning depolarization \( t_i \) which are shown in A, or following a period of recovery \( t_c3 \) in B, are expressed relative to control \( t_c1 \), filled black) contractures. Vertical bars show ±s.e.m.
Figure 5.11: (A&B) Examples of biphasic contractures observed in two separate preparations obtained from denervated EDL. Each tension response is an initial (t_c1) contracture in response to exposure to 200K. Vertical calibration bar equals 5 mN.
Table 5.5: Effects of denervation on the absolute and relative decay times of maximal 200K contractures in EDL fibres.

<table>
<thead>
<tr>
<th></th>
<th>90-50%</th>
<th>50-10%</th>
<th>80-50%</th>
<th>50-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute times:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.7±1.6</td>
<td>12.8±3.0</td>
<td>6.5±1.3</td>
<td>8.2±2.1</td>
<td></td>
</tr>
<tr>
<td>* 4.5±1.0 **</td>
<td>6.4±1.6 **</td>
<td>3.3±0.7 **</td>
<td>4.3±0.9 **</td>
<td></td>
</tr>
<tr>
<td>7.0±1.1</td>
<td>10.8±2.4</td>
<td>5.6±1.3</td>
<td>7.1±1.5</td>
<td></td>
</tr>
<tr>
<td><strong>Relative times:</strong></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.57±0.03 **</td>
<td>0.50±0.02 **</td>
<td>0.50±0.03 **</td>
<td>0.53±0.04 **</td>
<td></td>
</tr>
<tr>
<td>0.82±0.09</td>
<td>0.85±0.02</td>
<td>0.85±0.07</td>
<td>0.88±0.04</td>
<td></td>
</tr>
<tr>
<td><strong>Denervated (n=5)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute times:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.8±3.1</td>
<td>41.8±15.0</td>
<td>8.0±2.4</td>
<td>20.8±5.8</td>
<td></td>
</tr>
<tr>
<td>* 11.2±4.8</td>
<td>34.8±11.9</td>
<td>8.5±3.7</td>
<td>17.0±5.5</td>
<td></td>
</tr>
<tr>
<td>12.2±5.1</td>
<td>29.9±10.6</td>
<td>9.0±3.9</td>
<td>16.8±5.9</td>
<td></td>
</tr>
<tr>
<td><strong>Relative times:</strong></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>* 0.95±0.13</td>
<td>0.83±0.09</td>
<td>0.98±0.12</td>
<td>0.82±0.10</td>
<td></td>
</tr>
<tr>
<td>1.03±0.11</td>
<td>0.85±0.21</td>
<td>1.03±0.11</td>
<td>0.85±0.17</td>
<td></td>
</tr>
</tbody>
</table>

* \( t_i \): measured after 5 minute conditioning depolarization in 30K.
* * * \( p<0.1 \)
was significantly faster in normal EDL, while remaining relatively unaffected in
denervated preparations. This effect can be clearly seen in the histograms of
relative decay times for \( t_1 \) shown in Figure 5.12A. Times to decay from 50-20% in
denervated fibres were similarly not much affected by conditioning depolarizations
in contrast to normal fibres. The ability of denervated fibres to recover from the
conditioning depolarizations, as demonstrated by the slower relative decay times for
\( t_3 \) contractures, is shown in Figure 5.12B.

The most obvious differences in decay time between normal and denervated
EDL was seen for tension decay from below 50% of peak amplitude to zero.
Overall decay times for denervated EDL were always far larger than those for
normal EDL and demonstrated a remarkable resilience to the effects of a five
minute conditioning depolarization.

5.3.7 A five-state model to explain the time course of contractile inactivation.

A model to interpret events occurring during full contractile inactivation is
based on the five-state model describing the voltage sensor for E-C coupling
(General Introduction, section 1.11.8; Dulhunty, 1992). For convenience, the
model is presented again below:

\[ P \rightarrow Q \rightarrow A + Ca^{2+} \rightarrow I \rightarrow Y + Ca^{2+} \]
\[ \downarrow \]
\[ C \rightarrow O. \]

It is assumed that myoplasmic calcium is proportional to the concentration of
voltage sensor in the active (A) state. The concentration of A (or any other
conformational state for that matter) at any one time, \( t \), is dependent on the
movement of the voltage sensor into and away from all conformational states. That
is:

\[ A_t = A_0 \frac{Q(t-1)k_{QA}}{(I(t-1)k_{IA})} + \frac{(I(t-1)k_{IA})A(t-1)(k_{AQ}+k_{AI})}{k_{AQ}+k_{AI}} \]

The rate constants for the forward and reverse movement of voltage sensor
between each of the five conformational states can be manipulated to explain
contracture results in a number of different situations.

Case 1: In this situation the muscle fibres are initially polarized and completely at
rest. It can be assumed that close to 100% of the voltage sensor is initially in the P
Figure 5.12: Histograms describing relative times for the decay phase of maximal contractures for normal (hatched pattern) and denervated (rising diagonal) EDL. Decay times for $t_i$ (A) and $t_{c3}$ (B) are expressed relative to the $t_{c1}$ (filled black) contracture.

$** p<0.1$
configuration. Figure 5.13 shows changes in the concentration of P and A with time as a large depolarization converts P to A via Q. A hypothetical activator concentration of 1 must be reached before tension is generated.

The time course of activator formation shows that peak levels of activator are quickly achieved and then decay exponentially to subthreshold levels as conversion to the I state takes place. The rise and decay times for the activator concentration can be significantly affected by slight changes to the rate constants controlling conversion of the voltage sensor between different configurations. For example, altering $k_{AI}$ from 0.05 to 0.1 depresses the peak contracture height by 18%, and decreases the 90-10% decay time by 65%. The rate constants providing a contracture time course similar to that obtained under experimental conditions are given in Figure 5.13.

Case 2: Again, the muscle fibres are initially polarized and completely at rest. The time course of activator formation during conditioning and test depolarizations are shown in Figure 5.14A & B. Initially, the conditioning depolarization slowly converts a fraction of P to A via Q (Figure 5.14A). The rate at which this occurs is dependent on the strength of the conditioning depolarization since the conversion of P to Q is a voltage dependent step. In this case it is assumed that the conditioning depolarization does not convert all P to other conformations. There is a slightly elevated activator concentration prior to generation of the test contracture (Figure 5.14B) because the rate constants are such that a fraction of the voltage sensor remains in the A conformation. The peak concentration of activator in the test contracture is suppressed due to lower initial levels of precursor. As shown experimentally, the model predicts that time spent at levels above threshold activator is attenuated.

Case 3: In this situation the muscle fibres are initially polarized and completely at rest. In Case 1 above, the very small rate constants for the conversion of voltage sensor between the inactive states, I and Y, suggested that a second inactive state either did not exist, or was not exposed in the monophasic decay of activator concentration. Theoretically, it is possible that activator concentrations were subthreshold for contraction when the second inactive state influenced the decay of A. Experimentally, the monophasic decay of maximal contracture tension was typical of that seen in normal fast- and slow-twitch mammalian muscle while many of the maximal contractures for denervated EDL shown in section 5.3.6 demonstrated a biphasic decay response.
Figure 5.13: Predicted changes in the relative concentrations of voltage sensitive molecules in the active (continuous line) and precursor (heavy dashed line) states following a large depolarization (e.g., a $t_c$ contracture). A hypothetical activator concentration above 1 is required to maintain myoplasmic calcium levels above the threshold for contraction. Rate constants for the model described in the text are: $k_{PQ}$, 0.4 sec$^{-1}$; $k_{QA}$, 0.4 sec$^{-1}$; $k_{AI}$, 0.05 sec$^{-1}$; $k_{IY}$, 0.005 sec$^{-1}$; $k_y$, 0.0005 sec$^{-1}$; $k_{IA}$, 0.005 sec$^{-1}$; and 0.02 sec$^{-1}$ for both $k_{AQ}$ and $k_{QP}$. 
Figure 5.14: Predicted activator (continuous line) and precursor (heavy dashed line) concentrations generated by a 5 minute conditioning depolarization (A) (first 100 seconds shown) which is followed by a large depolarization (B). Note that initial precursor concentrations in B are significantly depleted, while activator levels are slightly elevated. Rate constants for the model described in the text were as follows: $k_{pQ}$, 0.08 sec$^{-1}$ in A and 0.4 sec$^{-1}$ in B; $k_{QA}$, 0.3 sec$^{-1}$ in A and 0.4 sec$^{-1}$ in B; $k_{AQ}$, 0.15 sec$^{-1}$ in A and 0.05 sec$^{-1}$ in B; $k_{Y}$, 0.005 sec$^{-1}$ in A and B; $k_{IA}$, 0.02 sec$^{-1}$ in A and 0.005 sec$^{-1}$ in B; $k_{AQ}$, 0.15 sec$^{-1}$ in A and 0.02 sec$^{-1}$ in B; $k_{QP}$, 0.4 sec$^{-1}$ in A and 0.02 sec$^{-1}$ in B.
The model shows a biphasic decay in A if there is an increase in the rate constant from I back to A. Therefore, while inactivation takes place, conversion of the voltage sensor back to the active state slows the decay of the level of activator (Figure 5.15A). For example, the $k_{IA}$ rate constant for a monophasic decay (see eg: Figure 5.13) has a value of 0.005, while the same rate constant for a biphasic decay (Figure 5.15B) has a value of 0.015. This rate constant sets the level of the pedestal tension. The role of the second inactive state, Y, is to alter the slope of the second phase of the activator/tension response (Figure 5.15B). Hence, if the rate constant of the conversion of I to Y is large enough, then the pedestal level of activator decays and the response becomes truly biphasic.
Figure 5.15: Examples of possible activator (continuous line) and precursor (heavy dashed line) concentrations which would explain experimental results in normal (A) and denervated (B) EDL. Note how a slight shift in activator concentrations transforms a monophasic decay in graph A into a biphasic decay in B. Rate constants were only altered for $k_{AI}$ (0.1 sec$^{-1}$ in graph A compared to 0.075 sec$^{-1}$ in graph B) and $k_{IA}$ (0.01 sec$^{-1}$ in graph A and 0.015 sec$^{-1}$ in graph B). All other rate constants were the same in both examples, i.e., $k_{P0}$, 0.4 sec$^{-1}$; $k_{QA}$, 0.4 sec$^{-1}$; $k_{Y}$, 0.08 sec$^{-1}$; and 0.02 sec$^{-1}$ for both $k_{AQ}$ and $k_{QP}$. 
5.4 DISCUSSION

An analysis of the effects of a conditioning depolarization on the time course of maximal potassium contractures has been carried out on normal and denervated mammalian skeletal muscle. The study provided results which could account for the reduced contracture time course seen by Lamb & Stephenson in skinned single muscle fibres of the frog. Mammalian skeletal muscle is unsuitable for the dissection of good quality single muscle fibres which are intact from tendon to tendon, so that bundles of muscle fibres must be used for experimental purposes. An important consideration in the use of bundles of fibres is that diffusion and equilibration times for newly applied solutions must vary according to preparation size and therefore might affect activation and inactivation rates. There was up to a threefold difference in the activation and inactivation parameters (ie rise and decay times) for different preparations, which could be due to biological variation between individual preparations or to diffusion times. It was unlikely that diffusion times were important because peak contracture height was not significantly reduced as it would be if there was asynchronous activation and inactivation of individual fibres. If preparations were so large that superficial fibres were inactivating before axial fibres were fully activated, then peak contracture height should be considerably less than peak tetanic amplitude. This was not seen. In addition, the effects of conditioning depolarizations suggest that diffusion times were not rate limiting since the time for equilibration of 200K should be the same in control and conditioned preparations. Therefore, it is likely that different contracture time courses were due to real differences between bundles in the activation and inactivation rates. Because the rise and decay times varied considerably, changes in normalized parameters were compared in control and conditioned fibres from normal and denervated soleus and EDL.

The activation and subsequent decay of maximal tension following a large depolarization can be adequately described by the model given in section 5.3.7. As previously described, the model assumes that the voltage sensor for E-C coupling can exist in any of five conformational states, the concentration of calcium in the myoplasm being proportional to the percentage of voltage sensor in the active state. The following sections show that the five-state model can explain many of the results.
(a) Effect of conditioning depolarization on activation times for maximal contractures.

With a percentage of the voltage sensor converted to the inactive state by a conditioning depolarization (steady-state inactivation), there is less precursor to be converted to the active conformation with a large depolarization. Given that threshold amounts of activator are required for tension generation, peak activator concentrations in conditioned fibres must be reached more rapidly than in control (tc1) contractures if the conversion of P to A occurs at a constant rate for a given depolarization.

Interestingly, a 5 minute conditioning depolarization had a lesser effect on denervated skeletal muscle than in normal muscle. That is, the time to reach peak contracture tension was similar for both tc1 and t1 contractures in denervated preparations. Therefore the question is raised as to what factors limit the rate at which tension can be generated? Hodgkin & Horowicz (1960a) showed that the rate of tension development is proportional to applied depolarization. That is, the rate constant for the conversion of Q to A increases with the strength of depolarization. The increase in A is also dependent on the rate at which A is removed by conversion to the I state (primarily) or back to the Q conformation of the voltage sensor. The results presented in this study suggest that, following a conditioning depolarization, the maximal relative rate of increase in A for t1 with respect to the tc1 contracture, is increased in denervated muscle, although not to the same extent as occurs in normal muscle.

(b) Effect of conditioning depolarization on inactivation times for maximal contractures.

In addition to the conclusions in (a), it is of interest to consider the rate at which activator is converted to the inactive state in the test contracture which follows a conditioning depolarization. Again, if the rate of conversion of A to I is constant for a given depolarization, then less instantaneous activator will be present at the peak of the contracture since there is less P to be converted to A when the test depolarization is applied. Obviously, the conversion of A to I, using the same rate constants as in the control situation, will result in a faster depletion to threshold of the smaller reserves of activator formed in conditioned preparations. The model predicts that peak activator and the time required to reach it is also very dependent on the rate of conversion to I.
The relative rate of conversion of A to I could be slower for a maximum depolarization in denervated muscle. This would account for slower relative rise and decay time parameters that were seen.

5.4.2 An explanation of the biphasic timecourse of maximal contractures induced by denervation.

One of the most interesting observations in this study was that denervation of mammalian muscle often resulted in a biphasic decay in maximal tension. In agreement with Dulhunty (1985), biphasic decays were particularly apparent in denervated EDL preparations. Since biphasic inactivation kinetics were not seen in normal muscle, this observation is another example of the specific effect that denervation has on mechanical inactivation.

A biphasic decay could be interpreted in a number of ways. Firstly, it is plausible that the biphasic decay was a consequence of two populations of fibres inactivating at different rates. Assuming a simple summation of tension between fibres of the two populations, a biphasic inactivation profile could be obtained. Evidence, however, suggests that the biphasic decay is not due to two populations of fibres. Of particular relevance to the present work on denervation is the interpretation of results from contracture studies using the perchlorate anion (Dulhunty et al. 1992) and low external calcium bathing solutions (Luttgau et al. 1987; Dulhunty & Gage, 1988). Perchlorate has been shown to induce a biphasic decay of contracture tension in preparations that normally monophasic. This response has been attributed to the unmasking of a second inactive state of the voltage sensor (Dulhunty et al. 1992). The observation is inconsistent with two populations of fibres since the biphasic response should normally be seen for all contractures - control and perchlorate treated - of the one preparation.

Inactivation, measured as test contracture height following a conditioning depolarization, decays in a biphasic manner which is dependent on the strength and duration of the conditioning depolarization. Since the mechanical inactivation of a maximal contracture reflects the onset of steady-state inactivation, it is reasonable to assume that, if the concentration of activator could remain above threshold for long enough, then the decay should be biphasic. However, under normal circumstances it is believed that the slow phase of inactivation occurs when activator levels are below the level required to generate tension.

The experimental observations with perchlorate provided excellent evidence for the unmasking of a second inactive configuration of the voltage sensor for E-C coupling. It was proposed that perchlorate, which shifts the voltage dependence of
charge movement (Luttgau et al. 1983; Csernoch et al. 1987), could raise the intracellular Ca^{2+} concentration to levels which remain above the threshold for contraction during the period at which the slow phase of inactivation is entered.

The present study on denervated skeletal muscle provides evidence of another process which unmasks the second inactive state of the voltage sensor. In terms of the model given in section 5.3.7, denervation must increase two rate constants to reveal a biphasic decay, i.e. the rate constants for the conversion of I to A and for I to Y. As for perchlorate, it is critical that denervation elevates activator concentrations such that the second inactive state can be unmasked. Indeed much of the interpretation of data discussed in section 3.4.3 for the activation curve for EDL following denervation was based on the finding by Kirby & Lindley (1981) that resting Ca^{2+} concentrations in this preparation were in fact elevated. This may also contribute to the unmasking of the second phase of decay.
CHAPTER 6

This chapter is divided into two sections. Part I looks at the effect of using the divalent anion sulphate on the activation curve for contraction in soleus muscle. Part II forms a section of work on perchlorate ions presented by Dulhunty, Zhu, Patterson & Ahern (1992) in the Journal of Physiology, 448, pp. 99-119.

Part I: USE OF METHANESULPHONATE AS THE MAJOR IMPERMEANT ANION IN BATHING SOLUTIONS.

6.1 INTRODUCTION

The use of impermeant anions as substitutes for chloride in biological solutions is common (see eg: Kenyon & Gibbons, 1977; Pollard, Creutz, Pazoles & Hansen, 1977; Dani, Sanchez & Hille, 1983). In the experiments presented in Chapters 3, 4, and 5, and in many other studies from this laboratory (see eg: Dunhunty, 1991; Chua & Dulhunty, 1988, 1989), chloride is substituted with the impermeant sulphate anion in order to avoid the effects of high chloride conductance on T-tubule depolarization and potassium contracture tension. For consistency and voltage control, a control low-chloride solution was used in voltage clamp experiments. However, the use of solutions containing sulphate as the major anion has some limitations which have been discussed in Chapter 2 (section 2.5)

The major obstacle to using solutions containing sulphate as the major impermeant anion is that there is a trade-off between (a) maintaining normal ionic strengths, (b) maintaining normal osmolarity for all solutions and (c) maintaining normal sodium concentrations. For example, in the solutions presented in Table 2.1 (Chapter 2), the ionic strength of the control solution is normal, but is considerably less than that for the other solutions in which the potassium concentration has been raised. In addition, [Na+] is 80.5 mM and well below that of 140 mM found in normal Krebs solutions, simply because raising [Na+] concentration by addition of Na₂SO₄ increases the ionic strength of the solution (see Chapter 2). Cairns (1991) has shown that the lower sodium content slightly depresses peak tetanic force.

The present experiments were designed to ascertain whether the use of elevated potassium solutions of high ionic strengths resulted in changes to the voltage dependence of activation. The divalent anion sulphate was replaced with a monovalent anion, methanesulphonate.
6.1.2. METHODS.

Rat soleus muscles were used in all experiments described in this section. Tissue dissection procedures and potassium contracture techniques for the construction of activation curves have been described in Chapter 2.

6.1.2.1 Solutions

The composition of solutions used in these experiments is shown in Table 6.1. The potassium concentration in each solution is prefixed by "M" to represent use of the impermeant methanesulphonate anion. In order to be consistent with the sulphate solutions, all methanesulphonate solutions contained 16mM chloride. The high potassium solutions with potassium concentration less than 200 mM were prepared by substituting a percentage of potassium methanesulphonate in the M200K solution for sodium methanesulphonate. In this way, ionic strength and osmolarity were kept constant. Therefore, the M40K solution had only 20 mM more sodium than M3.5K, and all elevated potassium solutions had a higher osmolarity than the sulphate solutions.

In a second series of experiments, the osmolarity of the 40K, 80K and 120K solutions was decreased to that of the sulphate solutions in order to examine the effects of high osmotic strength on activation data. The composition of these solutions is also given in Table 6.1

6.1.2.2 Potassium contractures.

Data for the construction of activation curves was prepared from results obtained and treated in the same manner as that outlined in Chapter 3.3.2. Briefly, isometric tension was recorded from small bundles of intact muscle fibres in response to stimuli presented electrically or by exposure to methanesulphonate solutions containing elevated potassium.
Table 6.1: Composition of bathing solutions used in methanesulphonate experiments. Ion concentrations are given in mM. Solutions in which the osmolarity was decreased are prefixed by "Lo Os".

<table>
<thead>
<tr>
<th>Solution</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Cl⁻</th>
<th>MeSO₃⁻</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3.5</td>
<td>140</td>
<td>3.5</td>
<td>2.3</td>
<td>16.6</td>
<td>133.5</td>
<td>32</td>
</tr>
<tr>
<td>M40</td>
<td>160</td>
<td>40</td>
<td>2.3</td>
<td>16.6</td>
<td>190</td>
<td>-</td>
</tr>
<tr>
<td>M80</td>
<td>120</td>
<td>80</td>
<td>2.3</td>
<td>16.6</td>
<td>190</td>
<td>-</td>
</tr>
<tr>
<td>M120</td>
<td>80</td>
<td>120</td>
<td>2.3</td>
<td>16.6</td>
<td>190</td>
<td>-</td>
</tr>
<tr>
<td>M200</td>
<td>-</td>
<td>200</td>
<td>2.3</td>
<td>16.6</td>
<td>190</td>
<td>-</td>
</tr>
<tr>
<td>Lo OsM40</td>
<td>120</td>
<td>40</td>
<td>2.3</td>
<td>16.6</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>Lo OsM80</td>
<td>80</td>
<td>80</td>
<td>2.6</td>
<td>16.6</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>Lo OsM120</td>
<td>40</td>
<td>120</td>
<td>2.6</td>
<td>16.6</td>
<td>150</td>
<td>-</td>
</tr>
</tbody>
</table>

In addition, all solutions contained: 1 mM Mg²⁺; 11 mM glucose; 2 mM TES (N-tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid) pH buffer. pH=7.40±0.05, adjusted with NaOH.
6.1.3 RESULTS

6.1.3.1 Effect of methanesulphonate anion on the voltage sensitivity of tension generation in soleus muscle.

The membrane potentials of soleus muscle fibres bathed in the MS solutions is given in Table 6.2. It can be seen that the membrane potential at each high potassium concentration is more negative in the MS solutions, than in the sulphate solutions.

The average 200K/tetanus ratio declined slightly in MS solutions (1.02±0.03, n=26) compared to that obtained in sulphate solutions (1.16±0.02, n=112). The implications of this observation for relative contracture studies are not particularly important, so long as the 200K/tetanus ratio remained constant throughout the experiment. However, since the contractile response is fully activated upon exposure to 200 mM potassium solutions, this observation indicates that peak tetanic amplitude is increased in the elevated sodium environment of the M3.5 solution. This is consistent with the results of Cairns (1991).

Figure 6.1 shows relative K-contracture tension upon exposure to high potassium solutions plotted against membrane potential in soleus fibres. It can be clearly seen that use of the MS solutions changes the voltage sensitivity of tension generation such that the activation curve (filled circles, broken line) is shifted to more negative membrane potentials compared to the activation curve in which sulphate (open circles, continuous line) is the major anion (see Chapter 3). Average data for this, and the following section, is presented in Table 6.3. Boltzmann curves fitted to the average data showed that the voltage for half-maximal activation was shifted by -11 mV following substitution of the sulphate anion with MS. The slope factor, k, remained unaltered at a value of 3 mV.

6.1.3.2 Effect of lower osmotic strength methanesulphonate solutions on the voltage sensitivity of the tension response.

The experiments described in the previous section, were performed with solutions in which the osmotic strength was significantly elevated with respect to the sulphate solutions used in other experiments in order to maintain a constant osmolality of the high K⁺ solutions equal to that of the 200K. The high K⁺ MS solutions had an osmotic strength of approximately 420 mosM compared to around 340 mosM for the sulphate solutions. It has been shown that solutions in which the osmolarities are elevated or depressed can alter the tension response of skeletal
Table 6.2: Membrane potentials measured in solutions containing normal and elevated potassium. The major impermeant anion in each solution is shown.

<table>
<thead>
<tr>
<th>K⁺ ion conc (mM)</th>
<th>Sulphate</th>
<th>MeSO₃⁻</th>
<th>MeSO₃⁻ (Lo Os)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>-85.9±0.3 (115)</td>
<td>-85.8±0.4 (62)</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>-40.3±0.6 (20)</td>
<td>-43.8±0.7 (20)</td>
<td>-43.9±0.8 (16)</td>
</tr>
<tr>
<td>80</td>
<td>-24.0±0.6 (20)</td>
<td>-32.8±0.7 (17)</td>
<td>-33.2±0.7 (16)</td>
</tr>
<tr>
<td>120</td>
<td>-16.0±0.4 (29)</td>
<td>-22.1±0.4 (22)</td>
<td>-23.2±0.5 (19)</td>
</tr>
<tr>
<td>200</td>
<td>-1.6±0.4 (21)</td>
<td>-10.4±0.5 (10)</td>
<td>-10.8±0.4 (8)</td>
</tr>
</tbody>
</table>
Figure 6.1.1: The effect of MeSO$_3$ on the activation of contraction in soleus muscle. Relative contracture tension, $T_a$, is plotted against $V_m$ measured in high-K$^+$ solutions. The curves show average results obtained in solutions in which MeSO$_3$ (filled circles, broken line) or sulphate (open circles, broken line) was the major anion.
Table 6.3: Average relative tension values (mean±SEM) for soleus muscle preparations upon exposure to elevated potassium concentrations. Data was normalized by applying Equation 2 (Chapter 3, section 2.1) to tension responses. Significant differences are with respect to control (sulphate).

<table>
<thead>
<tr>
<th>K⁺ ion conc (mM)</th>
<th>Sulphate</th>
<th>MeSO₃⁺</th>
<th>MeSO₃⁻ (Lo Os)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40K</td>
<td>0.03±0.01 (9)</td>
<td>0.19±0.04 (7) *</td>
<td>0.06±0.02 (5) **</td>
</tr>
<tr>
<td>80K</td>
<td>0.77±0.05 (10)</td>
<td>0.93±0.02 (7) †</td>
<td>0.84±0.05 (6)</td>
</tr>
<tr>
<td>120K</td>
<td>1 (8)</td>
<td>1 (4)</td>
<td>0.97±0.01 (5)</td>
</tr>
<tr>
<td>200K</td>
<td>1 (112)</td>
<td>1 (26)</td>
<td>1 (29)</td>
</tr>
</tbody>
</table>

* p < 0.001  
† p < 0.025  
** p < 0.1
muscle (see April & Brandt, 1973). To test the effect of elevated osmotic strength on the activation curve, the tonicity of the MS solutions was dropped to that of the sulphate solutions by replacing 40 m\( l \) of 1M sodium methanesulphonate with distilled water. A 200K solution with this osmolarity could not be prepared. The membrane potentials for soleus fibres bathed in the low osmotic strength solutions are also given in Table 6.2. There were no significant differences in membrane potentials measured in solutions with normal or elevated osmotic strength. Figure 6.1.2 shows differences in the activation curves obtained with the normal (filled circles, broken line) and high (open circles, continuous line) osmotic strength MS solutions. Boltzmann curves fitted to the average data show that the voltage for half-maximal activation is shifted by 2 mV in the positive direction for normal osmotic strength solutions compared to the curve for data obtained with the elevated osmotic strength solutions. In addition, the voltage sensitivity of K-contractures in normal osmotic strength MS solutions becomes slightly steeper, with \( k \) changing from 3 mV to 2.5 mV.

6.1.3.3 Time course of maximal potassium contractures upon exposure to the M200K solution.

The time course of maximal 200K contractures in sulphate Krebs solutions was examined in Chapter 5. To determine whether the activation or decay of contracture for soleus muscle was altered by the use of the MS solutions, full contractures were generated by one exposure of soleus preparations to the M200K solution.

In eight out of eight preparations, the time course of decay of contracture tension was monophasic. Table 6.4 shows that there were little, if any, differences in the rise and decay times for maximal contractures obtained with sulphate and MS 200 mM potassium solutions. The average rise times (10-90%, 20-80%) for MS contractures were slightly slower (significance t-test) while the average decay times (90-10%, 80-20%) were virtually unaffected.
Figure 6.1.2: Comparison of activation curves obtained with MeSO₃ solutions. The tonicity of solutions was either similar to that of the sulphate solutions (LoOs solutions - filled squares, continuous line), or hypertonic (filled circles, broken line).
Table 6.4: Comparison of absolute rise and decay times (seconds) for 200K contractures in which sulphate or methanesulphonate was the major impermeant anion in bathing solutions.

<table>
<thead>
<tr>
<th>Anion</th>
<th>10-90%</th>
<th>20-80%</th>
<th>90-10%</th>
<th>80-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO$_4^{2-}$ (n=8)</td>
<td>4.5±0.4</td>
<td>3.1±0.3</td>
<td>27.9±3.7</td>
<td>18.3±2.5</td>
</tr>
<tr>
<td>MeSO$_3^-$ (n=8)</td>
<td>5.1±0.4</td>
<td>3.6±0.2</td>
<td>27.4±3.4</td>
<td>19.5±2.7</td>
</tr>
</tbody>
</table>
6.1.4 DISCUSSION

The study was designed to examine whether the use of the impermeant divalent anion sulphate in bathing solutions altered the voltage dependence of K-contracture data tension in mammalian muscle. The use of normal and high potassium, sulphate solutions meant that the sodium concentration in the control solution (with normal ionic strength) was lower than in a normal Krebs solution, while the ionic strength of the high potassium solutions was greater than normal requiring that concessions needed to be made regarding the final ionic and osmotic strengths, and sodium concentrations for each solution. The impermeant monovalent anion methanesulphonate was used as a substitute for sulphate, thereby avoiding many of the problems associated with divalent anions. MS is a potentiating agent which belongs to the lyotropic series of anions (Sandow, 1965; Lorkovic, 1971). Because muscle fibre membranes are virtually impermeant to MS (Woodbury & Miles, 1973), it is unlikely that this anion has any effect on intracellular calcium stores (Lorkovic, 1971).

A negative shift in the activation curve for lumbricalis and white gastrocnemius muscles of the rat has previously been reported by Lorkovic (1971) following replacement of chloride with MS. This shift was attributed to additional depolarization in MS solutions (compared to chloride containing solutions) upon exposure to a given potassium concentration. However, a comparison between Lorkovic (1971) and this study becomes complicated since the compositions of solutions in each study are quite different, and Lorkovic measured membrane potentials within five seconds of solution changeover. Slow changes in internal chloride concentrations upon exposure of muscle fibres to high potassium solutions containing altered chloride concentrations can mean that membrane potential takes in the order of minutes to reach a stable level (Dulhunty, 1978). In the present study, a negative shift (-11 mV) in voltage for half-maximal activation of soleus muscle with MS occurred in conjunction with a less significant shift in membrane potential. Since these shifts were with respect to data obtained from soleus muscles bathed in sulphate Krebs solutions, a comparison with data from normal chloride solutions is not possible.

The reason for the observed hyperpolarization of membrane potentials in MS solutions is open to speculation. MS could alter potassium permeability, or it could stimulate the sodium/potassium pump to cause electrogenic hyperpolarization. MS has little effect on the voltage dependence of peak sodium permeability in frog skeletal muscle (Dani et al, 1983) and equilibration in low chloride solutions had
been undertaken prior to experimentation. Therefore the effect cannot be explained by actions on sodium or chloride permeabilities. A notable effect of MS was that it shifted the voltage sensitivity of activation. This would suggest that, in addition to changes to the membrane potential, the voltage sensor for E-C coupling was affected by either sulphate or MS. It is unlikely that this effect on the voltage sensor could be due to a modification of membrane surface charge. Sulphate would result in additional negative charges at the surface membrane, causing a "perceived" decrease in the membrane field and a depolarization. In the presence of elevated potassium solutions, the depolarization sensed by the voltage sensor would be to more positive potentials than that sensed in the presence of a monovalent anion (eg: MS). This would cause an apparent negative shift in the activation curve for the sulphate containing solutions compared to the MS solutions. The results shown in this study demonstrated the opposite effect. Therefore, it is likely that there was a specific effect of MS on the voltage sensor for E-C coupling.

To determine whether maximum relative tension was altered by hypertonic methanesulphonate solutions, a series of experiments, carried out in lower osmotic strength solutions, demonstrated that the activation curve was only slightly affected. That is, the voltage for half-maximal activation was shifted by just 2 mV in the positive direction and became slightly steeper. A minimal effect of very high osmotic strength MS solutions on the production of tension was also noted by Lorkovic (1971).

These experiments were basically inconclusive in terms of identifying problems associated with the use of sulphate solutions. The MS anion induced large changes in membrane potential and voltage sensitivity of activation with respect to results obtained with sulphate solutions. However, it is not clear whether the sulphate or MS data is closest to the true voltage dependence in chloride containing solutions. Unfortunately, lack of time prevented the continuation of this investigation. Minimal differences between MS and sulphate solutions were observed in the time course for maximal 200K contractures. The results obtained in this study demonstrated that the average rise times for MS contractures were slightly longer than those obtained in sulphate solutions, while the decay times were almost identical. The monophasic decay of maximal contractures for MS 200K solutions was consistent with a barely noticeable effect of MS on the contracture time course compared to data obtained with sulphate solutions (see Chapter 5). A similar result has been demonstrated for mouse soleus muscle, where Lorkovic (1983) and Dulhunty (1980) used MS and sulphate solutions respectively, and showed similar time courses for maximal contractures in small bundles of fibres.
6.2 ACTIONS OF PERCHLORATE ON THE MEMBRANE POTENTIAL FOR CONTRACTION THRESHOLD.

6.2.1 INTRODUCTION

The aim of this experiment was to study the effect of perchlorate on the strength-duration curve for contraction of mammalian soleus muscle. Perchlorate alters the activation of contraction and charge movement in amphibian muscle, while leaving other voltage dependent processes, such as mechanical inactivation and Na⁺ and K⁺ conductances, unaffected (Foulks et al. 1973a, b; Gomolla et al. 1983; Luttgau et al. 1983; Csernoch et al. 1987; Huang, 1987)

The negative shift seen in the activation curve for perchlorate-conditioned amphibian muscle (Foulks et al. 1973; Gomolla et al. 1983; Luttgau et al. 1983) results in a corresponding shift in the membrane potential for contraction threshold (Csernoch et al. 1987). In the study carried out by Dulhunty et al. (1992) on mammalian soleus muscle, a negative shift in the activation curve for contraction (obtained using K-contractures) in perchlorate-conditioned muscle fibres was also observed. However, mammalian (rat) muscle fibres were less sensitive to perchlorate than voltage clamped frog muscle fibres (Gomolla et al. 1983; Luttgau et al. 1983) since smaller shifts in the voltage for half maximal tension were obtained. The following study was undertaken to test whether this difference was due to the use of voltage clamp as opposed to potassium contractures. Contraction thresholds were measured using a two-microelectrode point voltage clamp.

6.2.2 METHODS

The soleus muscle preparation and methods for the visual determination of contraction threshold using two-microelectrode point voltage clamp have been outlined in Chapter 2.

Muscle preparations were bathed in the standard low-chloride control solution (Table 2.1) to which 2 mM or 10 mM perchlorate was added as the sodium salt. TTX (0.125 uM) was added to the bathing solution to ensure that contraction was in response to depolarizing pulses and not action potentials.
6.2.3 RESULTS

The contraction thresholds for mammalian soleus muscle fibres are significantly affected by treatment with the perchlorate anion (2mM & 10mM) at all pulse durations. Strength-duration curves for control and perchlorate-treated fibres are presented in Figure 6.2.1.

The results presented here clearly demonstrate that perchlorate induces a hyperpolarizing shift in the membrane potential for $V_t$ compared to that in control solutions. The shift was dependent on the concentration of perchlorate used; in this case, 2mM and 10mM. Average shifts away from control values for contraction thresholds recorded in perchlorate treated fibres at all pulse durations were $-6.0 \pm 1.2$ (n=13) for 2 mM perchlorate, and $-12.5 \pm 1.3$ (n=10) for 10 mM perchlorate. The result for the 100 msec pulse duration in 10mM perchlorate (average shift of $-14.4$ mV) is somewhat less than that quoted by Csernoch et al. (1987) for frog muscle, where an average shift of $-22.7$ mV was obtained in 8mM perchlorate. This finding is consistent with potassium contracture studies on the activation properties of perchlorate treated muscle fibres where it has been shown that the shift in the voltage for half maximal activation of tension ($V_a$) in mammalian muscle is less affected by perchlorate conditioning (Dulhunty et al. 1992) compared to amphibian muscle (see eg Gomolla et al. 1983). Changes in contraction threshold with perchlorate-treatment shown in this study confirm the shifts in $V_a$ observed by Dulhunty et al. (1992).
Figure 6.2.1: The effect of perchlorate treatment on the strength-duration curve for contraction threshold measured under two microelectrode voltage clamp conditions. The membrane potential for contraction threshold (in mV, vertical axis) is plotted against pulse duration (in msec, horizontal axis) for control (open squares) and 2 mM (filled squares) or 10 mM (open triangles) perchlorate treated soleus fibres. $(n = 13)$ $(n = 10)$
6.2.4 DISCUSSION

The actions of perchlorate on mammalian skeletal muscle are different to those described for amphibian muscle. The activation process in mammalian muscle is less sensitive to perchlorate and, in contrast to amphibian muscle, the voltage sensitivity of the inactivation process also undergoes a shift to more hyperpolarized membrane potentials (Dulhunty et al. 1992). In addition, mammalian soleus muscle undergoes a spontaneous "perchlorate contracture" when conditioned in solutions containing this anion. Implications of this contracture on the results presented here will be discussed subsequently.

The results in studies presented to date have concluded that perchlorate has a specific effect on the voltage sensor for E-C coupling. There are numerous pieces of evidence which support this conclusion. Perchlorate has been shown to induce a negative electrostatic potential on the surface of artificial phospholipid membranes (McLaughlin et al. 1975). This is important since charge movement, which reflects a conformational change in the voltage sensor for E-C coupling, is shifted to more negative membrane potentials in the presence of perchlorate (Luttgau et al. 1983; Huang, 1986; Csemoch et al. 1987). This shift in the voltage sensitivity of charge movement is not accompanied by a change in the threshold amount of charge required for contraction, the peak rate of release of calcium from the SR or on the binding of calcium to troponin C (Csernoch et al. 1987).

The present study adds further support to the premise that the primary effect of perchlorate occurs at the voltage sensor for E-C coupling. Strength-duration curves have been described as reflecting the kinetics of activator formation or calcium release from the SR (Adrian et al. 1969; Costantin, 1974). If the shape of the strength-duration curve reflects the time course of calcium release from the SR, then parallel shifts in contraction thresholds following treatment with different concentrations of perchlorate are not consistent with an action of the anion in changing the rate of calcium release from the SR.

To simply ascribe the hyperpolarizing shift in contraction threshold to an effect of perchlorate on the voltage sensor for E-C coupling is made difficult by the existence of the "perchlorate contracture". This contracture implies that there exists a supra-threshold amount of calcium in the myoplasm. The origin of the elevated calcium was unlikely to be voltage sensor mediated since perchlorate conditioned fibres are not depolarized in the rested state (in fact they are hyperpolarized by 3-5 mV). The perchlorate contracture was dependent on the external calcium concentration, but not on (a) a calcium influx through DHP-sensitive calcium
channels or (b) on a sodium-calcium exchange mechanism or (c) calcium-activated calcium release (Dulhunty et al, 1992). However, the contribution of this unknown source of calcium must be significant if tension generation is to occur. Indeed a 10-fold increase in myoplasmic calcium must occur for contraction threshold to be achieved (at 22°C). This is based on observations by Stephenson & Williams (1981) and assumes that resting myoplasmic calcium concentration is $10^{-8}$M.

As a consequence of the perchlorate contracture, an additional amount of calcium must be generated by a depolarizing pulse such that a contraction can be visually detected. Therefore, the negative shift in the observed contraction is due not only to an effect of perchlorate on the voltage sensor for E-C coupling, but also on a second unknown process which supplies additional calcium. Since the perchlorate contracture amplitude is suppressed by depolarization, this could be a divalent cation channel such as that described in cardiac muscle (Rosenberg, Hess & Tsien, 1988) and myotubes of human or mouse (Fong, Turner, Denetclaw & Steinhardt, 1990), in which the open probability of this channel is greatest in the -70 mV to -80 mV range and declines outside these potentials. However, it would be difficult to explain the existence of this channel with control data unless the channel is only activated in the presence of perchlorate.
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