THE EFFECTS OF β-ADRENOCEPTOR ACTIVATION ON CONTRACTION IN NORMAL AND FATIGUED RAT SKELETAL MUSCLE

by

Simeon Peter Cairns (BSc. Hons.)

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The work presented in this thesis was performed entirely by the candidate.

Simeon Cairns

This thesis is dedicated to the memory of

HILDA MARIANA KERR

Arohanui Haimona

ABSTRACT

The ability of catecholamines to modulate contractile force was examined in directly stimulated bundles of fibres isolated from rat skeletal muscle. Terbutaline, a selective β_2-adrenergic agonist, was used to activate the catecholamine receptors normally present in skeletal muscle.

Terbutaline (10 μM) potentiated non-fatigued isometric twitches and tetani by about 15% in the slow-twitch soleus and potentiated twitches by about 7% in fast-twitch sternomastoid fibres. Force potentiation was not modified by denervation indicating that terbutaline acted directly on the muscle fibres. Adrenaline, a physiological catecholamine, also increased contractile force suggesting that this response may be functionally important. Propranolol, a β-adrenoceptor blocker, abolished and dibutyryl-cAMP (DBcAMP), a membrane permeable derivative of cAMP, mimicked the force potentiation by terbutaline suggesting that β-adrenoceptor activation and myoplasmic cAMP were involved in this response.

In addition, terbutaline and DBcAMP accelerated relaxation in normal soleus fibres whereas relaxation in sternomastoid fibres was slowed. The acceleration of relaxation in soleus may be due to the cAMP-dependent phosphorylation of phospholamban, a sarcoplasmic reticulum (SR) protein, which increases the activity of the SR Ca^{2+} pumps and hence accelerates myoplasmic Ca^{2+} removal.

The positive inotropic effect of terbutaline did not involve: (i) enhanced sodium-potassium (Na-K) pump activity; (ii) enhanced action potential activation; (iii) accelerated carbohydrate metabolism; (iv) or an increased trans-sarcolemmal Ca^{2+} current. Caffeine (1 mM) produced similar effects to terbutaline on peak tetanic force but not on relaxation. Pretreatment with caffeine attenuated or abolished the potentiation of twitches and tetani by terbutaline. This suggests that the myofilaments are not involved in the force potentiation and it is postulated that terbutaline, via myoplasmic cAMP, causes additional Ca^{2+} release from the SR which increases force.

High-frequency fatigue (HFF) - the decline of force in prolonged tetani - occurred more rapidly in the fast-twitch than in the slow-twitch fibres. Maximal potassium contractures, produced during HFF, completely restored force which suggests that: (i) HFF was due to impaired SR Ca^{2+} release; (ii) HFF did not involve mechanical inactivation and (iii) the voltage-dependent mechanism of excitation-contraction coupling could still function maximally in HFF. The rate of HFF was increased by reducing the $[Na^+]$ and by small increases in $[K^+]$ in the bathing solution suggesting that action potential failure contributed to the impaired SR Ca^{2+} release. The rate of HFF increased with stimulation frequency and recovery was rapid indicating that HFF

may be caused by changes in the ionic concentrations in the T-system occurring as a direct consequence of action potential activity.

Terbutaline and DBcAMP both slowed HFF. This effect was not caused by a stimulation of the Na-K pump. Caffeine (1-5 mM) caused a concentration-dependent slowing of HFF and the terbutaline-induced increase in fatigue resistance was abolished in the presence of 1 mM caffeine. It is hypothesized that the terbutaline-induced slowing of fatigue was caused by modulation of Ca^{2+} release so that more Ca^{2+} is released at the low levels of voltage activation which prevail during HFF.

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ABBREVIATIONS

ATP	Adenosine trisphosphate
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular free calcium concentration
Ca-pump	Calcium pump (Ca^{2+}-ATPase)
cAMP	Cyclic adenosine monophosphate
cAMP-PK	Cyclic adenosine monophosphate protein kinase
Cl^-	Chloride ion
CNS	Central Nervous System
DBcAMP	Dibutyryl cyclic adenosine monophosphate
DHP-receptor	Dihydropyridine receptor
E-C coupling	Excitation-contraction coupling
EMG	Electromyogram
FI_x	Fatigue index at x seconds
HFF	High-frequency fatigue
IP_3	Inositol trisphosphate
K^+	Potassium ion
K-contracture	Potassium contracture
MBcAMP	Monobutyryl cyclic adenosine monophosphate
Na^+	Sodium ion
Na-K pump	Sodium-potassium pump (Na^+-K^+-ATPase)
pCa	$-\log_{10}([Ca^{2+}])$
SR	Sarcoplasmic reticulum
TEA	Tetraethylammonium ion
T-system	Transverse tubular system
TTX	Tetrodotoxin

DEFINITIONS

Contraction: refers to an increase in muscle force as as result of electrical stimulation, i.e. initiated by action potentials.

Contracture: refers to an increase in muscle force occurring independently of stimulation.

Excitation-contraction coupling: refers to those processes by which depolarization of the T-system membrane leads to Ca^{2+} release from the sarcoplasmic reticulum.

Fatigue: refers to any decline in the isometric force generating capacity of a muscle or fibre as a consequence of direct electrical stimulation.

Fibrillation: refers to the spontaneous force oscillations which are seen in denervated muscle. They are thought to be caused by twitches in individual fibres resulting from the spontaneous firing of action potentials.

Ionic shift: In this thesis refers specifically to the change in the trans-sarcolemmal ion gradients resulting from repetitive stimulation, i.e. K^+ accumulation and Na^+ depletion in the T-system, as well as increased $[Na^+]$ and decreased $[K^+]$ in the myoplasm.

Mechanical inactivation: refers to the spontaneous decay of force that occurs under conditions of maintained depolarization.

Relaxation: refers to the decay of force from the peak value back to the baseline level which occurs following the cessation of stimulation.

Tachyphylaxis: refers to progressively smaller drug-induced responses that may occur with repetitive drug appplication - it may be caused by receptor desensitization.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

The regulation of force production by contracting skeletal muscle is of prime importance for normal function in all species throughout the animal kingdom. Graded dynamic or static contractions are required for activities such as eating, breathing, lifting, locomotion - such as walking or running - or simply to maintain posture, to name a few. Maximal muscle performance is a necessity for the survival of animals in the wild, enabling them to capture prey or to escape from predators. Similarly, for human performance in athletic events, such as sprinting, it is important that the working muscles can generate a high maximum power output (i.e. product of the force produced and the shortening speed) and can also maintain this power output for as long as possible, i.e. minimize the development of fatigue.

The basic framework for the chain of events involved in the voluntary activation of contraction is well established (Figure 1.1). Action potentials, initiated in the motor cortex of the brain, are propagated along descending motor pathways in the spinal cord and then via peripheral motor nerves to the musculature. When an action potential invades and depolarizes a motoneuron terminal, the process of neuromuscular transmission is initiated and culminates in the release of acetylcholine to activate specific receptors in the endplate region of the sarcolemma. The subsequent local depolarization, i.e. endplate potential, spreads passively to the adjacent surface membrane to initiate an action potential. Muscle action potentials are propagated across the sarcolemma in all directions away from the centrally located neuromuscular junction, and then radially into the depths of the fibre along narrow invaginated tubular extensions of the surface membrane, known as the transverse (T-) tubular system. Then, in the process known as excitation-contraction coupling (E-C coupling), depolarization of the T-tubule membrane somehow triggers the release of calcium ions (Ca^{2+}) from the adjacent terminal cisternae of the sarcoplasmic reticulum (SR); the SR being a system of internal membraneous sacs which surround the myofibrils and release, sequester and store Ca^{2+}. The processes involved in signal transmission between the membranes of the T-tubule and the SR, and which control Ca^{2+} efflux through specialized SR Ca^{2+} channels are not well understood (see Chapter 1.2b). Myoplasmic Ca^{2+} then rapidly diffuses down its concentration gradient into the myofilament lattice where it binds to troponin-C, a thin filament regulatory protein, thereby removing the inhibitory influence of tropomyosin. This allows an ATP dependent interaction between the myosin crossbridges and actin filaments with crossbridge cycling and the generation of force.

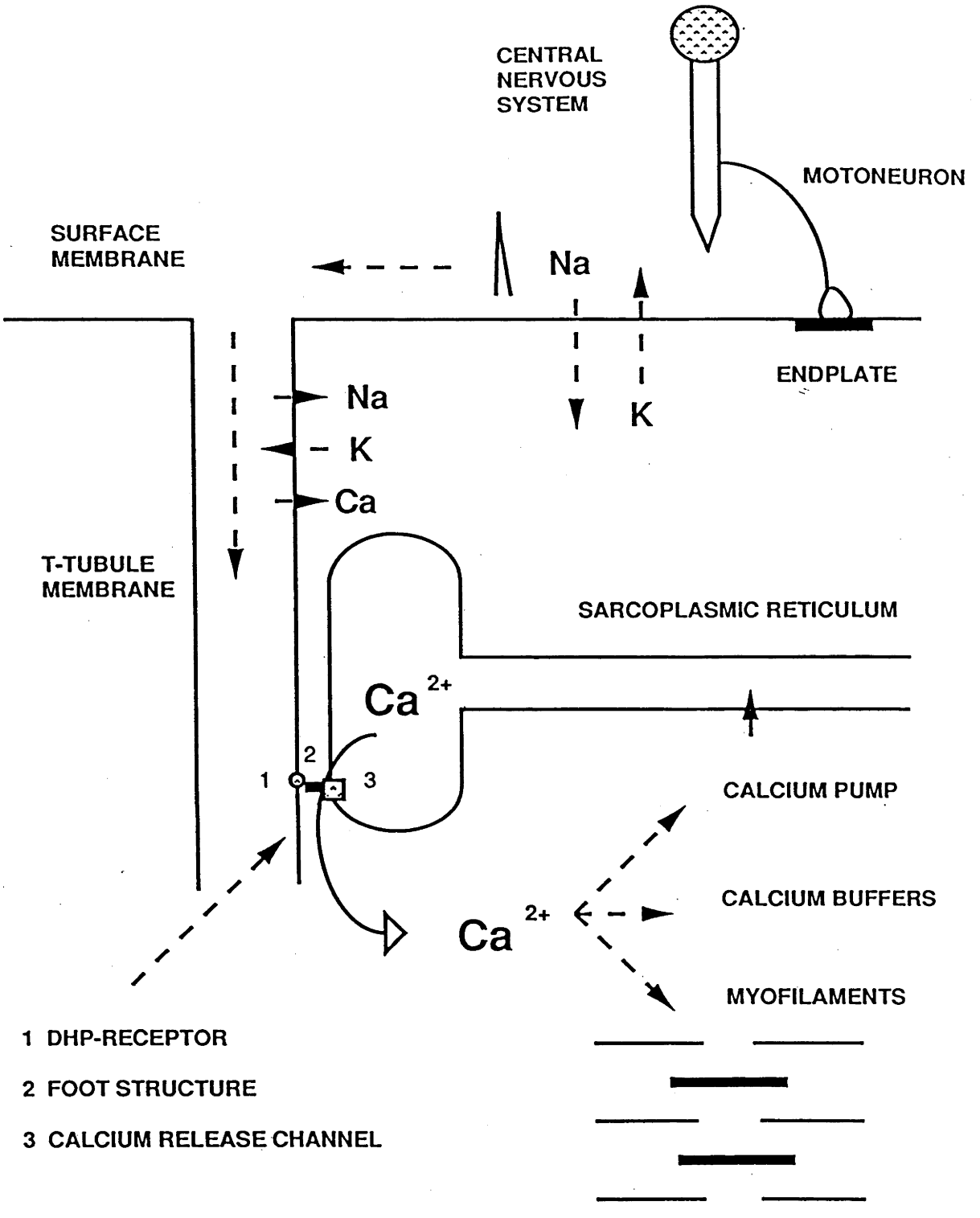


FIG. 1.1: Schematic diagram of the chain of events and cellular processes involved in the voluntary activation of contraction in skeletal muscle.

Relaxation occurs following termination of action potential activation, when $[Ca^{2+}]_i$ falls towards resting levels due to deactivation of Ca^{2+} release and the continued removal of Ca^{2+} from the myoplasm. Ca^{2+} is removed by an ATP dependent SR Ca^{2+}-pump (i.e. the Ca^{2+}-ATPase), which transports Ca^{2+} into the lumen of the SR, and possibly by myoplasmic Ca^{2+} binding proteins, such as parvalbumin, which act in parallel with the Ca^{2+}-pump. As myoplasmic $[Ca^{2+}]$ is reduced, Ca^{2+} dissociates from troponin-C, restoring the inhibitory influence of tropomyosin, and crossbridge cycling stops.

In this thesis two factors involved in the regulation of isometric muscle performance are examined: (i) the ability of a class of hormones, called catecholamines, to increase muscle force and to modulate the rate of relaxation and (ii) the effect of prolonged stimulation to reduce force production, i.e. fatigue. The development and modification of techniques involved with electron microscopy, patch and voltage clamping, molecular biology and biochemical methods, intracellular ion indicators, pharmacological tools, and skinned fibre studies have led to an increased, but as yet incomplete, understanding of the cellular mechanisms of muscle contraction. This introduction reviews studies on contractile activation and the regulation of muscle force in accordance with the theme of this thesis. Since the present study was performed on mammalian skeletal muscle fibres I have referred to research on mammalian preparations where possible. However, as less data are available from mammalian muscle compared to a wealth of information from elegant experiments on amphibian twitch fibres, the latter are discussed also.

1.2 CELLULAR MECHANISMS OF CONTRACTILE ACTIVATION

This section describes some of the processes involved in the activation of contraction, i.e. the action potential and E-C coupling. For further information the reader is referred to a number of excellent reviews (Stephenson 1981; Caputo 1983; Caillé et al. 1985; Lüttgau & Stephenson 1986; Dulhunty 1988; Ríos & Pizarró 1988; Caswell & Brandt 1989; Fleischer & Inui 1989). For reviews on the thin filament regulation of force see El-Saleh et al. (1986), Walsh (1987) and Zot and Potter (1987), and for crossbridge function see Squire (1983) and Huxley and Kress (1985).

(a) The muscle action potential

Excitation of the sarcolemma involves the initiation and propagation of action potentials along the surface and T-system membranes. The configuration of the intracellularly recorded muscle action potential is similar to that for the nerve axon (Nastuk & Hodgkin 1950; Gage & Eisenberg 1969; McArdle et al. 1980). Radio-isotope studies have revealed a net influx of sodium ions (Na^+), followed by a delayed net efflux of

potassium ions (K^+), during the action potential in muscle (Hodgkin & Horowicz 1959a). Voltage clamp experiments have revealed a fast inward current through depolarization-activated channels which has been identified as a Na^+ current since it is reduced by lowering extracellular [Na^+], has a reversal potential similar to the sodium equilibrium potential, and is antagonized by tetrodotoxin (TTX), a toxin which blocks the Na^+ channel (Adrian et al. 1970; Duval & Léoty 1978; Pappone 1980; Stefani & Chiarandini 1982). Similarly, the overshoot of the action potential approaches the sodium equilibrium potential and depends on the extracellular [Na^+] (Nastuk & Hodgkin 1950; McArdle et al. 1980) suggesting that a Na^+ current is responsible for the upstroke of the action potential (Nastuk & Hodgkin 1950; Adrian et al. 1970; Stefani & Chiarandini 1982; Lüttgau & Stephenson 1986). The repolarization of the action potential is thought to involve at least two mechanisms. Firstly, the Na^+ current is transient and is "turned off" by the fast inactivation of Na^+ channels during membrane depolarization (Adrian et al. 1970; Duval & Léoty 1978; Pappone 1980; Ruff et al. 1988). Secondly, a late outward K^+ current through delayed rectifier K^+ channels also contributes to membrane repolarization (Adrian et al. 1970; Duval & Léoty 1978; Pappone 1980). Notably, tetraethylammonium ions (TEA) which block the delayed outwards current (Pappone 1980; Stefani & Chiarandini 1982) also slow the repolarization of the action potential (Delbono & Kotsias 1987).

Transmission of surface membrane excitation into the interior of the fibre is less well understood. The requirement of an intact T-system is essential for this process and contraction, as shown in the classical experiments of Gage and Eisenberg (1969). They demonstrated that, following disconnection of T-tubules from the surface membrane by glycerol treatment, the fibres could conduct surface action potentials of normal amplitude but failed to contract. Transmission of the surface action potential along the T-tubular membrane is thought to be mainly due to the active propagation of action potentials rather than by the passive spread of depolarization (Adrian et al. 1969; Costantin 1970; Gonzalez-Serratos 1971; Bezanilla et al. 1972; Bastian & Nakajima 1974). Indeed, T-tubular action potentials have now been recorded optically with voltage sensitive dyes (Nakajima & Gilia 1980; Delay et al. 1986; Heiny et al. 1990). The ionic basis of the T-tubular action potential is thought to be similar to action potentials in the surface membrane. TTX-sensitive Na^+ channels and delayed rectifier K^+ channels are present in the T-tubule membrane (Jaimovich et al. 1976; Dulhunty 1979; Stefani & Chiarandini 1982; Sabbadini & Dahms 1989), although the density of these channels is less than in the surface membrane (Jamovich et al. 1976; Stefani & Chiarandini 1982). Mammalian T-tubule membrane also has a high chloride (Cl^-) conductance (Palade & Barchi 1977; Dulhunty 1979), which may be functionally important to prevent excessive K^+ accumulation and depolarization-induced repetitive firing (Adrian & Bryant 1974; Dulhunty 1979; Almers 1980). Recent optical evidence

suggests that a Cl^- current can contribute to repolarization of the T-tubular action potential (Heiny et al. 1990). Surprisingly, this result was obtained from amphibian muscle which is thought not to have a high T-tubular Cl^- conductance (Eisenberg & Gage 1969). Also present in the T-tubule membrane are voltage-activated Ca^{2+} channels (Stefani & Chiarandini 1982; Donaldson & Beam 1983; Borsotto et al. 1984; Flockerzi et al. 1986; Hosey et al. 1986; Lamb & Walsh 1987) and Ca^{2+}-activated K^+ channels (Latorre et al. 1982). Currents through these channels are too slow to be involved during a single action potential although they may become important during repetitive activation (Donaldson & Beam 1983; Fink et al. 1983; Lüttgau & Wetter 1983; Avila-Sakar et al. 1986; Lamb & Walsh 1987).

Although only a small number of ions move across the sarcolemma during a single action potential (Hodgkin & Horowics 1959a; Adrian & Peachley 1973; Clausen & Everts 1988), the change in trans-sarcolemmal ion concentrations may become large during prolonged activation (Bezanilla et al. 1972; Juel 1986; Clausen & Everts 1988). Sarcolemmal sodium-potassium (Na-K) pump proteins are thought to be responsible for maintaining the normal trans-sarcolemmal ion gradients (Clausen 1986; Sejersted 1988; Clausen & Everts 1989). The Na-K pump is a ouabain-sensitive Na^+-K^+-ATPase, an enzyme which utilizes ATP to translocate 3 Na^+ out of the fibre for 2 K^+ into the fibre (Clausen 1986). It is located in the surface and T-tubule membranes, although the density is less in the T-system (Venosa & Horowicz 1981; Clausen 1986; Sabbadini & Dahms 1989). This enzyme is activated by stimulation-induced increases in the extracellular $[K^+]$ or intracellular $[Na^+]$, the latter being the most important (Sejersted 1988; Clausen & Everts 1989).

(b) Excitation-Contraction Coupling

It is widely accepted that contraction is caused by an increase in the myoplasmic $[Ca^{2+}]$ (Caputo 1983; Lüttgau & Stephenson 1986; Stephenson 1988; Fleischer & Inui 1989). This is clearly demonstrated by the direct application of Ca^{2+} to the myofilaments in skinned fibre preparations whereby force is produced (Ashley & Moisescu 1977; Donaldson et al. 1978; Stephenson & Williams 1981; Godt & Nosek 1989). In the skinned preparation the sarcolemma is removed or disrupted either by mechanical peeling or chemical treatment, allowing the control of the ionic environment surrounding the SR and myofilaments. Furthermore, the activation of contraction in intact fibres by action potentials, depolarization by voltage clamp or high $[K^+]$ solutions, are all accompanied by an increase in the $[Ca^{2+}]_i$ (Ashley & Ridgway 1970; Blinks et al. 1978; Eusebi et al. 1980; Allen et al. 1989; Fryer & Neering 1989; Allen & Westerblad 1990). This Ca^{2+} signal, known as a Ca^{2+} transient, has been detected using a wide variety of intracellular Ca^{2+} indicators (Miledi et al. 1982; Baylor et al.

1983; Cannell & Allen 1984; Allen & Westerblad 1990). Moreover, electron microprobe studies indicate that most, if not all, of the increase in $[Ca^{2+}]_i$ is due to Ca^{2+} released from the SR (Somlyo et al. 1981).

Voltage-activation of contraction

Hodgkin and Horowicz (1960), in their well known experiments, demonstrated that rapid application of high $[K^+]$ solutions produced an increase in force (K-contracture) in single intact amphibian twitch fibres. These K-contractures increased in amplitude with the $[K^+]$, and hence the extent of membrane depolarization (Hodgkin & Horowicz 1959b). These results suggested that force production does not depend on the action potential *per se*, but on membrane depolarization. Similarly, K-contractures can be induced in small bundles of fibres from mammalian muscles (Dulhunty 1980; Anwyl et al. 1984; Gallant & Donaldson 1989), although equilibration in low $[Cl^-]$ solutions is required to produce large K-contractures (Dulhunty & Gage 1983, 1985; Chua & Dulhunty 1988). Force production can also be evoked by voltage-clamp depolarization in amphibian fibres (Caputo & De Bolaños 1979; Caputo et al. 1981; Ildefonse et al. 1985), or by depolarization of T-tubular membranes in skinned fibres induced by ionic substitution (Donaldson 1985; Donaldson et al. 1989; Lamb & Stephenson 1990a). Any study of contractile activation should, therefore, account for the normal voltage-dependent mechanism of E-C coupling.

The possibility that the SR is electrically coupled to the T-tubule membrane and that direct depolarization of SR membrane triggers Ca^{2+} release (Mathias et al. 1980) is unlikely as the SR does not appear to have a membrane potential (Somlyo et al. 1981; Oetliker 1982) and the membrane capacitance is too low to account for the much greater area of the SR membrane (Dulhunty 1988). Another hypothesis is that during membrane depolarization a T-tubular Na^+ current triggers contraction, i.e. Na^+-induced Ca^{2+} release (Potreau & Raymond 1982; Caillé et al. 1985). However, removal of external Na^+ or addition of TTX did not reduce the amplitude of Ca^{2+} transients evoked by voltage clamp depolarization (Miledi et al. 1984), and the amplitude of K-contractures were not influenced by TTX (Dulhunty & Gage 1985). These results suggest that a Na^+ current through TTX-sensitive Na^+ channels is not directly involved in triggering Ca^{2+} release. Therefore, it has been postulated that "voltage sensitive molecules" in the T-tubule membrane are responsible for the transduction of membrane depolarization into Ca^{2+} release from the SR (Schneider & Chandler 1973; Caputo 1983; Dulhunty 1988; Ríos & Pizarró 1988).

Asymmetric charge movement

In 1973, Schneider and Chandler depolarized frog fibres by the voltage clamp technique and, after elimination of ionic and linear capacitive currents, discovered a small voltage displacement which is now called asymetric charge movement. This charge movement was thought to represent the activity of a putative voltage sensor which is required to trigger Ca^{2+} release from the SR. In support of this hypothesis, charge movement is mainly generated in the T-system and is activated over the same membrane potential range as it is required for contraction (Schneider & Chandler 1973; Chandler et al. 1976a, 1976b; Gage & Dulhunty 1983, 1985; Rakowski et al. 1985; Lamb 1986). Moreover, changes in the amount of charge moved correlate well with Ca^{2+} release under a variety of experimental conditions (Kovács et al. 1979; Rakowski et al. 1985; Melzer et al. 1986; Ríos & Brum 1987; Brum et al. 1988a, 1988b). However, there is still doubt about the involvement of some components of charge movement in Ca^{2+} release (Lamb 1986; Melzer et al. 1986; Lamb & Walsh 1987; Huang 1988).

Dihydropyridines (DHP), such as nifedipine, which are antagonists of the voltage-activated Ca^{2+} channel of the sarcolemma, block a component of charge movement (Lamb 1986; Lamb & Walsh 1987; Ríos & Brum 1987) as well as "slow" Ca^{2+} currents (Ildefonse et al. 1985; Lamb & Walsh 1987; Ríos & Brum 1987). A close relation between the effects of nifedipine on slow Ca^{2+} currents and charge movement motivated Lamb and Walsh (1987) to propose that the nifedipine sensitive component of charge movement was a gating current for a T-tubular Ca^{2+} channel. Later in the same year, Ríos and Brum observed a close correlation between the effects of nifedipine on Ca^{2+} release and charge movement and they proposed that the DHP-receptor was identical to the putative voltage sensor of E-C coupling. The importance of the DHP-receptor for E-C coupling has recently been shown in the elegant experiments of Tanabe et al. (1988). They demonstrated that microinjection of an expression plasmid, containing the complementary DNA encoding the DHP-receptor, into muscle cells from mice with muscular dysgenesis restored both contraction and slow Ca^{2+} currents. Thus, the DHP-receptor is essential for E-C coupling.

T-tubule - SR communication

There are currently three main theories for transmission between the T-tubules and the terminal cisternae, the first involving mechanical coupling and the latter two chemical messenger processes.

(i) Mechanical model:

Chandler and coworkers proposed a relatively simple model in which charge movement initiated a conformational change in a long molecule which transversed the junctional gap and subsequently unplugged a channel in the terminal cisternae to allow Ca^{2+} efflux into the myoplasm (Schneider & Chandler 1973; Chandler et al. 1976b). This model has recently become more compelling following electron microscopy and biochemical studies (Caswell & Brandt 1989; Brandt et al. 1990).

Structures in the junctional gap between the T-tubular and terminal cisternae membranes - a distance of about 15 nm (Franzini-Armstrong 1970; Fleischer & Inui 1989) - which might be important in E-C coupling have been revealed by electron microscopy (Franzini-Armstrong 1970; Somlyo 1979; Eisenberg & Eisenberg 1982; Dulhunty 1988, 1989; Brandt et al. 1990). Franzini-Armstrong (1970) described structures designated as "feet" and other workers observed "bridges" (Somlyo 1979) and "pillars" (Eisenberg & Eisenberg 1982), although these latter structures may also be part of the foot structure (Dulhunty 1988). The importance of the junctional foot structure in E-C coupling has recently been confirmed by three experimental observations. (i) The alkaloid, ryanodine, has been shown to bind specifically to the Ca^{2+} release channel of the terminal cisternae (Fleischer et al. 1985). (ii) The isolated and purified ryanodine receptor has a morphology identical to the foot structure (Inui et al. 1987; Lai et al. 1988). (iii) The purified ryanodine receptor can function as a Ca^{2+} channel when reincorporated into lipid bilayers (Hymel et al. 1987; Imagawa et al. 1987; Lai et al. 1988), with properties similar to those for the Ca^{2+} release channel of SR membranes (Smith et al. 1985, 1986). Thus, it has been suggested that the ryanodine receptor, the SR Ca^{2+} release channel and the junctional foot protein are all parts of the same structure (Caswell & Brandt 1989; Fleischer & Inui 1989).

The foot structures are aligned with the DHP-receptor in the junctional gap (Dulhunty 1989). However, the molecular interaction between the DHP-receptor and junctional foot protein does not appear to be direct but via a glycolytic enzyme (Brandt et al. 1990). Thus the machinery for a mechanical coupling mechanism seems to be available, although signal transmission via the foot protein is not necessarily mechanical (Dulhunty 1989; Ikemoto et al. 1989; Lamb & Stephenson 1990c).

(ii) Calcium-induced calcium release:

In 1970, it was shown that application of Ca^{2+} to skinned fibres caused Ca^{2+} release from the SR (Endo et al. 1970; Ford & Podolsky 1970). Similarly, micromolar concentrations of Ca^{2+} stimulate Ca^{2+} release from isolated SR vesicles (Nagasaki & Kasai 1981; Meissner et al. 1986) and activate the isolated Ca^{2+} release channel (Smith

et al. 1985, 1986; Lai et al. 1988; Rousseau et al. 1988). The hypothesis that a trans-sarcolemmal Ca^{2+} influx occurs during membrane depolarization triggering Ca^{2+} release from the SR (Meissner et al. 1986) has recently become more attractive since the putative voltage sensor might also be a modified Ca^{2+} channel (Ríos & Brum 1987; Tanabe et al. 1988). However, there are several lines of evidence against such a mechanism in skeletal muscle. (i) Slow Ca^{2+} currents, recorded in the T-tubular membrane, are too slow to initiate twitches (Donaldson & Beam 1983; Lamb 1986; Lamb & Walsh 1987). (ii) There is no obvious relation between the amplitude of the Ca^{2+} current and the Ca^{2+} transient evoked by voltage clamp depolarization in frog fibres (Brum et al. 1987). (iii) Contraction still occurs in low extracellular [Ca^{2+}] (Armstrong et al. 1972; Blinks et al. 1978; Dulhunty & Gage 1988), although Ca^{2+} transients may be slightly reduced (Blinks et al. 1978; Miledi et al. 1984). It is important to note, however, that the voltage sensors require at least some Ca^{2+} to function normally (Brum et al. 1988a, 1988b; Dulhunty & Gage 1988).

Therefore, a Ca^{2+} influx is unlikely to be involved in the normal voltage-dependent activation of E-C coupling, but this does not eliminate the possibility that a Ca^{2+}-induced Ca^{2+} release mechanism might contribute to the total Ca^{2+} released. Indeed, the pharmacological blockade of Ca^{2+}-induced Ca^{2+} release by procaine or ruthenium red eliminates a component of voltage-activated Ca^{2+} release in skinned fibres (Donaldson 1985; Donaldson et al. 1989; Lamb & Stephenson 1990b). It has been proposed that Ca^{2+} might activate Ca^{2+} release in parallel with the voltage-dependent mechanism (Ríos & Pizzaró 1988) or further activate Ca^{2+} channels opened by voltage-activation (Lamb & Stephenson 1990b).

(iii) Inositol trisphosphate-induced calcium release:

It has been proposed that depolarization of T-system membrane stimulates the production of inositol 1,4,5-trisposphate (IP_3) which then diffuses across the junctional gap to cause Ca^{2+} release from the SR (Vergara et al. 1985; Volpe et al. 1985; Donaldson 1986; Vergara & Asotra 1987). This hypothesis is supported by several results: (i) IP_3 is produced in muscle on tetanic stimulation (Vergara et al. 1985); (ii) IP_3-induced Ca^{2+} release occurs in isolated triads (Valdiva et al. 1990) and (iii) injection of IP_3 causes Ca^{2+} release and contraction in skinned fibres (Vergara et al. 1985; Volpe et al. 1985; Donaldson 1986; Donaldson et al. 1987, 1988; Nosek et al. 1986). There are, however, a number of negative results (Lea et al. 1986; Mikos & Snow 1987; Hanon et al. 1988) and criticism of the evidence from skinned fibre studies on technical grounds (Hanon et al. 1988). A major argument against an IP_3-induced Ca^{2+} release mechanism in the normal voltage-activation of E-C coupling is that flash photolysis of caged IP_3 produces contractures which develop much too slowly (Walker

et al. 1987). Nevertheless, the machinery is available for an effect of IP_3 on Ca^{2+} release (Surez-Isla 1988; Brandt et al. 1990) so that this chemical messenger may have a modulatory influence.

In summary, it appears that DHP-receptors in the T-tubule membrane act as the "voltage sensors" for E-C coupling. The DHP-receptors somehow convert membrane depolarization into Ca^{2+} release from the SR, possibly by activating Ca^{2+} release channels in the terminal cisternae membrane via the foot structures. However, the exact mechanism by which this occurs is at present unclear. Calcium- and IP_3-induced Ca^{2+} release may have additional or modulatory effects on Ca^{2+} release.

1.3 REGULATION OF MUSCLE FORCE

The force developed by a whole muscle can be graded by controlling the number of fibres that are simultaneously contracting and the force developed by each individual fibre. In the first case, force can be regulated by varying the type and number of motor units activated in parallel, a process known as recruitment (Burke & Edgerton 1975; Buchthal & Schmalbruch 1980; Burke 1990; Kernell 1990). This discussion is concerned with the second mechanism, namely the regulation of force development at the cellular level. Three important relationships are discussed.

(a) The force-$[Ca^{2+}]$ relationship

The steady state relation between isometric force and $[Ca^{2+}]$ is well documented for skinned fibres (Ashley & Moisescu 1977; Donaldson et al. 1978; Stephenson & Williams 1981; Fuchs & Fox 1982; Cooke et al. 1988; Godt & Nosek 1989), and has also recently been described for intact fibres (Allen et al. 1989; Allen & Westerblad 1990). Application of Ca^{2+} from a resting concentration of 10 nM up to a maximal concentration of 30 μM, in Ca^{2+} buffered solutions, causes force production in skinned fibres (Stephenson & Williams 1981; Wendt & Stephenson 1983). This relation is usually presented as a semilogarithmical plot, between the steady state force and the pCa of the bathing solution ($pCa = -\log_{10}[Ca^{2+}]$). The force-pCa curve is typically sigmoidal, indicating that a threshold $[Ca^{2+}]$ is required to initiate contraction and that force reaches a plateau at high $[Ca^{2+}]$ (Lüttgau & Stephenson 1986). At intermediate Ca^{2+} concentrations force is graded and is related to the amount of Ca^{2+} bound to troponin-C (Fuchs & Fox 1982).

Maximum Ca^{2+}-activated force occurs when all of the Ca^{2+} binding sites on troponin molecules become saturated with Ca^{2+} (Fuchs & Fox 1982; Baylor et al. 1983; Cannell & Allen 1984). Further increases in force are, therefore, independent of increases in myoplasmic $[Ca^{2+}]$. Maximum Ca^{2+}-activated force is likely to be determined by the

maximal rate of crossbridge cycling or force produced per crossbridge. This force level is modified by factors such as temperature (Stephenson & Williams 1981) and metabolites (Donaldson et al. 1978; Cooke et al. 1988; Godt & Nosek 1989).

Force production at submaximal Ca^{2+} concentrations can be regulated, in principle, by two main mechanisms. Firstly, changing the myoplasmic [Ca^{2+}], due to processes that alter SR Ca^{2+} release or Ca^{2+} removal, would change force as represented by a shift along the force-pCa curve. Secondly, the slope of the force-pCa curve or its position relative to the [Ca^{2+}] axis may be altered. For instance, increasing the affinity of troponin-C for Ca^{2+}, so that more Ca^{2+} binds to troponin-C at each submaximal [Ca^{2+}], would result in a shift of the entire force-pCa curve towards lower [Ca^{2+}], i.e. an increase in the Ca^{2+} sensitivity of the myofilaments. The converse would also be true for a reduction in the Ca^{2+} affinity of troponin-C. Such changes in Ca^{2+} sensitivity have been shown for a number of metabolites (Ashley & Moisescu 1977; Donaldson et al. 1978; Cooke et al. 1988; Godt & Nosek 1989) and drugs (Wendt & Stephenson 1983; Walsh 1987). The position of the force-pCa curve is also shifted towards lower [Ca^{2+}] when submaximal crossbridge activity is increased following myosin light chain phosphorylation in fast-twitch muscles (Walsh 1987; Metzger et al. 1989).

(b) The force-membrane potential relationship

Hodgkin and Horowicz (1960) were the first experimenters to quantify the steady state relation between isometric force production and membrane potential from their K-contracture experiments in single frog twitch fibres. Force production started at 20-25 mM K^+ and thereafter was related to the logarithim of the external [K^+], or membrane potential, by a steep S-shaped curve that gave a maximal force output at about 100 mM K^+. A similar relation is observed between force and membrane potential in voltage clamp experiments in frog fibres (Caputo & De Bolaños 1979; Caputo et al. 1981), and between force and [K^+] in mammalian fibres - well described by a Boltzmann curve (Dulhunty 1980; Dulhunty & Gage 1983, 1985). The force-membrane potential relation shows that: (i) there is a voltage threshold for contraction; (ii) force is graded over a range of membrane potentials; and (iii) force reaches a maximum plateau level, i.e. maximum voltage-activated force.

The important question "How does membrane potential control the production of force?" has been addressed in several voltage clamp experiments. The relation between membrane potential and the amplitude of Ca^{2+} transients (Kovács et al. 1979; Miledi et al. 1983; Delay et al. 1986), or the peak rate of Ca^{2+} release (Rakowski et al. 1985; Melzer et al. 1986; Klein et al. 1990) was, in each case, found to be sigmoidal, indicating that Ca^{2+} release was at first graded but became saturated with large depolarizations. The amount of charge moved as a function of membrane potential is

similarly described by a sigmoidal curve and a Boltzmann function (Chandler et al. 1976a, 1976b; Dulhunty & Gage 1983, 1985; Lamb 1986; Melzer et al. 1986). When charge movement and $[Ca^{2+}]_i$ were recorded simultaneously over a range of membrane potentials, it was found that the amount of charge moved and the peak rate of Ca^{2+} release were tightly coupled, although some charge had moved prior to the first detection of Ca^{2+} release (Kovács et al. 1979; Rakowski et al. 1985; Melzer et al. 1986). Force could not be measured simultaneously, but it would appear that the coupling between membrane potential and charge movement resulting in Ca^{2+} release from the SR, is an important determinant of the force-membrane potential relation.

However, the above relation is not rigid since low concentrations of the drug caffeine cause the force-membrane potential curve to be shifted towards lower membrane potentials, i.e. to the left (Sandow et al. 1964; Caputo et al. 1981). When $[Ca^{2+}]_i$ was measured in the presence of caffeine at various membrane potentials, it became apparent that caffeine increased the rate of Ca^{2+} release with both submaximal and maximal depolarizations (Delay et al. 1986; Klein et al. 1990). This result has important implications on the regulation of Ca^{2+} release since caffeine does not affect either charge movement nor membrane potential (Kovács & Szucs 1983; Delay et al. 1986; Klein et al. 1990). Firstly, Ca^{2+} release can be regulated by a mechanism in addition to the normal voltage-dependent activation of E-C coupling during both submaximal and maximal depolarizations. Secondly, maximal voltage activation of E-C coupling does not produce maximal Ca^{2+} release from the SR. Thirdly, since caffeine does not affect maximum voltage-activated force in frog fibres (Sandow et al. 1964; Caputo et al. 1981) but increases the maximum $[Ca^{2+}]_i$, it appears that the myofilaments were saturated with Ca^{2+}.

A second important feature of the force-membrane potential relation is that a spontaneous decline of force occurs during prolonged depolarization, induced by either high $[K^+]$ solutions or voltage clamp (Hodgkin & Horowicz 1960; Lüttgau 1963; Caputo 1976; Caputo & De Bolañas 1979; Dulhunty & Gage 1983, 1985; Chua & Dulhunty 1988). This phenomenon, called mechanical inactivation, is dependent on the magnitude and duration of the maintained depolarization (Hodgkin & Horowicz 1960; Caputo & De Bolaños 1979; Chua & Dulhunty 1988). It is not due to a failure of the myofilaments (Axelsson & Thesleff 1958; Ludin et al. 1966; Lüttgau & Oetliker 1968) and is associated with a reduction in the Ca^{2+} transient (Blinks et al. 1978; Allen et al. 1989). Thus it seems to be a feature of E-C coupling. A popular theory is that mechanical inactivation is caused by inactivation of the voltage sensors of the T-system (Chandler et al. 1976b; Rakowski 1981; Chua & Dulhunty 1988), which is supported by the observation that the amount of charge moved becomes smaller with prolonged

depolarization (Chandler et al. 1976b; Rakowski 1981). The possible involvement of other processes in E-C coupling, however, has not been eliminated.

(c) The force-frequency relationship

Muscle fibres are normally activated by trains of action potentials rather than by continuous depolarization (Grimby et al. 1981; Hennig & Lomo 1985, 1987). Neural regulation of force production is thought to involve control of the number, frequency, and pattern of action potentials delivered to the muscle; a process known as rate modulation (Burke & Edgerton 1975; Burke 1990). Certainly, the peak force response to a single stimulus, i.e. a twitch, is less than in a tetanus which is induced by trains of action potentials. The tetanus-to-twitch ratio, however, does vary from about 2 to 15 depending on the species and fibre-type (Lännergren & Smith 1966; Blinks et al. 1978; Dulhunty & Dlutowski 1979; Luff 1985). When force is expressed as a function of stimulation frequency, there was initially no change in force above twitch height until about 5-10 Hz when twitches start to fuse and then force rises steeply to a plateau at 50-200 Hz (Truong et al. 1964; Blinks et al. 1978; Edwards 1981; Hennig & Lomo 1987; Allen & Westerblad 1990). The stimulation frequency required to produce maximum tetanic force is very much dependent on fibre-type and temperature (Truong et al. 1964; Hennig & Lomo 1985, 1987). Notably, the recording of action potential activation *in vivo* indicates that maximum force is generated at much lower frequencies than *in vitro* and that the stimulation pattern is more complex than constant frequency trains (Grimby et al. 1981; Hennig & Lomo 1985, 1987).

Measurement of myoplasmic [Ca^{2+}] during action potential stimulation reveals that the amplitude of Ca^{2+} transients increase with frequency, being two to three times greater for a fused tetanus than for a twitch (Blinks et al. 1978; Miledi et al. 1982; Baylor et al. 1983; Cannell & Allen 1984; Allen & Westerblad 1990). It should be noted, however, that twitch [Ca^{2+}] is unlikely to be in a steady state, and the Ca^{2+} transient represents only a small proportion of the total Ca^{2+} released (Cannell & Allen 1984) so that twitch and tetanic [Ca^{2+}] cannot be compared by relation to the force-pCa curve. It is likely that the greater force developed during tetani than twitches reflects a larger amount of Ca^{2+} bound to troponin-C. The control of Ca^{2+} release by membrane potential and charge movement during tetanic stimulation is unknown and is an important area for further investigation.

In summary, the regulation of force at the cellular level primarily occurs via control of the frequency and pattern of action potentials delivered to the muscle. The action potentials activate voltage sensitive molecules in the T-system to produce a graded amount of charge movement which indirectly opens Ca^{2+} release channels in the SR. Other mechanisms may also control Ca^{2+} movements and myoplasmic [Ca^{2+}]. The

ultimate force production depends on the amount of Ca^{2+} bound to troponin-C and the precise location on the force-pCa curve.

1.4 AIMS OF THE EXPERIMENTS

As already mentioned, the main objectives of this thesis were to investigate (i) the hormonal regulation of isometric contractions, namely the effect of catecholamines on force and relaxation, and (ii) the effect of fatiguing stimulation on force production. Throughout this thesis differences due to either fibre-type or modifications due to inactivity - following removal of the nerve supply (denervation) - were examined.

Chapter 3, is concerned with the ability of catecholamines to regulate force production and the rate of relaxation of twitches and tetani in non-fatigued preparations. These effects were quantified and the pharmacology behind these responses was examined, i.e. the receptors involved, concentration-response relations, the second messenger/s involved.

The main question in Chapter 4 is "What is the cellular mechanism for the β-adrenoceptor mediated potentiation of force?" The roles of various cellular processes associated with the action potential, myofilaments and E-C coupling were examined. The effects of terbutaline were compared with those of caffeine.

Chapter 5 deals with the reduction of force caused by prolonged continuous tetanic stimulation. The cellular mechanisms responsible for this "high-frequency fatigue" were examined with special emphasis on the action potential and changes in the trans-sarcolemmal ionic distribution. Effects on relaxation and differences due to fibre-type and denervation were investigated.

In Chapter 6 the ability of catecholamines to regulate force production during high-frequency fatigue was studied. A small increase in fatigue resistance was observed and the mechanism for this response was examined with special emphasis on the role of the Na-K pump and modulation of the Ca^{2+} release channel.

CHAPTER 2
GENERAL METHODS

All experimental protocols presented in this thesis were approved by the Animal Experimentation Ethics Committee of the Australian National University.

2.1 MUSCLE PREPARATIONS

Adult (4-10 months) male white Wistar rats (*Rattus norvegicus*) weighing 250-550 g were used in all experiments. The skeletal muscles chosen for study were the soleus, a slow-twitch hindlimb muscle and the sternomastoid, a fast-twitch superficial neck muscle. The sternomastoid was dissected into the visibly distinguishable white and red muscle segments. These muscles were chosen because (i) they can be used as models for different mammalian fibre-types and (ii) abundant data is available on them to assist the present study. Different methods of assaying for fibre-type show that the rat muscles used have the following composition: soleus (84-96% type I; 4-16% type IIa; Ariano et al. 1973; Edström et al. 1982), red-sternomastoid segment (55-76% type IIa; 13-21% type IIb; 11-21% type I; Dulhunty & Dlutowski 1979; Luff 1985) and the white-sternomastoid segment (82-92% type IIb; 8-18% type IIa; Dulhunty & Dlutowski 1979; Luff 1985).

Animals were killed by suffocation in either halothane or carbon dioxide gas. Rat body weight was measured prior to dissection. The gross muscle dissections were as follows:
(i) *Soleus muscle:* The rat was pinned in a prone position to a cork dissecting board. Skin and hair overlying the posterior surface of a hindlimb were removed using surgical scissors. The Archilles tendon was severed close to the heel bone where it inserted. This tendon was held with forceps and the connective tissue on medial and lateral aspects of the lower hindlimb was transected up to the knee, thereby exposing the deep surface of the soleus. The proximal tendon was severed and the intact muscle freed from surrounding tissues.
(ii) *Sternomastoid muscle:* The rat was pinned in a supine position and the skin and submandibular glands overlying each sternomastoid muscle were removed while ensuring not to damage the nearby arteries. The tendon on the mastoid process was cut close to its insertion and the muscle was freed from the connective tissue attached along its length up to the sternum. This procedure was repeated for the contralateral sternomastoid muscle. The thorax was then pierced below the top two ribs and the sternum with attached sternomastoid muscles was removed. Each sternomastoid muscle was separated into white and red segments by tendon-to-tendon dissection near their region of junction.

Once excised, the intact muscles were pinned by their tendons, at approximately resting length, into a Petri dish lined with Sylgard (Dow Corning). Muscles were usually bathed in a modified Krebs solution (Solution 1, Table 2.1) at room temperature (18-22°C).

Preparations for contraction studies

These preparations were small bundles of 5-50 intact fibres. Fine dissection of each fibre bundle was performed under a low powered stereomicroscope (Wild Heerbrugh, 12-50 X magnification), with the muscle illuminated from below (Wild - light source). Bundles of fibres were obtained by tendon-to-tendon dissection using fine tipped forceps and microsurgical scissors (Carl Teufel, D-7201). Sufficient tendon was left attached to the fibres (> 7-8 mm) to enable the preparation to be mounted in the muscle bath without damage. Pathways for cutting the connective tissue around groups of fibres were readily identifiable whereas this was not so for single fibres. This factor and the length of these fibres (up to 35 mm) meant that isolation of single intact fibres, at least from these muscles, proved unsuccessful. Preparations with broken fibres were rejected although small fragments of cut fibres sometimes remained attached at the tendons; these fragments had no noticable effect on the performance of the remaining fibres. Each preparation had at least one diameter of less than 500 μm. The small cross-sectional dimensions of these preparations was thought to be adequate to prevent the major diffusion limitations of isolated whole muscles (Goldberg et al. 1975).

Preparations for membrane potential studies

These studies were performed on single fibres located on the surface of thin sheets of fibres, intact whole muscles or muscle segments. The surrounding connective tissue was carefully removed to assist with a clean microelectrode penetration of fibres. Thin sheets of fibres were obtained by dissecting away layers of fibres on one side of the preparation to reduce the chance of fibre damage on the other side of the preparation.

Denervation Procedure

Preparations were also obtained from soleus muscles that had been chronically denervated by peripheral nerve section. The reasons for using denervated muscles are described in Chapters 3.3c, 4.2 and 5.3b. Denervation surgery was performed on rats under ether anaesthesia and carefully maintained with an ether filled nose cone. Anaesthetized animals were placed prone on a dissection board and hair was removed from the left lumbar back region. Surgery commenced when the animal failed to exhibit a hindlimb flexion reflex in response to a toe pinch stimulus. A small incision of 1-2 cm was made through the skin to reveal the underlying musculature. The left sciatic nerve

TABLE 2.1: Composition of bathing solutions. Ion concentrations are in mM

SOLUTION	Na^+	K^+	Ca^{2+}	Cl^-	SO_4^{2-}	Sucrose
Solution 1	80.5	3.5	7.6	16	42.6	170
Solution 2	196.5	3.5	7.6	16	100.6	---
10K	190	10	7.6	16	100.6	---
60K	140	60	7.6	16	100.6	---
200K	---	200	7.3	16	100.3	---
Normal Krebs Solution	150	3.5	2.5	159.5	---	---

In addition all solutions contained: 1 mM Mg^{2+}; 11 mM glucose; 2 mM TES (*N*-tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid) pH buffer. pH = 7.4 ± 0.1, adjusted with NaOH. The measured ionized [Ca^{2+}] in Solution 1 was 2 mM.

was located just distal to the sciatic notch. Closed scissors were inserted between the surrounding musculature and opened thereby exposing the sciatic nerve. Using forceps and scissors, a 4-5 mm section of nerve was removed. A violent twitch of the hindlimb was usually observed at this time indicating a successful nerve section. The skin incision was sutured and the animal allowed to recover.

Soleus muscles were used from animals that had been denervated and maintained for 2-14 weeks. The rat usually carried its left hindlimb in a retracted position following denervation. Otherwise it appeared normal. Successful denervations were confirmed by: (i) the presence of fibrillations in the low chloride solutions; (ii) whole muscle atrophy and (iii) a lower than normal tetanus-to-twitch ratio (Table 2.3). Only two out of about ninety animals failed to meet these criteria.

2.2 SOLUTIONS AND DRUGS

In most experiments, preparations were bathed in a modified low [Cl^-] Krebs solution (Solution 1, Table 2.1). The main reasons that Solution 1 was used were that (i) force run-down (see Chapter 2.4) occurred more slowly in this solution than in normal Krebs solution - especially with sternomastoid preparations and (ii) a low extracellular [Cl^-] is required to produce large potassium contractures in mammalian fibres (Dulhunty & Gage 1985). Since at least one hour was required for the preparations to equilibrate (stable tetanic force) in the low [Cl^-] solutions (Dulhunty & Gage 1985; present study) the fine dissection and all isometric contraction measurements were usually made in the low [Cl^-] Solution 1.

In Solution 1, the impermeable sulphate ion was used as a substitute for Cl^-. Because the sulphate ion is a divalent ion, a lower than normal [Na^+] was used so that the ionic strength was similar to that of normal Krebs solution. Sucrose was added to maintain the osmolarity of Solution 1 at about 330 mosmol. TES (*N*-tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid) was used as the pH buffer. pH was set to 7.4 ± 0.1 pH units by adjustment with sodium hydroxide (measured with a T.P.S. digital pH meter). In most studies the solutions were bubbled with 100% oxygen gas (Med Grade, CIG). The absence or presence of 100% O_2 did not modify the contractile properties of these preparations.

Mammalian fibres normally have a high Cl^- conductance in the T-system membrane (Palade & Barchi 1977; Dulhunty 1979). Equilibration in a low extracellular [Cl^-] solution reduces the modifying influence of the high Cl^- conductance on membrane potential when challenged by high [K^+] solutions (Dulhunty 1979, 1980; Dulhunty & Gage 1983). Keeping the [K] x [Cl] product constant in normal and high [K^+] solutions was not sufficient to control membrane potential adequately (Dulhunty 1979; Dulhunty

& Gage 1985). The larger K-contractures produced in low rather than high [Cl^-] solutions are thought to be due to a relatively bigger tubular length constant in low Cl^- solutions (Dulhunty et al. 1984). Hence, this condition would allow a more rapid and synchronous passive spread of depolarization along the T-tubule membrane resulting in a larger K-contracture (Dulhunty & Gage 1985).

The use of Solution 1 does have some limitations. The lower [Na^+], relative to that in the normal Krebs solution (Table 2.1), although not influencing peak twitch force, does depress peak tetanic force slightly (see Chapter 5.3d(i)). Hence, it was necessary to use the normal Krebs solution in some experiments (see Chapters 3.3c, 5.4b(iii)). It should be noted that by using the normal [Na^+], but at a higher ionic strength than in Solution 1, there results a faster run-down of force in these preparations (Dulhunty pers. comm.).

2.3 FORCE RECORDING AND STIMULATION

A small volume (1.4 ml) Perspex muscle bath was used. The maximum rate of solution flow through the bath was (2 ml/s) giving a solution change over time of 700 ms. Solutions entered the bath via an inlet at one end and were removed at the opposite end of the bath by a motor driven suction pump (Charles Austen Pumps Ltd., model DYMAX MF1). Platinum plate electrodes (40 mm long) were mounted parallel on each side of the bath and separated by 4 mm. These electrodes were designed to extend beyond the entire length of the relatively long sternomastoid fibres as well as the shorter soleus fibres. The muscle bath was mounted on to a temperature controlled water jacket. Water temperature was thermostatically controlled by a Peltier element (thermomix 1420). Solutions were passed through small coiled tubing located in the water jacket and applied to the muscle bath through the solution inlet. A thermistor, calibrated immediately prior to each experiment, was placed close to the preparation, next to the solution outlet.

The muscle bath and thermal water jacket were mounted on to an air table (Vibraplane Kinetic system, model 1201-01-12, Boston) and surrounded by a Faraday cage, to reduce electrical noise. Spring-loaded clamping forceps, attached to a micromanipulator, were mounted perpendicular to the bath at the solution inlet end. A piezo-resistive force transducer (AME, A 857, 0-6 g, Horton, Norway) was mounted at the opposite end of the bath. The force transducer was connected to an amplifier. Contractions were viewed on a cathode ray oscilloscope (model RM 565, Beaverton, Oregon, USA or Hitachi V202) and simultaneously recorded with a chart recorder (Hewlett Packard 7402A). Force records selected for further analysis were transferred via an A to D converter (LS1-11 ANALOGUE SYSTEM AXV-11) to a laboratory computer (DIGITAL PDP11/03) and stored on floppy disks.

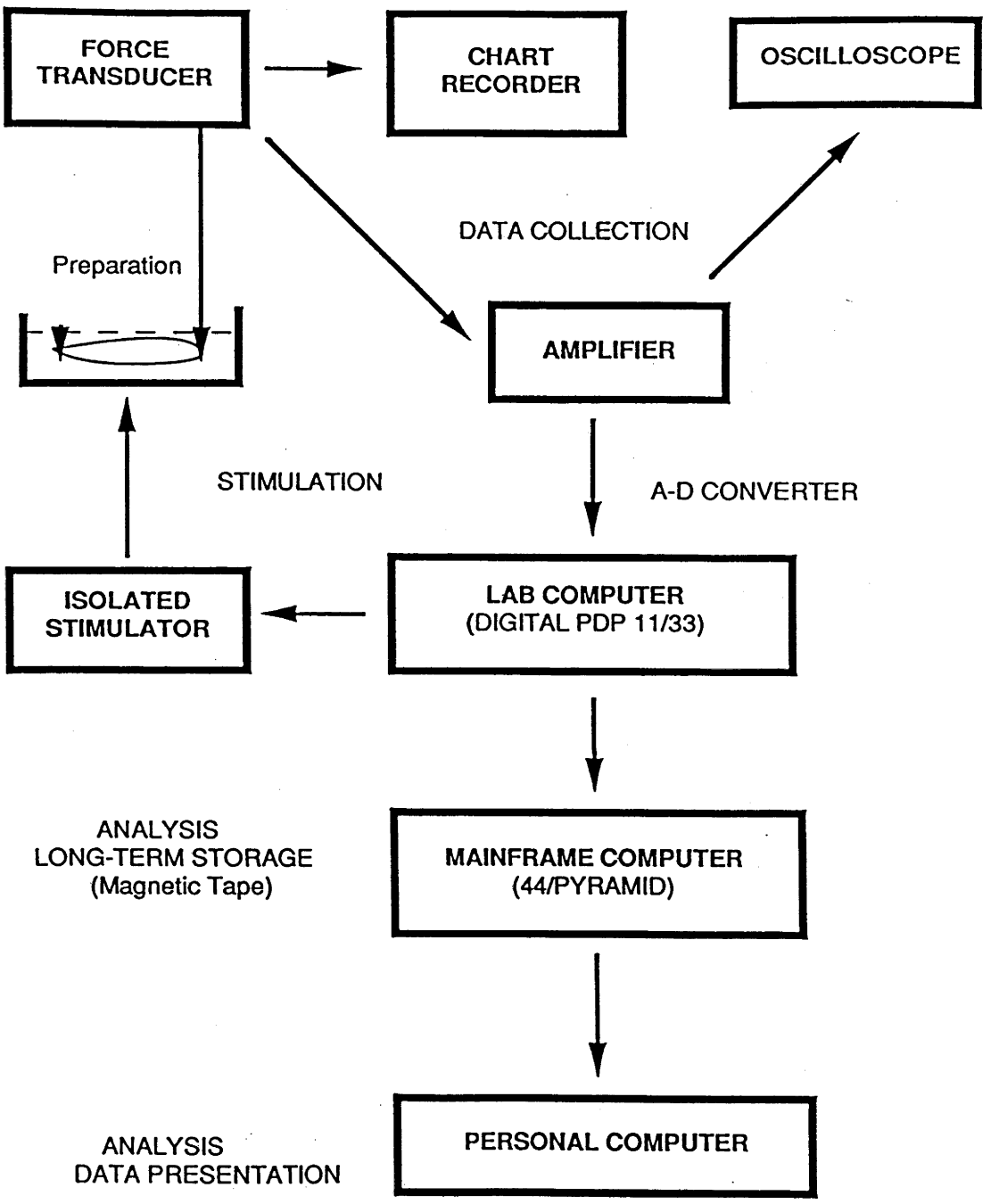


FIG. 2.1: Diagram illustrating the main features involved in the stimulation, recording and analysis of force in contraction studies.

Stimulation patterns were initiated from the laboratory computer using software previously developed for this laboratory (by R. Malbon, J.C.S.M.R., A.N.U.). This program was capable of initiating twitches and tetani at different stimulation frequencies, durations and delays. The pattern of stimulation was transmitted to the platinum electrodes via an isolated Grass stimulator (Type 2533) which was used to set the voltage pulse parameters. The general features involved with stimulation and the recording of force are shown in Figure 2.1.

2.4 EXPERIMENTAL PROTOCOL

Preparations were mounted horizontally to their long axis in the muscle bath, ensuring they were fully immersed in solution and was not contacting the sides of the bath. One tendon was fixed between the clamping forceps and the other tendon attached via a small stainless steel hook to the force transducer. The bathing solution was maintained at 24.0 ± 0.5°C unless otherwise stated. This temperature was chosen in an attempt to minimize the run-down of force seen at higher temperatures (Segal & Faulkner 1985). Isometric contractions were evoked by massive transverse electric field stimulation applied via platinum electrodes. Contraction was thus initiated by a uniform surface membrane action potential. Curare was usually not used. Pulse parameters were adjusted to ensure supramaximal stimulation for the twitch. Rectangular voltage pulses were set at a duration of 0.5 ms and at 1.1-1.3 X, the amplitude that first produced the maximum twitch force response. The length of the preparation was adjusted to that optimal for twitch force. From a relatively slack length, the fibres were stretched by fine adjustment of a micromanipulator attached to the clamping forceps until there was no further increase in peak twitch force with further stretch. The pulse parameters were checked again before the experiment commenced.

Isometric Contractions

Following equilibration, when stable reproducible twitches and tetani were produced, some basic contractile properties were measured (see Chapter 2.7, Tables 2.2 and 2.3). Fused tetani were induced at a stimulation frequency of 100 Hz in all types of preparation at 24°C. The frequency required to produce the maximum force in each preparation depended on the muscle fibre-type composition, the presence or absence of innervation, the bathing solution and the temperature. At higher than the optimal frequency, peak tetanic force was often depressed (Chapter 5.3e). The stimulation duration was adjusted to ensure peak tetanic force had been reached. At 100 Hz the duration was usually 1 s in normal and denervated soleus fibres and 250-300 ms in sternomastoid fibres. In fatigue studies, the preparations were tetanically stimulated for a duration in which force was reduced to less than 50% of peak tetanic force. This ranged from 1-2 s in sternomastoid fibres to 30 s in denervated soleus fibres.

Preparations that displayed a rapid run-down of peak tetanic force, i.e. > 0.5% per min, or large force undershoots following tetani were regarded as being unhealthy and were rejected.

Potassium Contractures

K-contractures were induced by rapidly exposing the preparation to solutions containing high [K^+] (60K or 200K, Table 2.1). These solutions were applied by gravitational flow via polystyrene tubing at a maximal rate of 2 ml/s. A high [K^+] solution was applied until contracture force had just started to decline from its peak (i.e. at the start of mechanical inactivation). It was then washed out with the control Solution 1. The contracture was not due to the higher ionic strength of 10K, 60K, 200K than Solution 1 since all high [K^+] solutions had the same ionic strength and 10K did not produce a contracture. K-contracture amplitude was normalized to peak tetanic force to eliminate variations due to preparation size. Following wash-out of high [K^+] solutions, no further contractures were induced until peak tetanic force had recovered to a steady force level close to the pre-contracture force. Normalized K-contracture force was usually reproducible to within 5% for a given preparation. 200K normally produces maximal contractures in normal and denervated soleus fibres (Chua & Dulhunty 1988). In the present study the 200K-contracture amplitude was close to or greater than peak tetanic force. However, it was important that at least one diameter of the preparation was only one or two fibres thick to achieve this ratio.

Drug Administration

Drugs were usually added to solutions just prior to commencement of experiments. Drugs were administered to the fibres by bath application using either (i) gravitational flow as for K-contractures, via the water jacket or directly into the muscle bath, or (ii) glass micropipettes when rapid exposure to drugs was not required.
Concentration-response relations were tested in two ways. Drugs that did not readily wash-out or caused tachyphylaxis (i.e. smaller responses on successive applications) were added cumulatively whereas other drugs were added in a random order.

Run-down

A problem with mechanical studies in isolated mammalian preparations is that of a run-down of force with time. Run-down was the fastest in the sternomastoid, slower in the normal soleus and the slowest in the denervated soleus preparations. It is unlikely that run-down was due to the presence of physically damaged fibres or the loss of individual fibres as discrete steps of force loss would have been expected, especially in the smaller bundles, but this was not seen. Preparation thickness, bubbling the solution

with 100% O_2 and a temperature of 24°C were all optimised to reduce force loss due to hypoxia (Goldberg et al. 1975; Segal & Faulkner 1985). The ratio of 200K-contracture force to peak tetanic force stayed much the same during run-down indicating that the action potential is not impaired. Run-down may be related to the use-dependent decline in voltage activated force seen in skinned fibres and attributed to an impairment of E-C coupling *in vitro* (Lamb & Stephenson 1990a).

2.5 MEMBRANE POTENTIAL STUDIES

Standard glass microelectrodes (tubing from Clark Electromedical Instruments), filled with 2.5 M potassium chloride and with tip resistances of 5-8 MΩ were used. Muscle preparations were mounted in a Perspex bath lined with Sylgard (Dow Corning), at approximately resting length, in Solution 1 at 22 ± 1°C. Individual surface fibres were impaled only once to avoid depolarization, seen in preliminary experiments, when fibres were impaled several times. Membrane potential was measured using standard procedures, as the potential difference between a microelectrode in a fibre and a reference microelectrode in the bathing solution adjacent to the preparation. The signal from the microelectrode was fed into an amplifier and displayed simultaneously on a digital voltmeter and an oscilloscope (TEKTRONIX S223 digitizing oscilloscope).

Individual fibres were impaled by fine adjustments with a micromanipulator. Only those fibres displaying a sharp increase in membrane potential on microelectrode penetration, a stable membrane potential, and less than a 2 mV potential difference on microelectrode withdrawal were regarded as viable. Fibres with a resting potential of less than -70 mV were regarded as depolarized and were not used. The stable potential following the initial penetration was recorded and taken to be the membrane potential for that fibre. Impalement of the microelectrode deeper into the fibre usually increased the potential by 5-15 mV. However, this was thought to be due to placement of the microelectrode tip into the myofilament lattice where the ion concentrations are different from that of the myoplasm (Stephenson et al. 1981). Membrane potentials were recorded in different fibres by moving the intracellular microelectrode laterally across the preparation, in small steps, and ensuring that the same fibre was not impaled twice.

2.6 DATA ANALYSIS AND PRESENTATION

The general scheme for data analysis, storage and presentation is shown in Figure 2.1. Data files, recorded in digital form on floppy disks on the laboratory computer, were transferred to mainframe computers (44/Pyramid) for analysis. Software (developed by E. Elekessy, J.C.S.M.R., A.N.U.) was used to graphically display each force record and then measure contractile properties such as force amplitude, time-course and fatigue

parameters (see Chapter 2.7). Data were acquired as one point every 0.5, 1 or 2 ms when time-course measurements were required and every 10 or 30 ms when only peak force or fatigue measurements were required. Files were transferred to magnetic tapes or cartridges for long term storage. Selected files were transferred to a personal computer (Fox 2010M, 16BIT) and converted to analogue form. These data files were loaded onto macros, developed using Lotus 123 software, which allowed measurement of some additional contractile parameters. The macro was also used to recreate, normalize and superimpose force traces for graphical presentation. Final graphs were produced with Sigmaplot software and plotted using a Hewlett Packard plotter or laser printer. Tables and diagrams were produced on a MacIntosh computer using MacDraw and Microsoft Word 4.0 software. All wordprocessing was performed using Microsoft Word 4.0. on personal computers.

Drug-induced effects on force were expressed relative to the immediate preceding control force record. Small systematic errors could occur with this measurement due to force run-down. However, a post-wash control was not used to calculate an average control value because it was often difficult to distinguish between drug wash-out and force run-down. To best quantify the effect of a drug on peak force the value for each preparation was taken as that for the first application of the drug. This was to avoid cumulative drug-induced effects or tachyphylaxis. When investigating non drug-induced effects such as with changes in frequency or solutions, the average response for the preparation was used. Statistical analysis was performed using Lotus 123. Some statistics (analysis of variance with combined paired and unpaired data) was performed by Mr. R. Cunningham, Department of Statistics, A.N.U..

2.7 DEFINITIONS

Contractile properties were measured at the optimal fibre length for the twitch, using supramaximal stimulation (0.5 ms pulse duration, 1.1-1.3 X the pulse strength required to produce maximum peak twitch force) at 24°C, unless stated otherwise in the text. The following definitions and methods of measurement were used:

Force Parameters:

Peak twitch force (TW): The difference in amplitude between the resting force, recorded at the onset of stimulation, and the peak isometric force resulting from a single stimulus.

Peak tetanic force (TET): The difference in amplitude between the resting force, recorded at the onset of stimulation, and the peak isometric force resulting from a train

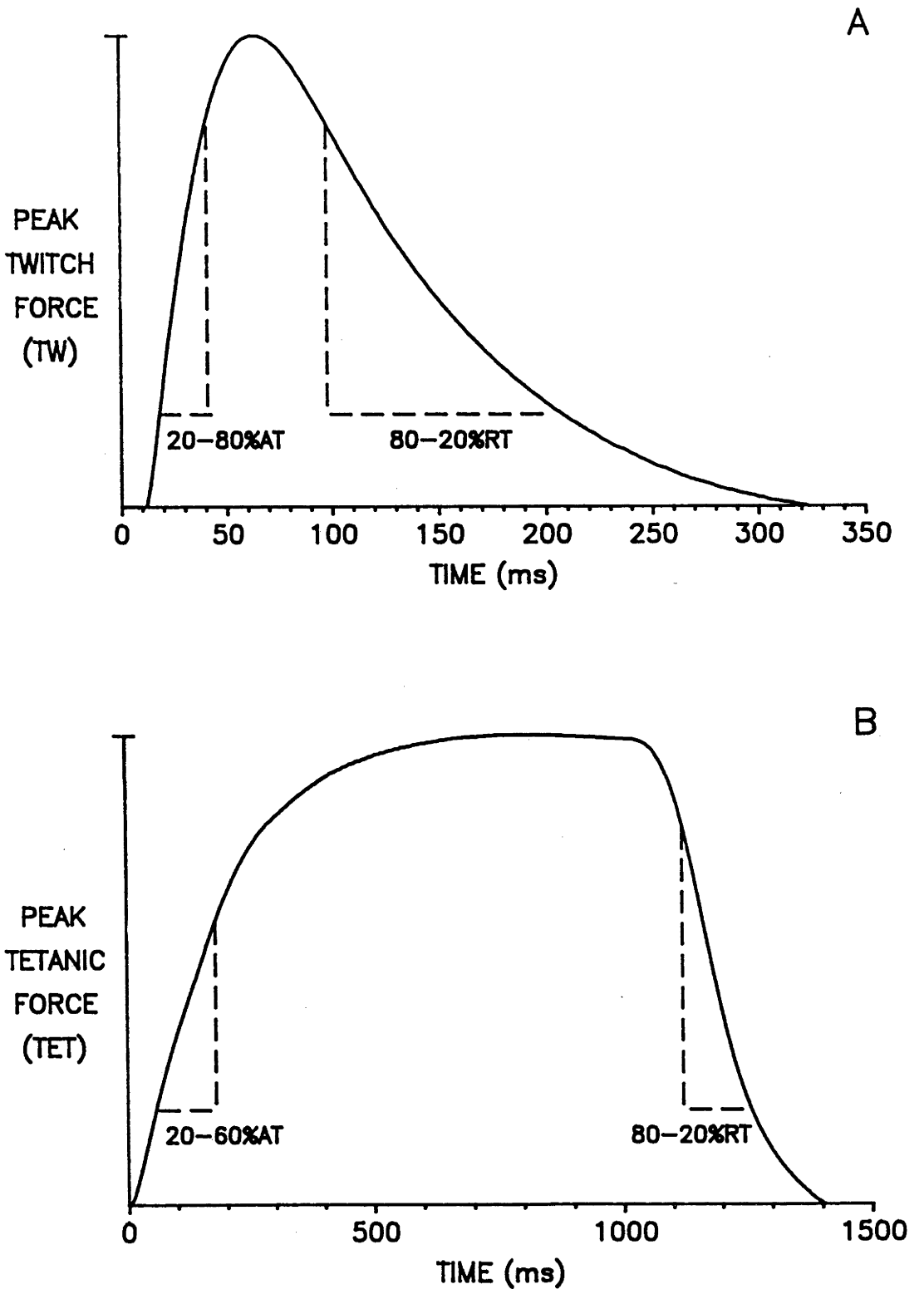


FIG. 2.2: Force records showing (A) twitch and (B) tetanic contractions (100 Hz for 1 s) in a normal soleus preparation. The contractile properties measured were: TW; TET; 20-60%AT; 20-80%AT; 80-20%RT (defined in the text).

TABLE 2.2: Basic contractile properties in preparations composed of different fibre-types

CONTRACTILE PROPERTY		White-Sternomastoid	Red-Sternomastoid	Normal Soleus
Twitch	20-80%AT (ms)	21.0 ± 3.0 * (n = 28)	19.5 ± 0.5 * (n = 13)	53 ± 2 (n = 18)
	80-20%RT (ms)	43.0 ± 2.5 (n = 39)	58.5 ± 4.5 (n = 16)	157 ± 5 (n = 42)
Tetanus	20-60%AT (ms)	27.5 ± 2.5 (n = 15)	41.0 ± 5.0 (n = 14)	84 ± 4 (n = 22)
	80-20%RT (ms)	35.0 ± 2.5 (n = 17)	70.0 ± 9.5 (n = 7)	154 ± 7 (n = 33)
TET/TW		8.9 ± 0.8 ** (n = 17)	10.1 ± 0.8 ** (n = 9)	14.1 ± 0.6 (n = 49)

Shown are the mean values (± s.e.m.), n = number of preparations. The contractile properties (20-80% rise time (20-80%AT), 20-60% rise time (20-60%AT), 80-20% relaxation time (80-20%RT), tetanus-to-twitch ratio (TET/TW)) were measured as described in the text. Statistical differences between the different types of preparation were examined by unpaired *t*-test. $P < 0.02$ except for * and ** where $P > 0.1$.

TABLE 2.3: Basic contractile properties in preparations from normal and denervated soleus muscles

CONTRACTILE PROPERTY	NORMAL SOLEUS	DENERVATED SOLEUS				
		2W	3W	4W	5-6W	8-9W
Twitch						
20-80%AT (ms)	53 ± 2 (n = 18)	30 ± 2 (n = 7)	40 ± 4 (n = 25)	---	44 ± 4 (n = 20)	26 ± 2 (n = 12)
80-20%RT (ms)	157 ± 5 (n = 42)	147 ± 12 (n = 6)	134 ± 6 (n = 31)	141 ± 7 (n = 17)	123 ± 7 (n = 23)	108 ± 6 (n = 13)
Tetanus						
20-60%AT (ms)	84 ± 4 (n = 22)	35 ± 3 (n = 6)	40 ± 2 (n = 15)	36 ± 2 (n = 17)	41 ± 4 (n = 8)	57 ± 5 (n = 10)
80-20%RT (ms)	154 ± 7 (n = 33)	101 ± 8 (n = 6)	101 ± 3 (n = 31)	103 ± 4 (n = 16)	99 ± 5 (n = 15)	76 ± 4 (n = 12)
TET/TW	14.1 ± 0.6 (n = 49)	2.5 ± 0.2 (n = 7)	3.1 ± 0.1 (n = 32)	2.8 ± 0.1 (n = 17)	3.1 ± 0.1 (n = 20)	3.2 ± 0.1 (n = 13)

Shown are the mean values (± s.e.m.), n = number of preparations. The contractile properties were measured as described in the text. W indicates the weeks of denervation.

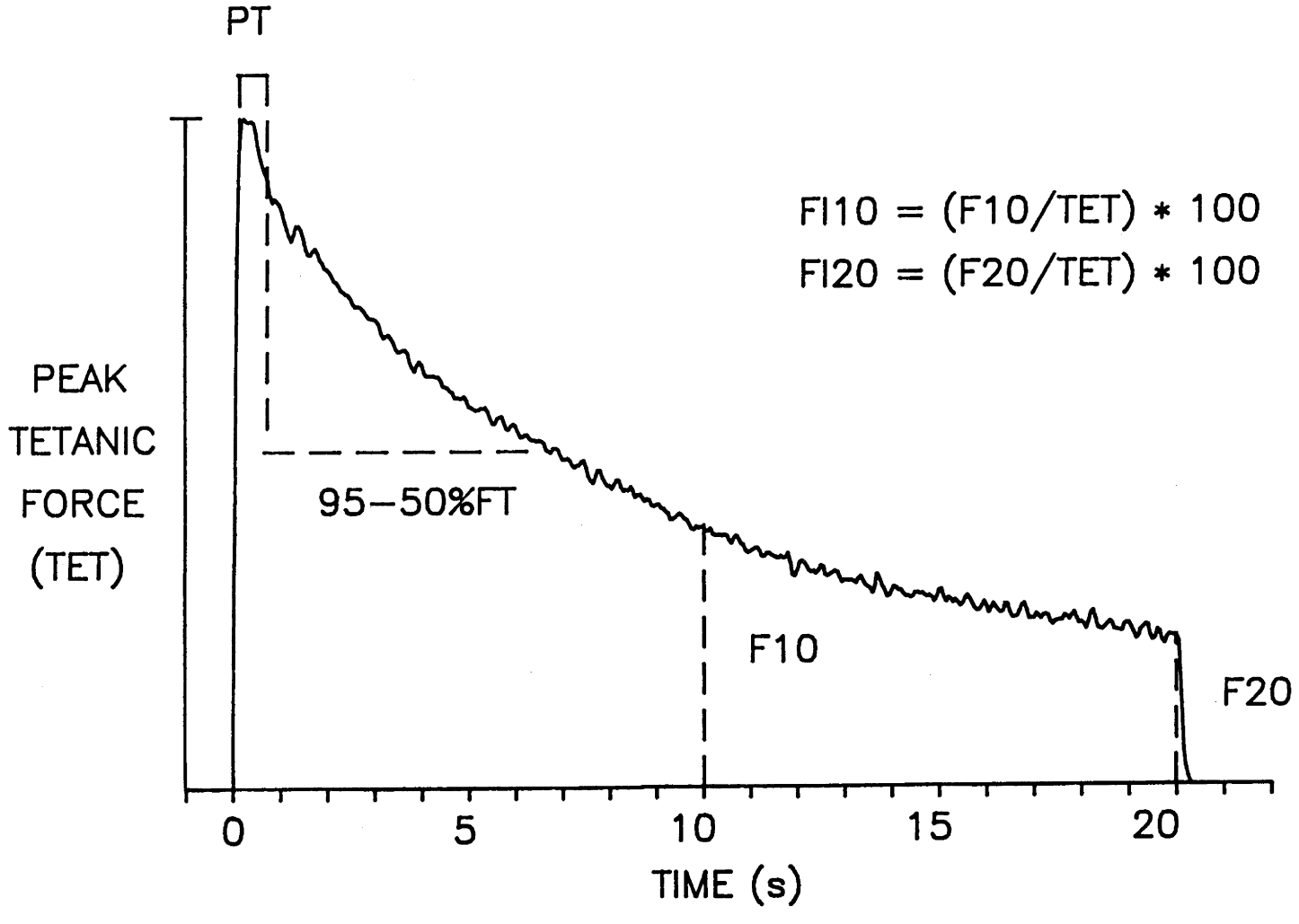


FIG. 2.3: Force record showing high-frequency fatigue induced by continuous stimulation at 50 Hz for 20 s in a denervated soleus preparation. The contractile properties measured were: PT; 95-50%FT; FI_{10} and FI_{20} (defined in the text).

of high frequency stimuli. The frequency used was 100 Hz or as otherwise defined in the text.

Tetanus-to-twitch force ratio (TET/TW): The ratio of peak tetanic force (100 Hz) to the peak force for the preceding twitch, with twitch stimulation at 0.067 Hz.

Force was expressed in units of mN. Some calculations of specific force (force per cross-sectional area) were made. Cross-sectional area was determined from bundle diameters (Dulhunty & Gage 1973). Values for specific peak tetanic force (200-300 mN/cm^2) were similar to those reported previously indicating that the preparations were healthy (Dulhunty & Dlutowski 1979; Dulhunty 1985; Luff 1985). Some values were up to 50% lower which may have resulted from an overestimation of cross-sectional area due to spaces between fibres within these bundles.

Time-course Parameters:

20% to 80% rise (or activation) time (20-80%AT): The time for force to rise from 20% to 80% of peak force, recorded for the twitch (units ms) (Figure 2.2A).

20% to 60% rise (or activation) time (20-60%AT): The time for force to rise from 20% to 60% of peak tetanic force (units ms). This is the early faster phase of force development prior to a slower gradual climb to a plateau (Figure 2.2B).

These rise time parameters were chosen instead of the "time to peak twitch force" and the "maximum rate of rise" for the tetanus because their method of measurement are clearly defined and reduces possible systematic errors associated with the other rise time parameters.

80% to 20% relaxation time (80-20%RT): The time for force to decay from 80% to 20% of peak or end stimulation force following the cessation of stimulation (units ms).

Figure 2.2 shows twitch and tetanus records from a soleus preparation and how the contractile properties were measured. Average peak force and time-course parameters for preparations composed of different fibre-types and from denervated muscles are shown in Tables 2.2 and 2.3.

Fatigue Parameters:

High-frequency fatigue (HFF) refers to any decline of peak tetanic force induced by prolonged continuous tetanic stimulation at a constant frequency.

Fatigue index (FI_*): The force generated at a stimulation duration of $*$ seconds expressed as a percentage of peak tetanic force (e.g. FI_{10} = force at 10 s/ peak tetanic force x 100).

Plateau time (PT): The time from 95% of peak tetanic force on the rising phase to 95% of peak tetanic force on the falling phase (units ms).

95% to 50% fatigue time (95-50%FT): The time for force to decline from 95% to 50% of the peak force generated during continuous tetanic stimulation at constant frequency (units ms).

Figure 2.3 illustrates how the fatigue parameters were measured in a denervated soleus preparation.

CHAPTER 3

EFFECTS OF β-ADRENOCEPTOR ACTIVATION ON CONTRACTION IN NON-FATIGUED SKELETAL MUSCLE

3.1 INTRODUCTION

It is well established that catecholamines increase the force of contraction (positive inotropic effect) and accelerate relaxation in cardiac muscle (Tsien 1977; Katz 1979; Kurihara & Konishi 1987; Endoh & Blinks 1988). Most of this force potentiation and all of the acceleration of relaxation are thought to follow activation of beta-(β)-adrenoceptors and are mediated by an increased concentration of myoplasmic cyclic-adenosine monophosphate ($[cAMP]_i$) (Tsien 1977; Handa et al. 1982; Scholtz 1986; Endoh & Blinks 1988). In addition, activation of alpha-(α)-adrenoceptors can induce small increases in force, without an acceleration of relaxation (Brückner et al. 1978; Endoh 1986; Endoh & Blinks 1988), due to a cAMP-independent mechanism (Brückner et al. 1978; Handa et al. 1982; Brückner & Scholz 1984; Endoh 1986).

Since the pioneering work of Oliver and Schäefer (1895), many studies have confirmed that catechol- and other sympathomimetic-amines can also modulate the contraction of mammalian skeletal muscle (for reviews see Bowman & Nott 1969; Tomita 1975; Bowman 1980; Williams & Barnes 1989b). The contractile response to sympathomimetic-amines depends on the fibre-type composition of the muscle studied. In fast-twitch muscles, sympathomimetics induce positive inotropic responses. Peak twitch force is increased by 10-20% and twitch relaxation is slowed (Brown et al. 1948; Goffart & Ritchie 1952; Bowman & Zaimis 1958; Bowman & Raper 1964; Tashiro 1973). The peak force of unfused tetanic contractions may be increased by up to 60% and there is an increase in the degree of fusion (Bowman & Zaimis 1958; Holmberg & Waldeck 1977; Bowman et al. 1985). In contrast, slow-twitch muscles usually display negative inotropic responses to sympathomimetics. Peak twitch force is depressed by 10-25% and there is an acceleration of twitch relaxation (Bowman & Zaimis 1958; Tashiro 1973; Bohmer & Raper 1976; Olin 1987). The peak force of unfused tetani is decreased by up to 60% and there is a reduction in the degree of fusion (Bowman & Nott 1970; Waldeck 1977). However, under some conditions slow-twitch muscles respond with an increase in force (Tashiro 1973; Tomita 1975; Holmberg & Waldeck 1980). Sympathomimetics have little or no effect on peak tetanic force in either type of muscle *in vivo* (Goffart & Ritchie 1952; Bowman & Zaimis 1958; Lewis & Webb 1976; Bowman & Nott 1970; Marsden & Meadows 1970) and *in vitro* (Juel 1988).

It is generally accepted that skeletal muscles, except possibly for the extraocular muscles (Bach-y-Rita & Ito 1966), do not receive any direct sympathetic innervation

(Bowman 1980). However, adrenoceptors are present in the surface and T-tubule membranes (Caswell et al. 1978; Grefrath et al. 1978) allowing responses to circulating catecholamines or to noradrenaline released from sympathetic nerves to skeletal muscle blood vessels. These receptors are predominantly of the β_2-adrenoceptor subtype (Hirata et al. 1986; Elfellah & Reid 1987). Consequently terbutaline, a selective β_2-adrenergic agonist (Bergman et al. 1969), was chosen as the principal drug for this study.

The main aims of this study were: (i) to systematically quantify the effects of terbutaline on isometric contractile properties of different types of rat skeletal muscle *in vitro*; (ii) to investigate any modifying influence of denervation on these responses; (iii) to establish the pharmacological mechanisms responsible for such changes, especially with regards to the role of cAMP.

3.2 METHODS

The muscle preparations and methods for mechanical studies are described in Chapter 2. Preparations were usually bathed in Solution 1 (Table 2.1) at 24°C. In a few experiments, the temperature of the bathing solution was either raised to 31°C or normal Krebs solution was used, as indicated in the text. All tetani were evoked by direct stimulation at 100 Hz for a duration that ensured peak force had been reached. This duration was usually 1 s in normal and denervated soleus fibres or 250-300 ms in sternomastoid fibres. Some data are included following tetanic stimulation for up to 20 s (Table 3.1).

Drugs were applied to the preparation via the bathing solution. Contractions were monitored on a chart recorder until the maximum drug-induced effect was seen and the drug was then washed out with control solution. The contractile properties measured were: peak twitch and tetanic force; 80-20% relaxation time (80-20%RT); 20-80% rise time for twitches (20-80%AT) and 20-60% rise time for tetani (20-60%AT). Maximum drug-induced effects on peak force or rise times are expressed as a ratio or percentage change relative to the immediately preceding control. Effects on relaxation times are expressed as the ratio of the maximum drug-induced response to the average of controls obtained immediately prior to drug administration and following wash-out. This was required to account for a time-dependent prolongation of relaxation times with the duration of the experiment, observed in preliminary experiments. Values presented in the text are the mean (± s.e.m.) and n is the number of preparations used. Statistical tests included ANOVA (using combined unpaired/paired data) and Students *t*-test.

Drugs used were: (i) terbutaline sulphate (kindly donated by ASTRA pharmaceuticals); (ii) adrenaline hydrogen tartate (BDH chemicals Ltd); (iii) N^6,-2'-O-dibutyryl

adenosine 3':5'-cyclic adenosine monophosphate sodium salt (DBcAMP) (Sigma); (iv) DL-propranolol hydrochloride (Sigma); (v) n-butyric acid sodium salt (Sigma); (vi) ethylenediaminetetra-acetic acid sodium salt (EDTA) (AJAX); (vii) D-tubocurarine chloride (Boehringer). Terbutaline, adrenaline, DBcAMP and propranolol were all stored in anhydrous form in light-resistant containers at 4°C. EDTA (50 μM) was added to all solutions to prevent the oxidation of sympathomimetic-amines (Carmon 1982). EDTA had no direct effect on force. The contractile responses to terbutaline were independent of the type of anaesthetic used to kill the experimental animals. This was important as halothane has been shown to prevent some actions of adrenaline (Kendig & Bunter 1972).

3.3 RESULTS

(a) Effects of terbutaline on isometric contractions in soleus muscle

Terbutaline (10 μM) consistently potentiated the peak force of isometric twitches and tetani evoked by direct stimulation in the slow-twitch soleus preparation. Contractures (increases in resting force) were not observed. Figure 3.1A shows a typical twitch response to terbutaline. The average increase in peak twitch force was 14.6 ± 2.9% (n = 15) (Table 3.1). There was a considerable variation in the magnitude of this response (SD = 11.0%, range 0-35.2%). Two preparations failed to show twitch potentiation although their tetani were potentiated. Twitch potentiation was time-dependent and reversible. Maximum twitch potentiation was usually seen 5-15 min after the drug was first applied and the effect washed out in 10-15 min. Holmberg and Waldeck (1980) reported that terbutaline induced a biphasic time-dependent twitch response, with an initial depression being followed by a potentiation of peak twitch force. An initial depression was observed in three preparations but it was never more than a 4% decrease and reversed into an augmentation by 5 min.

Figure 3.1B shows a typical potentiation of peak tetanic force with terbutaline in a soleus preparation. The average increase with terbutaline was 14.6 ± 1.9% (n = 30) as shown in Table 3.1. This was a surprising result as a sympathomimetic-induced potentiation of peak tetanic force of this magnitude has not been seen previously in mammalian skeletal muscle (Bowman 1980). The time-dependence of tetanus potentiation was similar to that for the twitch, with maximum effects seen 5-15 min after the drug was first applied. The maximum force increase by terbutaline was usually maintained until wash-out. The percentage increase in peak twitch and peak tetanic force were not significantly different from each other ($P > 0.1$, ANOVA).

The considerable variation in the potentiation of peak tetanic force with terbutaline is illustrated in the frequency-histogram in Figure 3.2A. This variation could not be

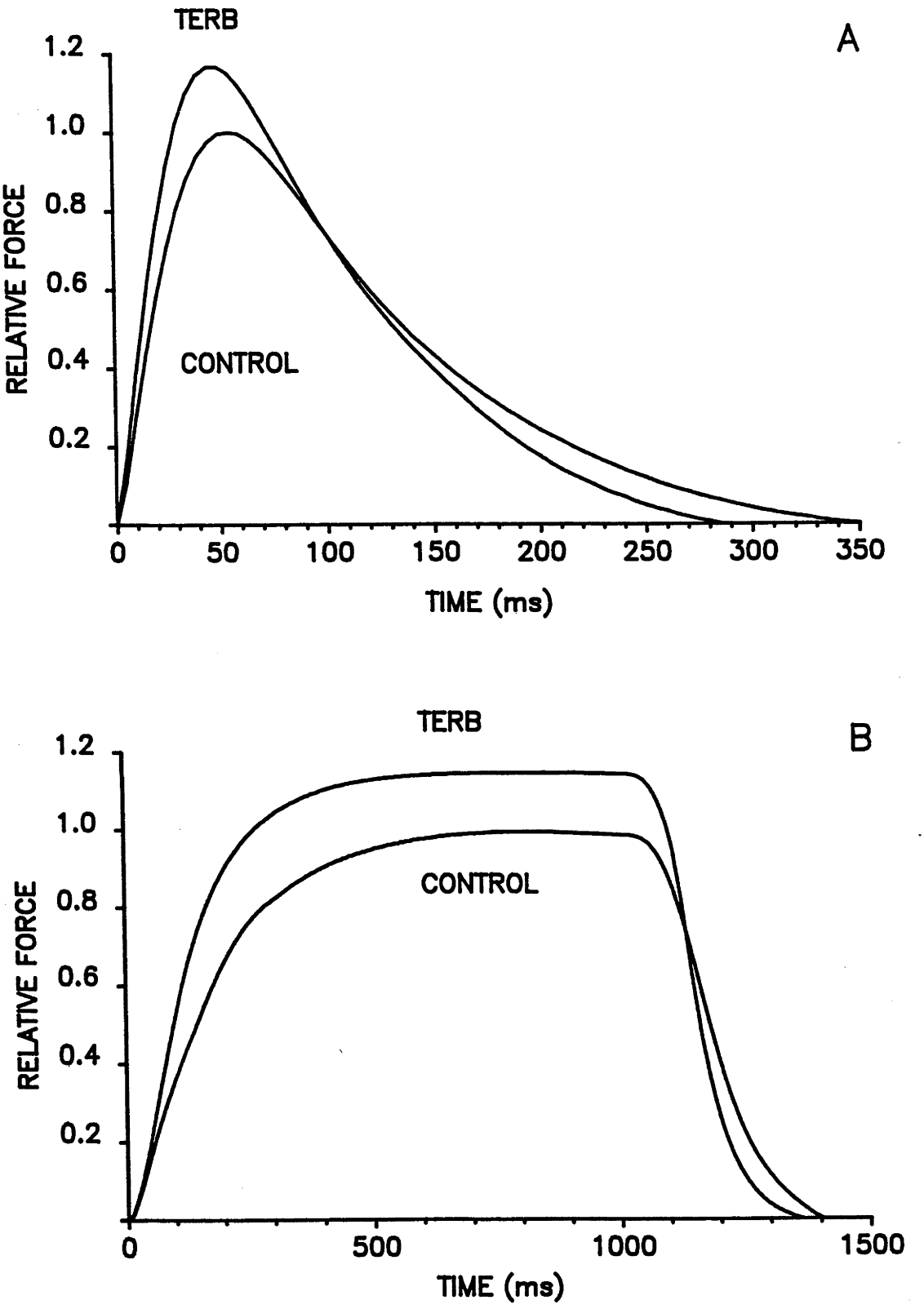
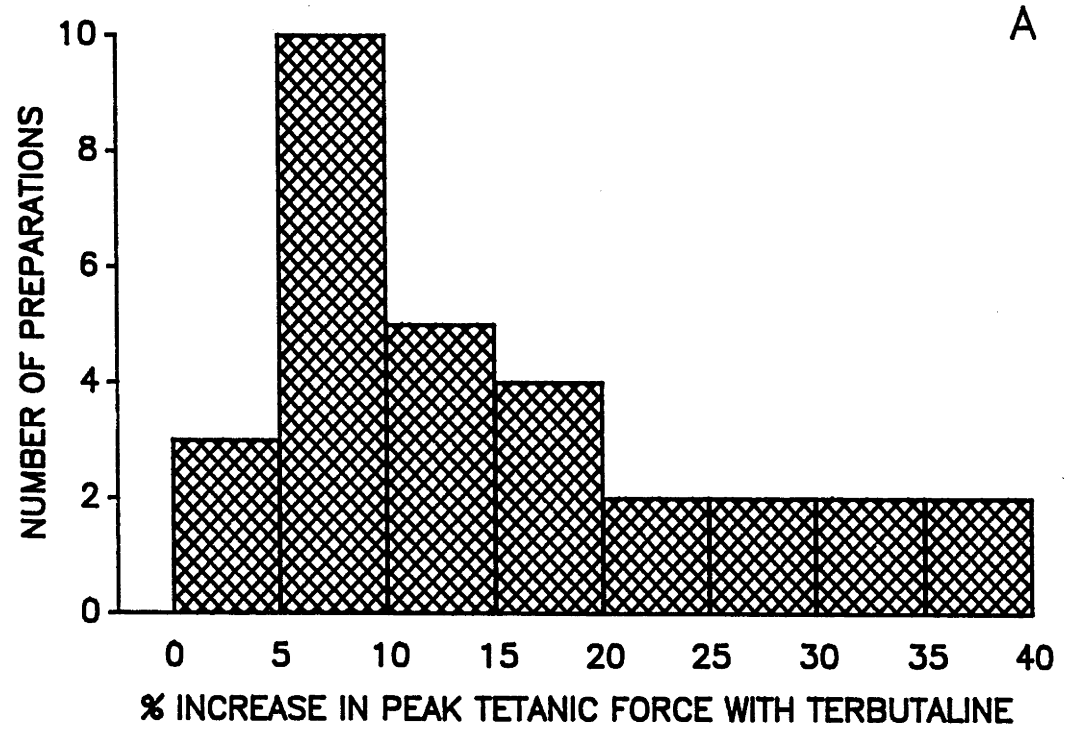


FIG. 3.1: Effects of 10 μM terbutaline on isometric contractions in soleus fibres at 24 °C. (A) twitch force and (B) tetanic force (100 Hz for 1 s). The superimposed records are from individual preparations that show average force potentiation with terbutaline. CONTROL is the force record obtained immediately prior to the addition of terbutaline. TERB is the maximum force response to terbutaline.

TABLE 3.1: Effect of 10 μM terbutaline on peak twitch and tetanic force in preparations composed of different fibre-types

MUSCLE PREPARATION	PEAK TWITCH FORCE (% INCREASE)		PEAK TETANIC FORCE (% INCREASE)	
	mean ± s.e.m.	SD	mean ± s.e.m.	SD
Normal Soleus	14.6 ± 2.9 (n = 15)	11.0	14.6 ± 1.9 (n = 30)	10.5
White-Sternomastoid	7.3 ± 2.1 (n = 8)	5.9 *	_____	
Red-Sternomastoid	7.2 ± 2.8 (n = 5)	6.2 **	_____	
Denervated Soleus	13.7 ± 2.1 (n = 23)	9.9	13.5 ± 1.1 (n = 44)	7.4

The maximum increase in peak twitch or tetanic force with terbutaline was expressed as a percentage increase relative to the immediately preceeding control twitch or tetanus. SD = one standard deviation. n = number of preparations. The value for each preparation was for the first application of terbutaline. Tetani were evoked at 100 Hz. Solution 1, 24°C. Effects of terbutaline were tested by paired *t*-test. For all values $P < 0.001$ except * $P < 0.025$, ** $0.1 < P < 0.05$.



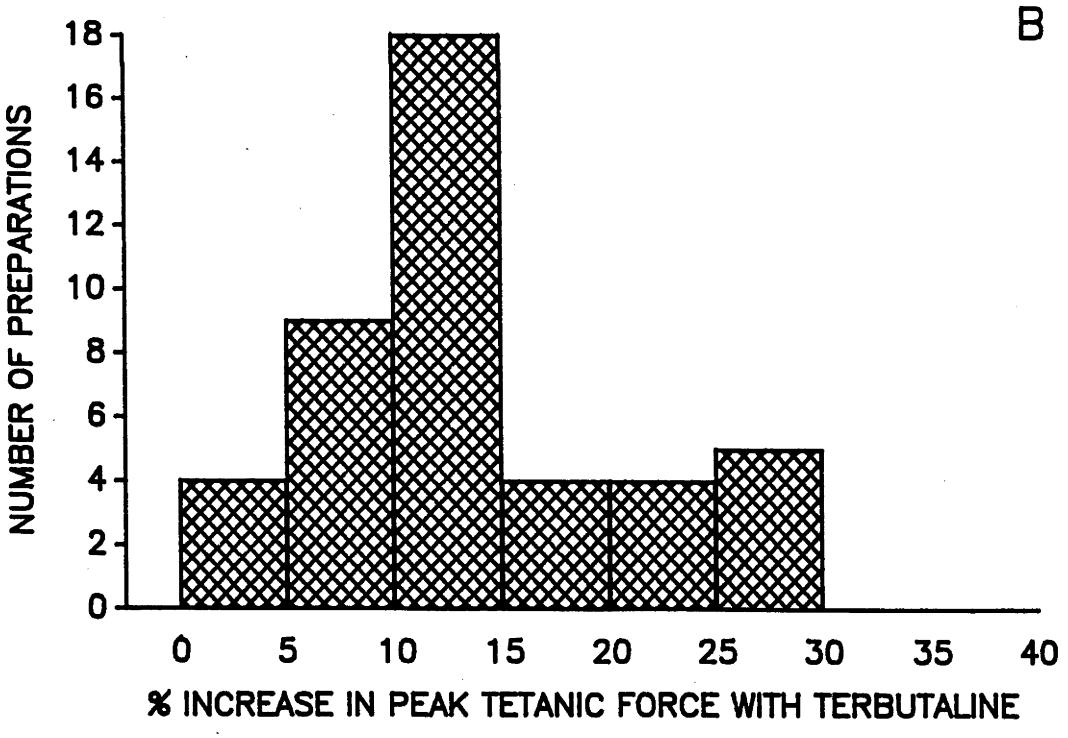


FIG. 3.2: A comparison of the distributions for the effect 10 μM terbutaline on peak tetanic force (100 Hz) in (A) normal soleus and (B) denervated soleus preparations at 24 °C. The magnitude of force potentiation is shown as a percentage increase above control force.

TABLE 3.2: Effect of 10 μM terbutaline on the rate of force relaxation in preparations composed of different fibre-types

MUSCLE PREPARATION		RELATIVE RELAXATION RATE (TERB/CONT)	n	80-20% RELAXATION TIME			
				CONT (ms)	TERB (ms)	DIFF (ms)	
Normal Soleus	- Twitch	1.14 ± 0.07	9	169 ± 15	153 ± 17	-16 ± 7	P <0.05
	- Tetanus	1.32 ± 0.05	20	156 ± 8	119 ± 6	-37 ± 5	P<0.001
White-Sternomastoid	- Twitch	0.89 ± 0.03	8	44.5 ± 3.5	50.0 ± 3.0	5.5 ± 1.5	P<0.01
Red-Sternomastoid	- Twitch	0.93 ± 0.02	5	60.0 ± 11.0	64.0 ± 11.0	4.0 ± 1.0	P<0.05
Denervated Soleus	- Twitch	0.76 ± 0.02	19	114 ± 6	151 ± 9	37 ± 4	P<0.001
	- Tetanus	0.97 ± 0.03	37	92 ± 3	95 ± 3	3 ± 2	P>0.1

Shown are the mean values (± s.e.m.). n = number of preparations. The 80-20% relaxation time (80-20%RT) was measured as described in Chapter 2. The relative relaxation rate was calculated as 1/(80-20%RT), for terbutaline relative to the control. CONT is mean of values obtained immediately prior to and following wash-out of terbutaline. TERB is the maximum effect with terbutaline. DIFF is the difference in the 80-20%RT between TERB and CONT. Tetani were evoked at 100 Hz for 1 s. Solution 1, at 24°C. Effects of terbutaline were tested by paired *t*-test.

explained by submaximal adrenoceptor activation as additional force could not be obtained by increasing the concentration of terbutaline above 10 μM. The magnitude of force potentiation showed no correlation with preparation thickness, age of the animal, season, or the extent of run-down: as seen with isolated cardiac muscle preparations (Kurihara & Konishi 1987). Indeed, the same variation was observed when different preparations were used from the same muscle. A likely explanation of this spread is that of a true biological variation.

Previous mechanical studies on the action of sympathomimetics on slow-twitch muscles have often shown that a maximum force depression occurs in unfused tetani (Bowman & Nott 1970; Waldeck 1977). The effect of 10 μM terbutaline was therefore investigated on unfused tetani evoked at 20 Hz for 1 s, at 31°C (n = 2) or 15 Hz for 1 s, at 24°C (n = 1). All preparations responded with an augmentation of peak force.

Although the muscle fibres were directly stimulated, it was possible that terbutaline augmented force by the known facilitatory action of sympathomimetic-amines on neuromuscular transmission (Bowman & Nott 1969; Tomita 1975; Carmon 1982). The prior addition of 1.5 μM tubocurarine, which completely blocks neuromuscular transmission (Carmon 1982), failed to abolish the potentiation of force by terbutaline (n = 2), suggesting that terbutaline acts directly on the muscle fibres.

Figure 3.1 shows that terbutaline also abbreviated the time-course for twitch and tetanic relaxation in soleus fibres. Terbutaline increased the 80-20% relaxation rate (1/(80-20%RT) by 14 ± 7% (n = 9) and 32 ± 5% (n = 20) for twitches and tetani respectively (see Table 3.2). The effect on tetanic relaxation was significantly greater than that on twitch relaxation ($P < 0.05$, ANOVA). Terbutaline had no significant effect on the rate of development of force for either the twitch or tetanus.

(b) Effects of terbutaline on isometric contractions in sternomastoid muscles

The effect of catecholamines on contraction in the different types of fast-twitch fibre are not well documented. Terbutaline (10 μM) potentiated peak twitch force in both types of fast-twitch sternomastoid preparation as illustrated in Figure 3.3. Peak twitch force was increased on average by 7.3 ± 2.1% (n = 8) and 7.2 ± 2.8% (n = 5) in white- and red-sternomastoid fibres respectively (Table 3.1). There was no significant difference in the magnitude of these effects ($P > 0.1$, ANOVA). Twitch potentiation in the pooled fast-twitch sternomastoid fibres, of 7.3 ± 1.6% (n = 13), was significantly smaller than that in the slow-twitch soleus fibres ($P < 0.05$, ANOVA).

The effect of terbutaline on peak tetanic force in the sternomastoid fibres was difficult to quantify due to a progressive run-down of force, especially with repetitive tetanic

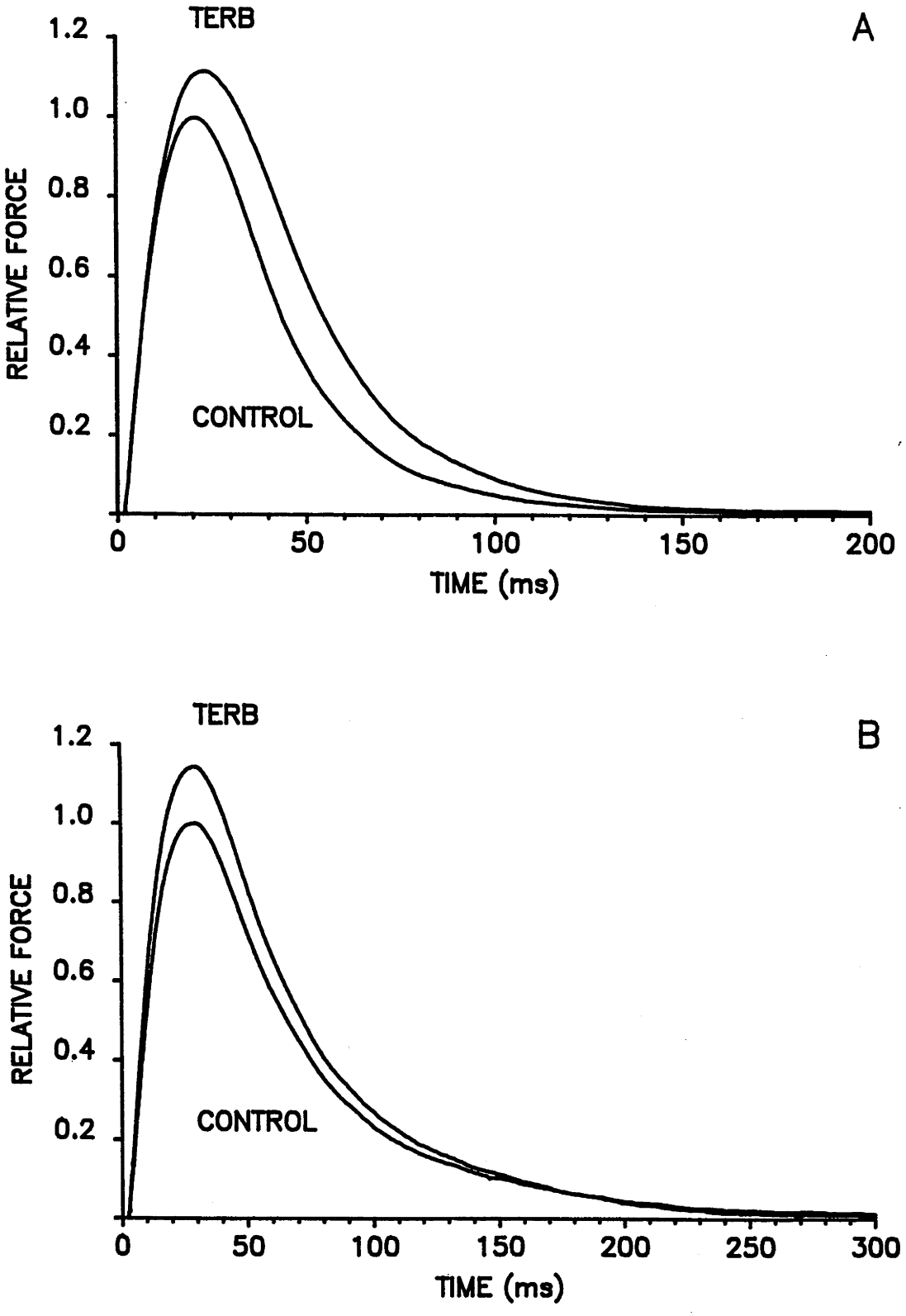


FIG. 3.3: Effects of 10 μM terbutaline on isometric twitches in (A) white-sternomastoid and (B) red-sternomastoid fibres at 24°C. These records are from individual preparations that show larger than average twitch potentiation with terbutaline. Note the prolonged relaxation in A but no change in the relaxation time-course in B. CONTROL and TERB are defined in the legend for Fig. 3.1.

stimulation. Nevertheless, peak tetanic force was increased in two sternomastoid preparations (5.1% and 7.5%) while in other preparations the rate of run-down appeared to be reduced.

Figure 3.3 shows that, in bundles of sternomastoid fibres, terbutaline-induced twitch potentiation was associated with either a prolongation, or no change, in the relaxation time-course. This was in marked contrast to the acceleration of twitch relaxation in soleus. On average, the rate of relaxation decreased by 11 ± 3% (n = 8) and 7 ± 2% (n = 5) for white- and red-sternomastoid fibres respectively. These were changes of only a few milliseconds (Table 3.2).

(c) Effects of terbutaline on isometric contractions in denervated soleus muscle

Following denervation, skeletal muscle becomes supersensitive to acetylcholine (Axelsson & Thesleff 1959). The sensitivity of muscle to catecholamines following chronic denervation has been investigated and contractures have been reported (Bowman & Raper 1965; Yamada & Harigaya 1974; Bohmer & Raper 1975; Evans & Smith 1976). It was of interest to see if terbutaline also produced contractures or if force potentiation was enhanced in denervated soleus muscle.

Terbutaline (10 μM) did not induce contractures. Instead, it induced a small depression of resting force in about 50% of the denervated soleus preparations. This depression was associated with a reduction in the amplitude of fibrillations - asynchronous twitches produced by the spontaneous firing of action potentials in some fibres (Smith & Thesleff 1976; Dulhunty 1985) - that contribute to the resting force in denervated muscle.

Terbutaline modulated contractions evoked by direct stimulation that were superimposed on fibrillations. Figure 3.4A shows a typical twitch response to terbutaline in a denervated soleus preparation. On average, peak twitch force was increased with terbutaline by 13.7 ± 2.1% (n = 23) (Table 3.1) which was not significantly different from the twitch potentiation in normal soleus fibres ($P > 0.1$, ANOVA). Maximum twitch potentiation usually occurred 10-15 min after the drug was first applied and the effect readily washed out. Note that twitch relaxation was markedly prolonged by terbutaline (Figure 3.4A). This was a surprising contrast to the acceleration of relaxation in normal soleus fibres (see Table 3.1).

A representative example of the effect of terbutaline on peak tetanic force in a denervated soleus preparation is shown in Figure 3.4B. Terbutaline potentiated peak tetanic force by 13.5 ± 1.1% (n = 44) and this increase was not different from that in normal soleus ($P > 0.1$, ANOVA). Thus, a change in the sensitivity to terbutaline

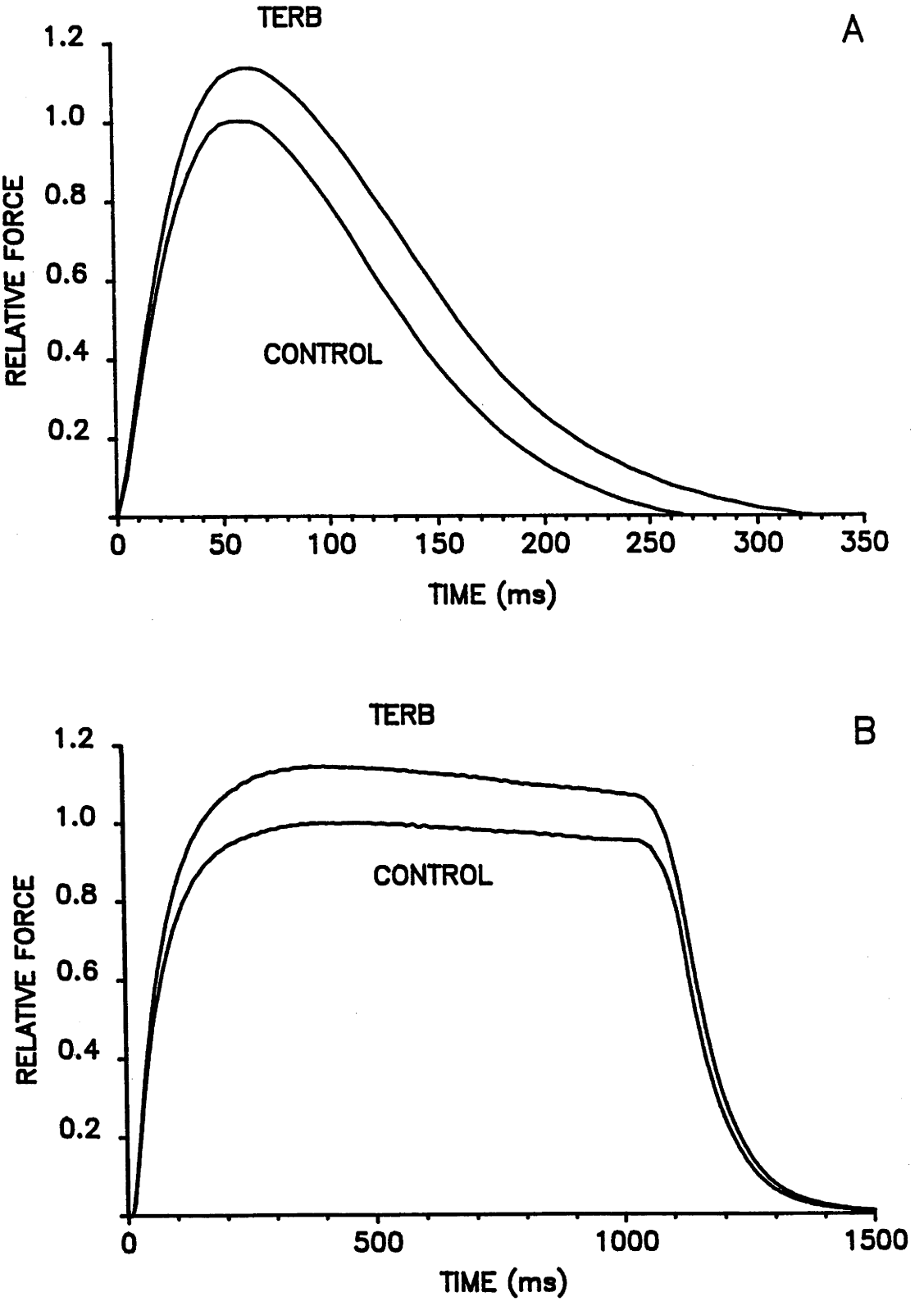


FIG. 3.4: Typical effects of 10 μM terbutaline on isometric contractions in denervated soleus fibres at 24 °C. (A) twitch force and (B) tetanic force (100 Hz for 1 s). CONTROL and TERB are defined in the legend for Fig. 3.1.

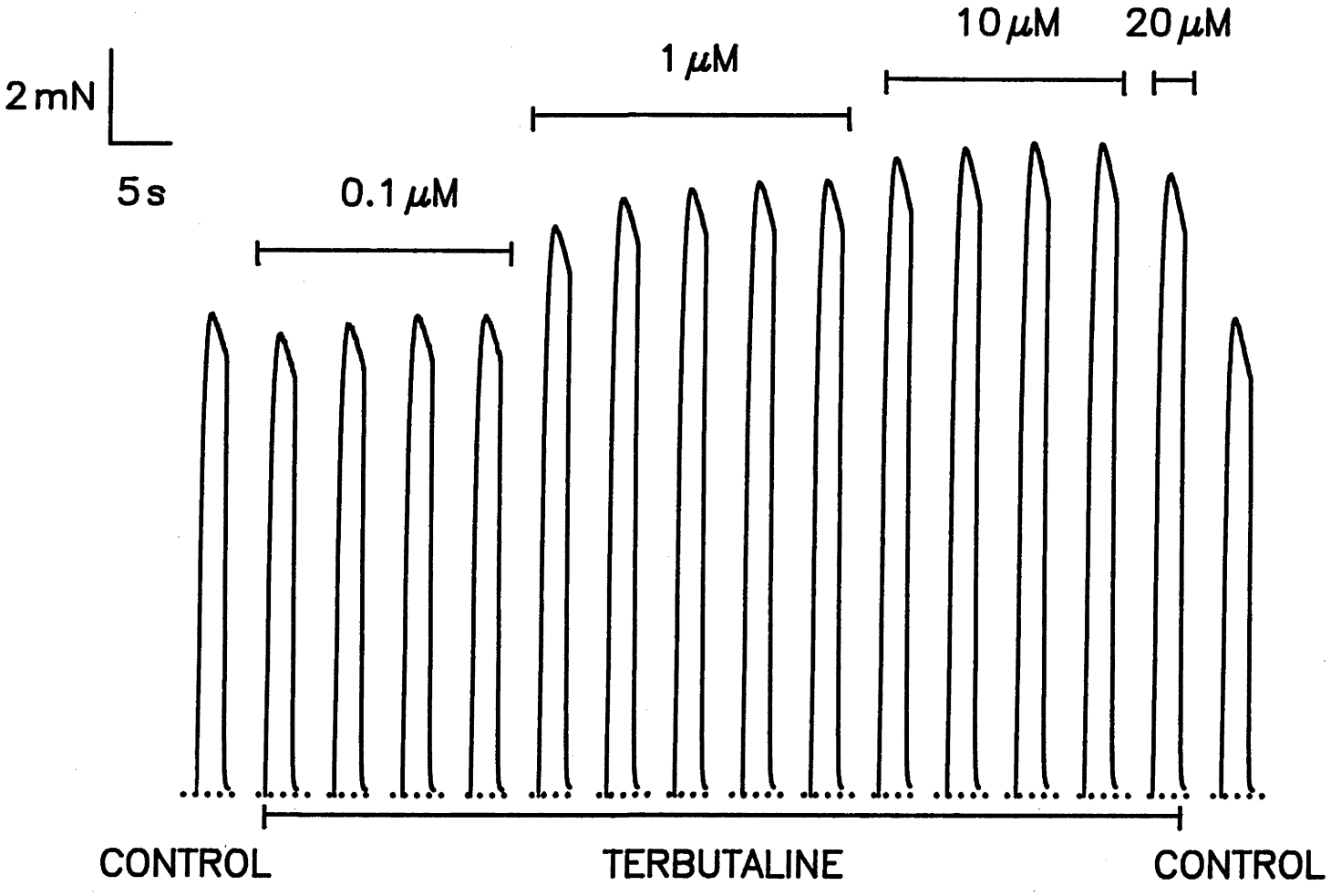


FIG. 3.5: Concentration- and time-dependent effects of terbutaline on peak tetanic force in denervated soleus fibres at 24 °C. Records are from a single preparation showing a large maximum increase of peak tetanic force with terbutaline (35%). Tetani were recorded every 5 min. The preparation was exposed to each concentration of terbutaline until the peak force had reached a steady level and then the concentration was increased as indicated. Tetani were evoked at 50 Hz for 2 s. Resting force is represented by the dots at the base of the traces.

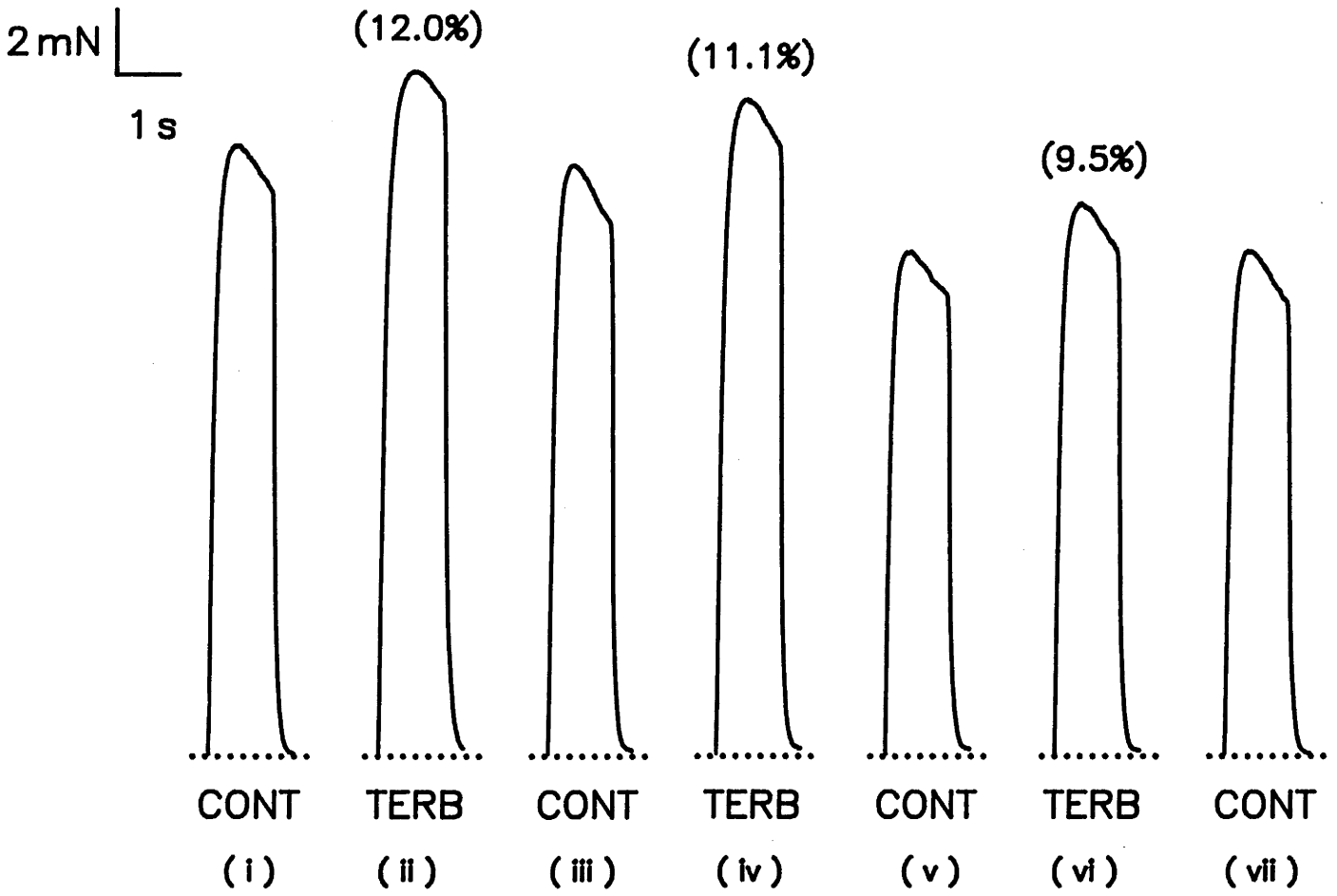


FIG. 3.6: Effect of repetitive application of 10 μM terbutaline on peak tetanic force (100 Hz) in a denervated soleus preparation at 24 °C. CONT is the record obtained immediately prior to each application of terbutaline or following wash-out. TERB is defined in the legend for Fig. 3.1. Terbutaline increased peak tetanic force by 12.0% (ii), relative to the first control (i). The second control was obtained 45 min after wash-out (iii), and then terbutaline increased peak tetanic force by 11.1% (iv). Another experimental intervention followed (not shown) prior to recording the third control 155 min after the second wash-out (v). The final application of terbutaline resulted in a potentiation of 9.5% (vi), and it reversed on wash-out (vii).

following denervation was not reflected in the potentiation of force. The spread in the extent of force potentiation by terbutaline is illustrated in Figure 3.2B. Clearly the distributions for the terbutaline-induced force potentiation were similar for normal and denervated soleus fibres.

Figure 3.5 shows the concentration- and time-dependence for the effect of terbutaline on peak tetanic force in an individual denervated soleus preparation. Qualitatively similar responses were seen in four preparations. Terbutaline (0.1 μM) induced a biphasic force response with an initial depression, followed by an augmentation. Higher concentrations of terbutaline further increased peak tetanic force; the maximum effect was with 10 μM terbutaline.

Figure 3.6 shows that repetitive applications of terbutaline induced quantitatively similar effects on peak tetanic force in a denervated soleus preparation. These effects were independent of the extent of force run-down. In contrast, normal soleus fibres displayed progressively smaller responses with successive applications of terbutaline, a phenomenon known as tachyphylaxis. The effect of terbutaline on peak tetanic force on the third application was 80 ± 5% (n = 12) of the increase on the first application in denervated soleus fibres, compared with only 44 ± 7% (n = 5) in normal soleus fibres. This indicates that denervation induces some protection from tachyphylaxis.

Since preparations were bathed in a modified Krebs solution (Solution 1, Table 2.1), the altered ionic environment might have allowed the potentiating effect of terbutaline to be seen. In five experiments performed in normal Krebs solution, 10 μM terbutaline still increased peak tetanic force. In three denervated soleus preparations, where paired data were obtained, terbutaline potentiated peak tetanic force by 19.4 ± 10.1% in normal Krebs solution and then 15.2 ± 4.7% in Solution 1.

(d) Effects of adrenaline on isometric contractions

It was of interest to see if adrenaline, a physiological catecholamine, induced similar contractile effects to that of terbutaline. Adrenaline (10 μM) potentiated isometric contractions in both normal and denervated soleus fibres (Figure 3.7 and Table 3.3). The effect of adrenaline on peak tetanic force was significantly smaller than with terbutaline in denervated soleus fibres ($P < 0.05$, ANOVA), but there was no difference in the normal soleus fibres ($P > 0.1$, ANOVA). Adrenaline potentiated twitches by 17.3 ± 3.4% (n = 6) (pooled normal and denervated soleus preparations), an effect similar to that for terbutaline. There was no consistent effect on the relaxation rate of the few normal soleus preparations tested.

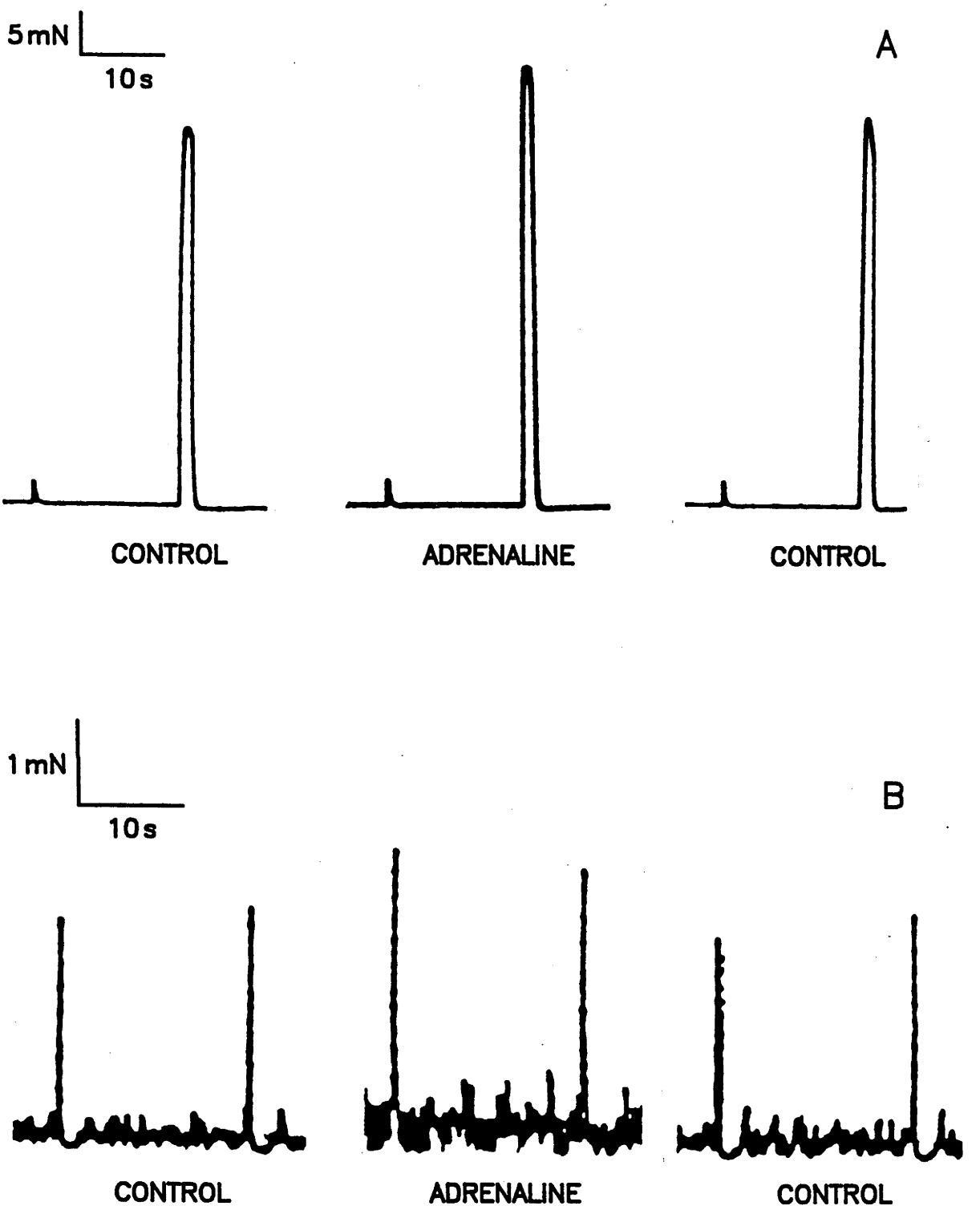


FIG. 3.7: Chart recordings showing the effects of 10 μM adrenaline on isometric force at 24°C. (A) Twitches (small responses) and tetani (large responses) in a normal soleus preparation. Adrenaline potentiated peak tetanic force (100 Hz) by 14.9%, the largest increase seen in these preparations. (B) Adrenaline increased the amplitude of twitches and fibrillations in a denervated soleus preparation.

TABLE 3.3: Effects of terbutaline, adrenaline and dibutyryl-cAMP on peak tetanic force in normal and denervated soleus preparations

DRUG	PEAK TETANIC FORCE (% INCREASE)			
	Normal Soleus		Denervated Soleus	
	mean ± s.e.m.	SD	mean ± s.e.m.	SD
Terbutaline (10 μM)	14.6 ± 1.9 (n = 30)	10.5	13.5 ± 1.1 (n = 44)	7.4
Adrenaline (10 μM)	7.8 ± 2.6 (n = 4)	5.3 *	6.9 ± 2.3 (n = 6)	5.6 *
DBcAMP (2 mM)	15.2 ± 2.0 (n = 5)	4.5	16.0 ± 2.8 (n = 7)	7.3
Terbutaline	12.7 ± 4.6	10.4 **	17.0 ± 3.8	10.0 **

The percentage increase in peak tetanic force was expressed as described in Table 3.1. SD = one standard deviation. n = number of preparations. Tetani were evoked at 100 Hz. Solution 1, at 24°C. Drug induced effects were tested by paired *t*-test. All values were significant for $P < 0.001$ except * $P < 0.05$. ** paired with DBcAMP.

In denervated soleus preparations, adrenaline induced small contractures in association with an increased amplitude of fibrillations (Figure 3.7B). This response to adrenaline contrasted with that of terbutaline where a decrease in resting force was seen. Adrenaline did not produce contractures in normal soleus preparations.

(e) Effects of propranolol and dibutyryl-cAMP on isometric contractions

In this section two major questions are asked regarding the pharmacological mechanism/s by which terbutaline modulates contraction: (i) does terbutaline potentiate force by activating β-adrenoceptors? and (ii) does cAMP act as an intracellular messenger in mediating these contractile effects?

(i) Role of β-adrenoceptors:

Terbutaline is considered to be a selective agonist for β_2-adrenoceptors (Bergman et al. 1969). Some β_2-adrenergic agonists, however, may produce effects that are independent of β-adrenoceptors (Maltin et al. 1989) and, since a high concentration of 10 μM terbutaline was used, there may have been non-selective effects on α-adrenoceptors that are also present in rat skeletal muscle (Hirata et al. 1986; Rattigan et al. 1986; Carlsen & Walsh 1987). Thus it was important to investigate whether β-adrenoceptors were activated during force potentiation by terbutaline.

Propranolol, a general β-adrenoceptor blocker, was used to see if it antagonized the force potentiation by terbutaline. Figure 3.8 illustrates a representative experiment in which terbutaline potentiated peak tetanic force in control conditions but the effect of terbutaline was completely abolished following a 15 min exposure to 0.1 μM propranolol. Similar responses were seen in four preparations (three denervated and one normal soleus). Propranolol (0.1 μM) alone had no direct effect on force. These results suggest that β-adrenoceptor activation is required for the force potentiation by terbutaline.

In preliminary experiments, 10 μM propranolol was used as this concentration of propranolol blocks the catecholamine-induced hyperpolarization in rat skeletal muscle (Clausen & Flatman 1977; McArdle & D'Alonzo 1981). However, 10 μM propranolol alone depressed peak force. This was possibly due to a membrane depolarization seen in high concentrations of propranolol (McArdle & D'Alonzo 1981; McArdle, pers. comm.).

(ii) Role of cAMP:

Dibutyryl-cAMP (DBcAMP) was used to test the hypothesis that cAMP mediated the contractile effects of terbutaline. DBcAMP is more membrane permeable and less

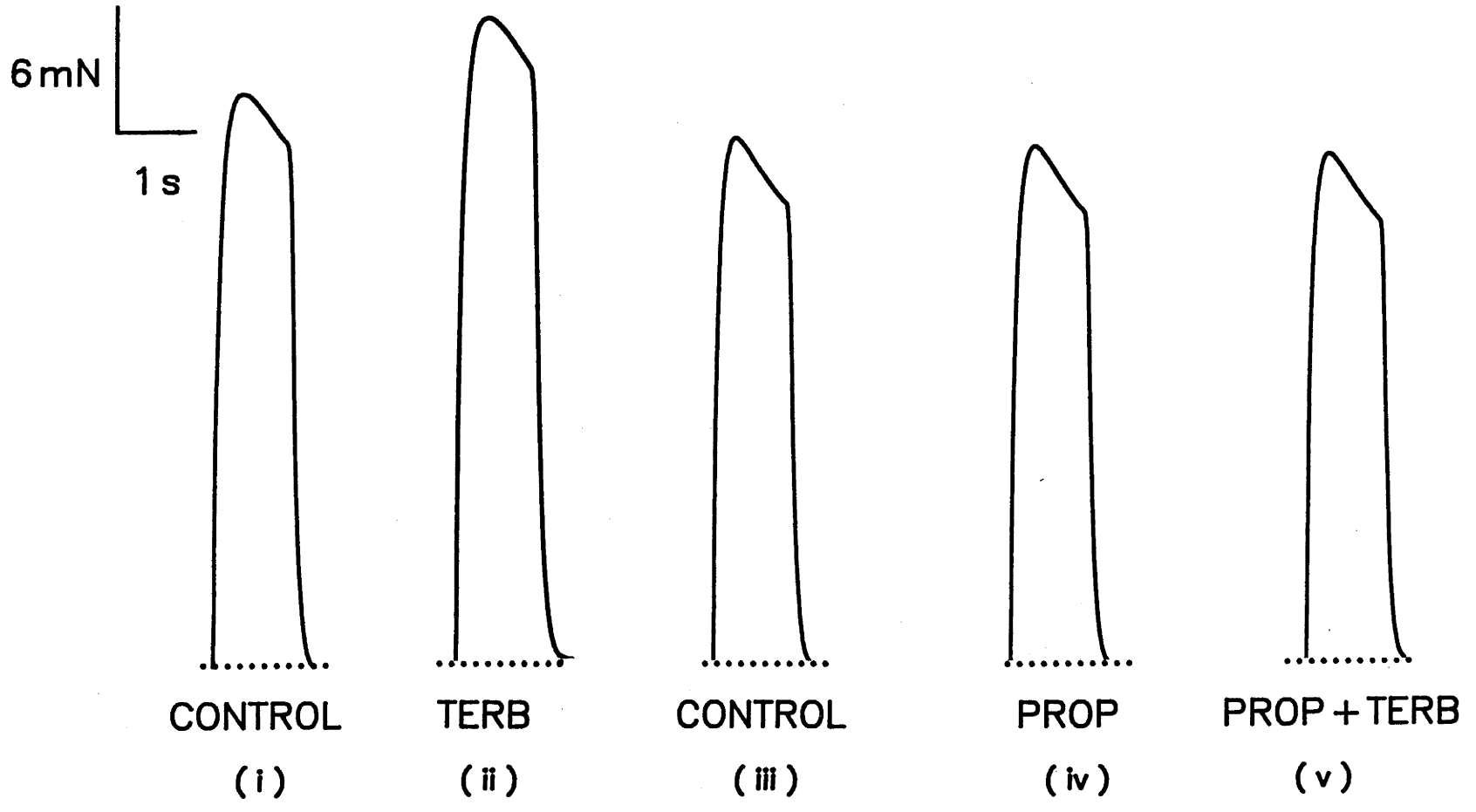


FIG. 3.8: Effect of 10 μM terbutaline on peak tetanic force (100 Hz) in the absence and presence of 0.1 μM propranolol in a denervated soleus preparation at 24 °C. CONTROL and TERB are defined in the legend for Fig. 3.1. PROP is the force response to 0.1 μM propranolol. PROP + TERB is the force response with terbutaline in the presence of propranolol. Terbutaline increased peak tetanic force by 10.7% (ii), relative to the first control (i). The second control was obtained 30 min after wash-out (iii). Propranolol, added for 15 min, had no direct effect on peak force (iv). Terbutaline did not potentiate force when added with propranolol (v).

susceptible to intracellular hydrolysis than cAMP (Skelton et al. 1970; Drummond et al. 1974; Tsien 1977). Inside the fibre DBcAMP is deacylated to monobutyry-cAMP (MBcAMP) and butyrate (Drummond et al. 1974). MBcAMP is thought to activate cAMP-dependent protein kinases (cAMP-PK) and hence mimics the intracellular events following β-adrenoceptor activation (Meinertz et al. 1973; Drummond et al. 1974). DBcAMP (2 mM) was used as similar concentrations are required for maximal twitch potentiation in cardiac muscle (Skelton et al. 1970; Meinertz et al. 1973; Tsien 1977).

Figure 3.9 shows a typical example of the effects of 10 μM terbutaline and then 2 mM DBcAMP on peak tetanic force in a normal soleus preparation. DBcAMP mimicked the tetanus potentiation by terbutaline although development of the maximum response took longer in DBcAMP than in terbutaline. The effect of DBcAMP did not readily wash-out. Consequently, terbutaline was always added first and post-wash controls for DBcAMP were not obtained. Table 3.3 shows the average data where the tetanus potentiation by terbutaline and DBcAMP was not different ($P > 0.1$, ANOVA). In addition, DBcAMP mimicked the terbutaline-induced acceleration of tetanic relaxation in normal soleus fibres (Figure 3.10 and Table 3.4).

DBcAMP also mimicked the tetanus potentiation by terbutaline in denervated soleus fibres (Figure 3.11). On average, DBcAMP increased peak tetanic force by 17.5 ± 2.9% (n = 9). The paired data for terbutaline and DBcAMP (Table 3.3) indicate that these effects were the same ($P > 0.1$, ANOVA). DBcAMP, like terbutaline, did not change the rate of tetanic relaxation in denervated soleus fibres (Table 3.4). Unexpectedly, DBcAMP initially depressed peak twitch force in about 50% of the experiments. This is illustrated in Figure 3.12 where peak twitch force was initially depressed by 9%, followed by a maximal potentiation of 22%. Twitch depression was seldom seen with terbutaline, suggesting that DBcAMP exerted an additional effect to that of terbutaline. Twitch depression with DBcAMP could occur at the same time as tetanus potentiation (Figure 3.11). Maximum twitch potentiation with DBcAMP (10.3 ± 4.1%) was quantitatively similar to that with terbutaline (10.9 ± 2.2%) (n = 9, $P > 0.1$, ANOVA). Twitch relaxation was slowed by DBcAMP, as in terbutaline, but small quantitative differences were apparent (Table 3.4).

There is evidence that some effects of DBcAMP are mediated or modified by butyrate (Yusta et al. 1988). Butyrate is a biproduct of commercial DBcAMP and is formed following deacylation of DBcAMP (Skelton et al. 1970; Tsien 1977; Kurihara & Konishi 1987). Consequently, the effect of 2 mM butyrate was examined on isometric force. Butyrate depressed peak twitch force by about 5% (n = 3) and either depressed or had no effect on peak tetanic force. Thus force potentiation by DBcAMP could not be attributed to butyrate.

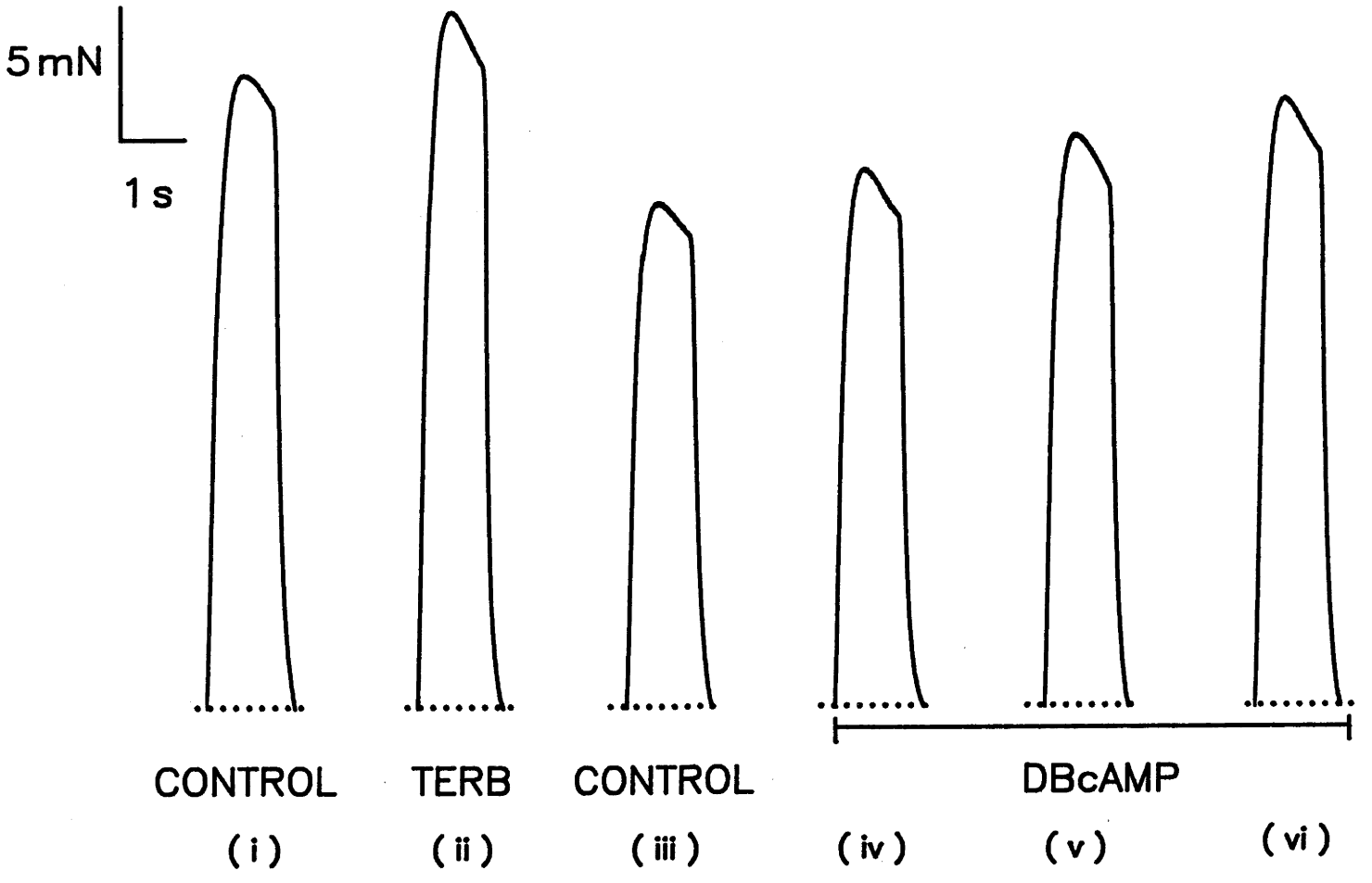


FIG. 3.9: Representative effects of 10 μM terbutaline and 2 mM dibutyryl-cAMP on peak tetanic force (100 Hz) in a normal soleus preparation at 24 °C. CONTROL and TERB are defined in the legend for Fig. 3.1. DBcAMP is a force response to dibutyryl-cAMP. Terbutaline induced a maximal increase of peak tetanic force of 9.8% in 6 min (ii), relative to the first control (i). The second control was obtained 27 min after wash-out of terbutaline (iii). Dibutyryl-cAMP increased peak tetanic force by 6.4% in 6 min (iv), 13.2% in 11 min (v), and then maximally by 19.4% in 16 min (vi).

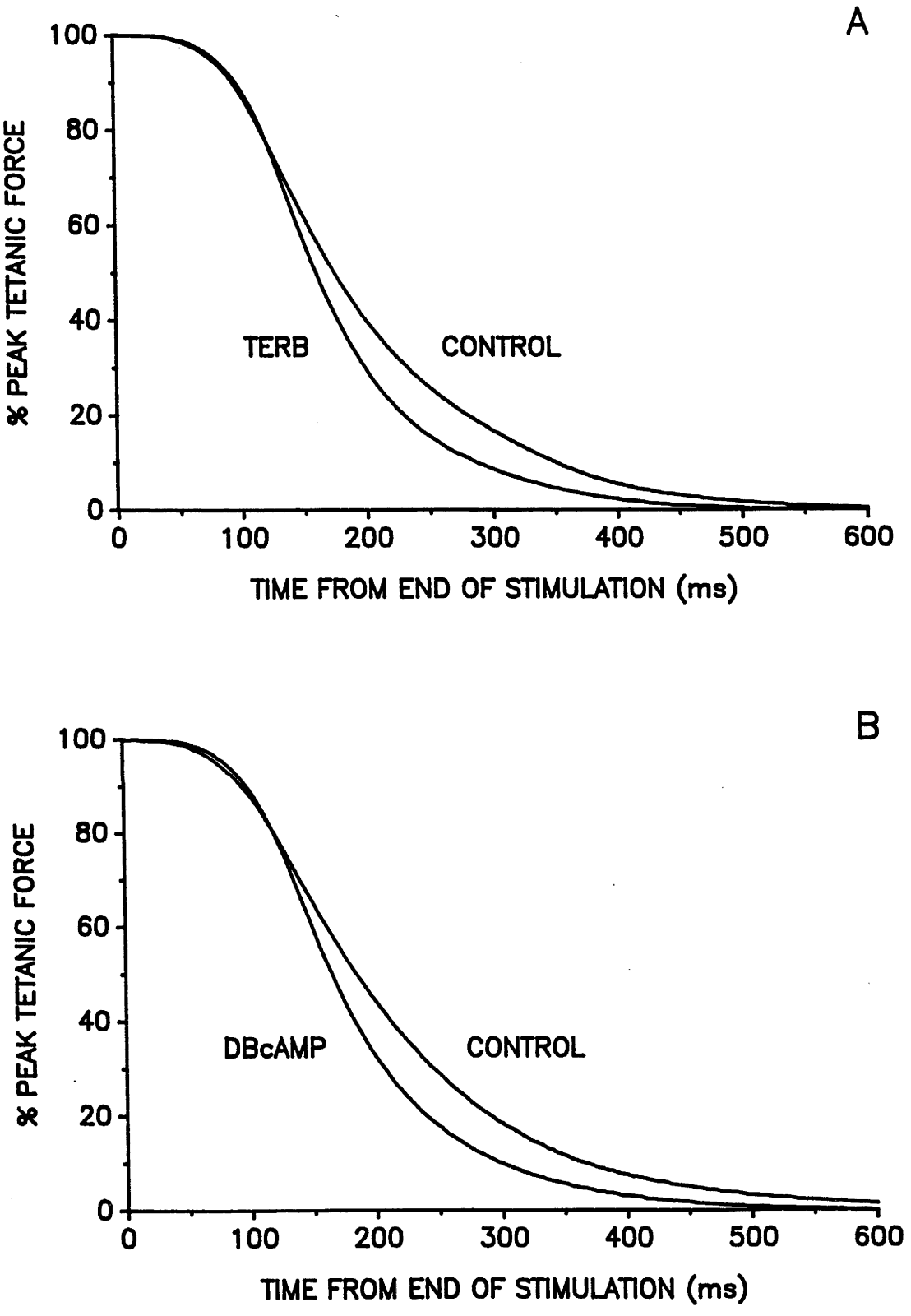


FIG. 3.10: Representative effects of (A) 10 μM terbutaline and (B) 2 mM dibutyryl-cAMP on tetanic relaxation in a normal soleus preparation at 24°C. The relaxation portion of the force traces, (100 Hz for 1 s), were normalized and superimposed. CONTROL is the relaxation response immediately prior to drug administration. The control response in B was obtained after wash-out of terbutaline in A. TERB and DBcAMP are the maximum relaxation responses to terbutaline and dibutyryl-cAMP respectively.

TABLE 3.4: Effects of 10 μM terbutaline and 2 mM dibutyryl-cAMP on the rate of force relaxation in normal and denervated soleus preparations

MUSCLE PREPARATION			RELATIVE RELAXATION RATE (TERB/CONT)	n	80-20% RELAXATION TIME		
					CONT (ms)	TERB (ms)	DIFF (ms)
Normal Soleus	- Tetanus	Terbutaline	1.35 ± 0.09	5	166 ± 15	123 ± 7	-43 ± 12
	- Tetanus	DBcAMP	1.31 ± 0.11 *		165 ± 15	126 ± 7	-39 ± 13
Denervated Soleus	- Tetanus	Terbutaline	0.95 ± 0.03	7	92 ± 5	97 ± 6	5 ± 3
	- Tetanus	DBcAMP	0.95 ± 0.02 *		87 ± 5	93 ± 7	5 ± 2
	- Twitch	Terbutaline	0.77 ± 0.03	8	101 ± 7	132 ± 9	31 ± 5
	-Twitch	DBcAMP	0.85 ± 0.02 **		97 ± 7	114 ± 8	17 ± 3

Shown are the mean values (± s.e.m.). n = number of preparations. Terbutaline and DBcAMP were applied to the same preparation. The 80-20% relaxation time, relative relaxation rate, TERB and DIFF are described in Table 3.2. CONT is the value obtained immediately prior to drug application. Tetani were evoked at 100 Hz for 1s. Solution 1, at 24°C. Differences between the effect of terbutaline and DBcAMP were tested by ANOVA * $P > 0.1$, ** $P < 0.05$.

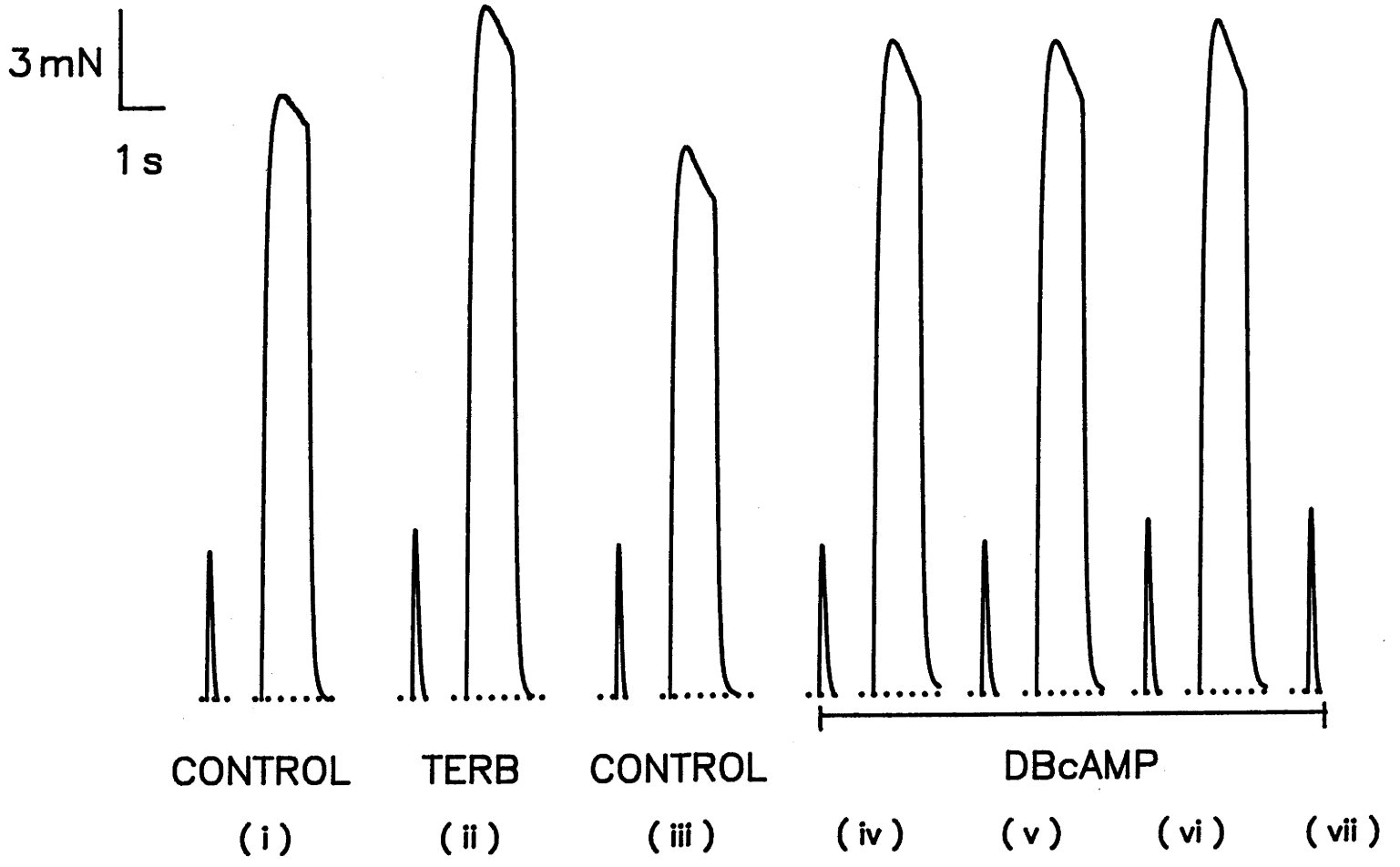


FIG. 3.11: Effects of 10 μM terbutaline and 2 mM dibutyryl-cAMP on twitches and tetani (100 Hz) in denervated soleus fibres at 24°C. CONT, TERB and DBcAMP are defined in the legends for Figs. 3.1, 3.6 & 3.9. Terbutaline increased peak twitch and tetanic force by 13% in 10 min and 15% in 12 min respectively (ii), relative to the first controls (i). The second control responses were obtained about 40 min after wash-out (iii). Dibutyryl-cAMP induced time-dependent changes in force (iv) & (v), and the maximal increase of peak twitch and tetanic force were 19.4% in 48 min (vii) and 22.1% in 40 min (vi) respectively.

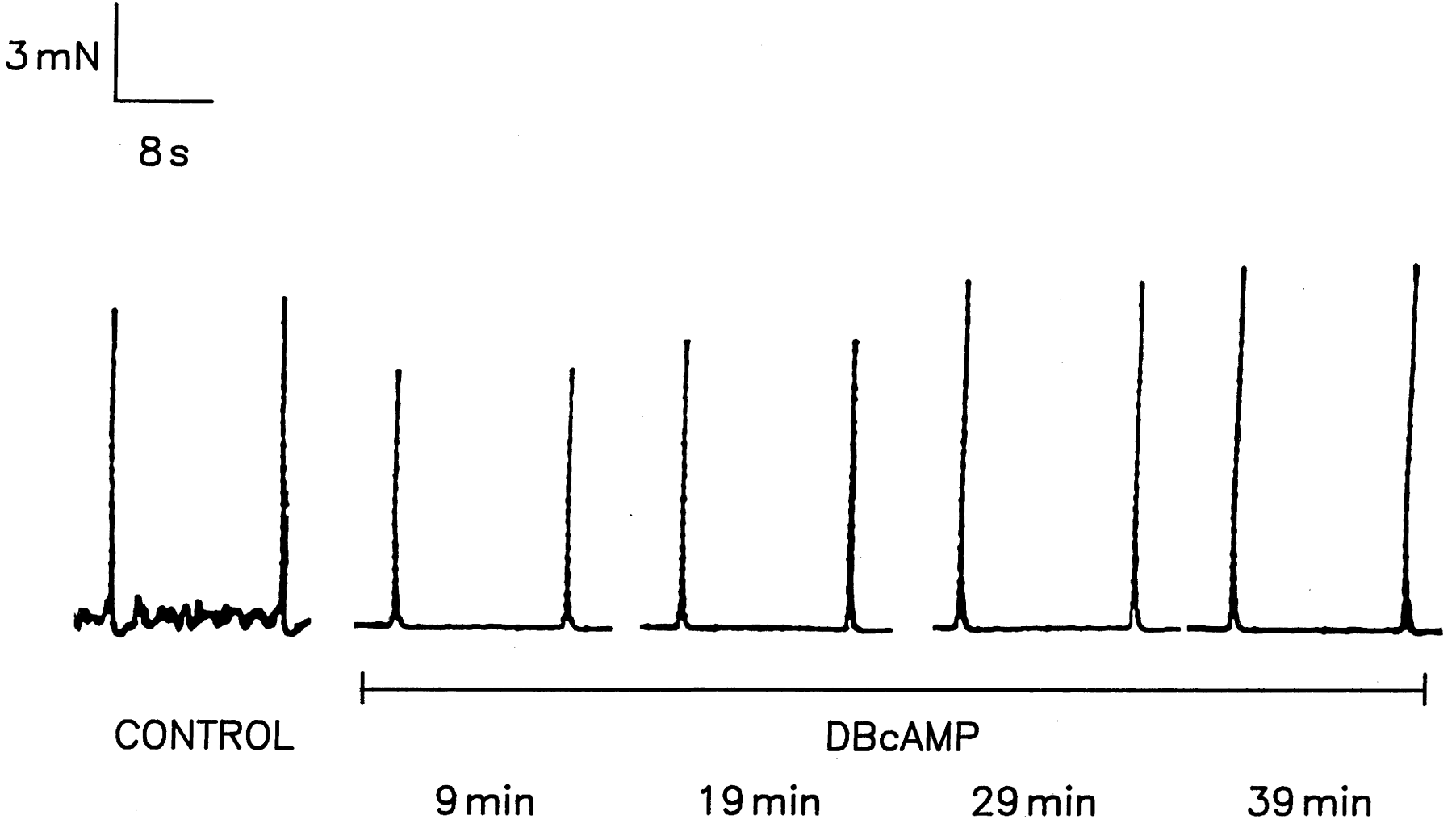


FIG. 3.12: Effect of 2 mM dibutyryl-cAMP on twitches and fibrillations in a denervated soleus preparation, in Solution 1, at 24°C. Dibutyryl-cAMP depressed fibrillations. Peak twitch force was initially reduced but reversed into a potentiation as exposure to dibutyryl-cAMP was prolonged.

3.4 DISCUSSION

This study has shown that terbutaline can modulate isometric contractions in fast- and slow-twitch muscles of the rat. These effects differed between preparations composed of different fibre-types, and following denervation, and could be mimicked by various pharmacological agents as discussed below.

(a) Effects of β-adrenoceptor activation on isometric contractions in different fibre-types

Terbutaline induced a maximum twitch potentiation of about 15% in soleus preparations and 7% in both types of sternomastoid preparations. The increases in peak twitch force were of a similar magnitude to that seen in most other studies on mammalian fast-twitch muscle (Goffart & Ritchie 1952; Bowman & Zaimis 1958; Bowman & Raper 1964; Tashiro 1973), and amphibian muscle (Gonzalez-Serratos et al. 1981; Arreola et al. 1987; Williams & Barnes 1989a). However, the positive inotropic responses for twitches and unfused tetani in the soleus were in striking contrast with most previous studies which showed that sympathomimetics depressed force in slow-twitch muscles (Bowman & Zaimis 1958; Tashiro 1973; Waldeck 1977).

Maximum twitch potentiation by terbutaline was significantly greater in soleus than in sternomastoid fibres (Table 3.1). In addition, dose-response studies suggest that slow-twitch muscles are more sensitive than fast-twitch muscles to catecholamines (Bowman & Zaimis 1958; Marsden & Meadows 1970; Holmberg et al. 1979). The greater maximum twitch potentiation and sensitivity to catecholamines may be attributed to a greater density of β-adrenoceptors, and a greater degree of enhancement of adenylate cyclase activity by catecholamines in slow-twitch muscles (Festoff et al. 1977; Williams et al. 1984; Buckenmeyer et al. 1990).

Another new finding was that terbutaline potentiated peak tetanic force by about 15% in soleus fibres. In previous studies, adrenaline decreased or had no effect on peak tetanic force in muscles in anaesthetised mammals (Goffart & Ritchie 1952; Bowman Zaimis 1958; Lewis & Webb 1976). The depression was associated with a vasoconstriction and reduced muscle blood flow, and was not seen in the presence of α-adrenoceptor blockers or when selective β-adrenergic agonists were used (Brown et al. 1948; Bowman & Zaimis 1958; Bowman & Nott 1970). Bowman & Nott (1970) showed that isoprenaline, a β-adrenergic agonist, induced small increases in peak tetanic force in soleus muscles of anaethetised cats. The small response (1-3%) required high stimulation frequencies and they suggested that it was due to either vasodilatory effects of isoprenaline or to contributions from fast-twitch fibres. In frog twitch fibres,

adrenaline causes a small increase of peak tetanic force of up to 10% (Gonzalez-Serratos et al. 1981; Arreola et al. 1987; Williams & Barnes 1989a).

Terbutaline accelerated twitch relaxation in soleus preparations and slowed twitch relaxation in both types of sternomastoid preparation in agreement with previous studies on fast and slow-twitch muscles (Bowman & Zaimis 1958; Tashiro 1973; Bohmer & Raper 1976). However, terbutaline induced a much more dramatic acceleration of relaxation for the tetanus than for the twitch. This suggests that either terbutaline more effectively modulates tetanic relaxation, or that different mechanisms determine the rate of tetanic and twitch relaxation.

(b) Effects of β-adrenoceptor activation on isometric contractions in denervated muscle

The increase of peak twitch and tetanic force by terbutaline and adrenaline in soleus fibres was unaltered by denervation and similar distributions for force potentiation were seen. This suggests that the force potentiation mechanism was the same in normal and denervated soleus fibres. Denervation did, however, modify two effects of terbutaline. Firstly, twitch relaxation was slowed by terbutaline in contrast to an increased rate of relaxation in normal soleus. Secondly, denervation reduced tachyphylaxis which might be due to (i) the β-adrenoceptors being less susceptible to desensitization in denervated fibres or (ii) enhanced adenylate cyclase activity in denervated fibres (Hashimoto et al. 1989) so that, even with receptor desensitization, the increase in $[cAMP]_i$ is sufficient to produce maximal force potentiation.

Catecholamines have been shown to produce contractures in skeletal muscle following denervation (Bowman & Raper 1965; Yamada & Harigaya 1974; Bohmer & Raper 1975; Evans & Smith 1976). In the present study neither terbutaline nor DBcAMP produced contractures. Instead, resting force was reduced with an associated depression of fibrillations (Figure 3.12). In contrast, adrenaline produced a small contracture at the same time as increasing fibrillations (Figure 3.7). Both effects on resting force may be related to the mechanism causing fibrillation (Bowman & Raper 1965). The frequency of fibrillatory potentials - spontaneous membrane potential oscillations which initiate action potentials - is increased by adrenaline (Bowman & Raper 1965; Smith & Thesleff 1976). This could synchronize fibrillations in different fibres to cause a contracture in the preparation. The hyperpolarization induced by terbutaline and DBcAMP (Chapter 4.3c) may prevent fibrillatory potentials reaching threshold for action potentials. The resulting desynchronization of fibrillations could then explain the reduced resting force (Bowman & Raper 1965).

(c) Pharmacological mechanism of action of terbutaline

The positive inotropic response was not exclusive to terbutaline since adrenaline potentiated contractions in a qualitatively similar manner to terbutaline. This suggests that the force potentiation was a common characteristic of catecholamines.

Propranolol abolished the tetanus potentiation by terbutaline, suggesting that activation of β-adrenoceptors was required for the response to terbutaline. These adrenoceptors are presumably of the β_2-subtype due to the selectivity of terbutaline (Bergman et al. 1969). Previous mechanical studies with sympathomimetics have also shown antagonistic effects with propranolol, or other β-blockers, indicating the involvement of β-adrenoceptors (Bowman & Nott 1970; Marsden & Meadows 1970; Holmberg & Waldeck 1977, 1980; Oota & Nagai 1977; Waldeck 1977).

The time-course for development of maximum twitch or tetanus potentiation by terbutaline suggests that a second messenger is involved. Since sympathomimetics increase $[cAMP]_i$ in mammalian skeletal muscle (Al-Jeboory & Marshall 1978; Fellenius et al. 1980; Merican & Nott 1981; Bowman et al. 1985; Chasiotis 1985) the messenger could be cAMP. DBcAMP quantitatively mimicked the effect of terbutaline on the peak force and relaxation of tetani in normal and denervated soleus fibres. Since butyrate did not potentiate force, it is likely that the force potentiation by DBcAMP is mediated by MBcAMP through the activation of cAMP-PKs (Drummond et al. 1974). Thus it is likely that cAMP is the intracellular messenger responsible for the contractile effects of terbutaline. However, maximum effects with DBcAMP developed more slowly than with terbutaline. A similar slow response in cardiac muscle has, however, been attributed to a slow rate of entry of DBcAMP into the fibres (Drummond et al. 1974).

DBcAMP has been shown to potentiate twitches in mammalian fast-twitch muscle (Kentera & Varagic 1975; Varagic & Kentera 1978) and frog fibres (Oota & Nagai 1977). In the present study, DBcAMP initially depressed peak twitch force and then potentiated force to finally mimic the effect of terbutaline. Butyrate depressed twitches and had little effect on tetani, whereas DBcAMP potentiated tetani when twitches were depressed (Figure 3.11). This suggests that DBcAMP induces twitch-depression which is possibly independent of butyrate. It might be related to the sympathomimetic-induced depression of force seen previously in slow-twitch muscles (Bowman & Zamis 1958; Tashiro 1973; Holmberg & Waldeck 1977).

Two other pharmacological interventions have been used to ascertain the role of cAMP. Firstly, forskolin, an activator of adenylate cyclase (Seamon et al. 1981) induces twitch potentiation in rat diaphragm (Prostran & Varagic 1986). However, forskolin had no

effect on contraction, although it increased $[cAMP]_i$ in guinea pig soleus muscle (Bowman et al. 1985; Waldeck & Widmark 1985). This result challenges the idea that cAMP is the myoplasmic messenger involved in force potentiation. Secondly, phosphodiesterase inhibitors, which prevent the degradation of cAMP, potentiate sympathomimetic-induced effects on contraction (Bowman & Nott 1974; Merican & Nott 1981).

(d) Physiological mechanisms for the force potentiation by terbutaline

Force potentiation by terbutaline, or DBcAMP, was due to a direct effect on the muscle fibres as it was seen during direct stimulation either in the presence of curare in normal soleus fibres or in denervated soleus fibres. Positive inotropic effects were only seen during electrical stimulation since these drugs did not trigger contractures. This suggests that terbutaline and DBcAMP both act by modulating contractions initiated by voltage-activated processes. Furthermore, twitch potentiation by terbutaline occurred independently of changes in the rate of relaxation as seen in the different fibre-types and following denervation. This suggests that at least two intracellular processes are affected by terbutaline: one determining peak force and the other the rate of relaxation. If the rate of twitch relaxation reflects the rate of Ca^{2+} removal by the Ca^{2+}-pump of the SR (Stein et al. 1982; Fryer & Neering 1986) then twitch potentiation must be independent of Ca^{2+}-pump activity. Also, the potentiation of force by terbutaline was not an artefact of the solutions, as twitches and tetani were potentiated in normal Krebs solution.

Why were subtetanic contractions potentiated by terbutaline in soleus whereas previous studies with sympathomimetics show a depression in slow-twitch muscles?. Species differences might have a role. However, Holmberg & Waldeck (1980), using isolated guinea pig soleus, observed that terbutaline induced an initial twitch depression that reversed with time into a potentiation of similar magnitude to that seen in the present study. This result suggests that twitch force can be modulated by terbutaline by at least two different mechanisms: one that depresses and one that potentiates force. In most previous studies, the sympathomimetic drugs were washed out soon after maximum force depression and hence subsequent force potentiation would not have been seen. The initial twitch depression by DBcAMP indicates that a force depressing mechanism may also be present in rat muscle, if the DBcAMP-induced twitch depression was not due to butyrate.

Why has a potentiation of peak tetanic force not been seen before? It has long been thought that the myofilaments are saturated with Ca^{2+} during tetanic stimulation and hence peak tetanic force could not be modulated by hormones that increase myoplasmic $[Ca^{2+}]$. Certainly, the amplitude of the tetanic Ca^{2+} transient continues to rise long

after force had reached a plateau in frog fibres, suggesting that the contractile apparatus was saturated with Ca^{2+} (Blinks et al. 1978; Cannell & Allen 1984). However, there are observations which are inconsistent with this conclusion. (i) Peak tetanic force can be augmented by low concentrations of caffeine (Isaacson et al. 1970; Rudel & Taylor 1971; Fryer & Neering 1989; Chapter 4.3h) and nitrate (Taylor 1976), and these interventions also increase $[Ca^{2+}]_i$ (Blinks et al. 1978; Fryer & Neering 1989). (ii) The amplitude of maximum potassium contractures is greater than peak tetanic force (Hodgkin & Horowics 1960; Sandow et al. 1964; Dulhunty & Gage 1985; Chua & Dulhunty 1988; Chapter 5.3c). All these results suggest that force can in fact be increased by further Ca^{2+} activation of the myofilaments.

The lack of effect of sympathomimetics on fused tetani *in vivo* might be due to anaesthetics, which directly potentiate force (Holmberg & Waldeck 1979), and may mask the force potentiation by sympathomimetics. Furthermore, Fellenius et al. (1980) showed that $[cAMP]_i$ was considerably higher in resting muscle *in vivo* than *in vitro*, so the potential to increase $[cAMP]_i$, and force, may be created by the *in vitro* condition.

A more detailed study of the mechanism of force potentiation by terbutaline is presented in the next chapter.

(e) Physiological mechanisms for the effects of terbutaline on relaxation

The terbutaline-induced acceleration of relaxation in soleus was qualitatively similar to that seen with β-adrenergic agonists in cardiac muscle. Hence, comparisons are made. It has been proposed that the faster relaxation in cardiac muscle might involve the myofilament proteins, troponin-I (Kranias & Solaro 1982; Robertson et al. 1982; Karczewski et al. 1990) or C-protein (Hartzell 1984), or the SR protein phospholamban (Katz 1979; Kranias & Solaro 1982; Lindemann et al. 1983; Lindemann & Watanabe 1985; Tada et al. 1988). Phosphorylation studies provide evidence for and against an involvement of each of these proteins (Hartzell 1984; Kranias & Solaro 1982; Lindemann et al. 1983; Garvey et al. 1988; Karczewski et al. 1990).

Phosphorylation of troponin-I reduces the sensitivity of myocardial troponin-C for Ca^{2+} (Robertson et al. 1982) resulting in a shift to the right in the force-pCa relation (Endoh & Blinks 1988; Garvey et al. 1988; McIvor et al. 1988). This would increase the relaxation rate if Ca^{2+} dissociation from troponin was rate limiting. However, McIvor et al. (1988) elegantly demonstrated that biochemical manipulation of the myofilament Ca^{2+}-sensitivity did not alter the rate of cardiac relaxation, nor the effect of isoprenaline on relaxation, suggesting that troponin-I was not involved. It is unknown whether troponin-I is phosphorylated in slow-twitch muscle; it is not phosphorylated in fast-twitch muscle (Stull & High 1977). Moreover, both troponin-I and C-protein

remain phosphorylated when the effect of catecholamines on cardiac relaxation had washed out (Garvey et al. 1988).

There is considerable evidence supporting the suggestion that the cAMP-dependent phosphorylation of phospholamban is involved in the acceleration of cardiac twitch relaxation by β-adrenergic agonists. Firstly, exposure to cAMP or cAMP-PK results in the phosphorylation of phospholamban (22 kD) and a simultaneous increase in the rate of Ca^{2+} uptake and Ca^{2+}-ATPase activity in isolated SR vesicles (Tada et al. 1974, 1975; Kirchberger & Tada 1976). Secondly, isoprenaline, or DBcAMP, induce a phosphorylation of phospholamban in intact mammalian cardiac muscle (Lindemann et al. 1983; Lindemann & Watanabe 1985) which correlates in concentration- and time-dependence with the increased Ca^{2+}-ATPase activity, and increased rate of relaxation (Lindemann et al. 1983). Thirdly, catecholamines and DBcAMP increase the rate of decay of the cardiac Ca^{2+} transient (Allen & Blinks 1978; Callewaerts et al. 1988; Endoh & Blinks 1988; Kurihara & Konishi 1988; McIvor et al. 1988). Thus a popular hypothesis is that the cAMP-dependent phosphorylation of phospholamban results in a stimulation of the SR Ca^{2+}-pump (by removing the inhibitory effect of unphosphorylated phospholamban on the Ca^{2+}-pump Tada et al. (1988)) to produce a faster decline of myoplasmic [Ca^{2+}] and consequently an acceleration of cardiac twitch relaxation (Katz 1979; Tada & Katz 1982; Tada et al. 1988). The cAMP-phospholamban system is illustrated in Figure 3.13.

The cAMP-phospholamban system might also be responsible for the terbutaline-induced acceleration of relaxation in soleus muscle. There is good evidence supporting this proposal. (i) Antibodies to cardiac phospholamban bind selectively to slow-twitch, but not fast-twitch, skeletal muscle fibres (Inui et al. 1985; Jorgensen & Jones 1986; Tada et al. 1988), (ii) The Ca^{2+}-ATPase of adult slow-twitch and cardiac muscle are coded for by identical genes and mRNAs, whereas different forms have been found in fast-twitch muscle (Brandl et al. 1986, 1987). Therefore, there could be a common regulatory system for the Ca^{2+}-ATPase of slow-twitch and cardiac muscle. (iii) Application of exogenous cAMP-PK results in the phosphorylation of a 22 kD protein and an increased rate of Ca^{2+} uptake in isolated SR vesicles from slow-twitch, but not fast-twitch, muscle (Kirchberger & Tada 1976; Shigekawa et al. 1976; Salviati et al. 1982). Thus β-adrenergic activation of the cAMP-phospholamban system might account for the acceleration of relaxation in soleus. The absence of phospholamban might explain the inability of terbutaline to accelerate relaxation in the sternomastoid fibres. The inability of terbutaline and DBcAMP to accelerate relaxation in denervated soleus fibres might mean that phospholamban, or the ability of phospholamban to modulate Ca^{2+}-ATPase activity, is lost following denervation. The terbutaline-induced acceleration of relaxation was greater for the tetanus than for the twitch in normal

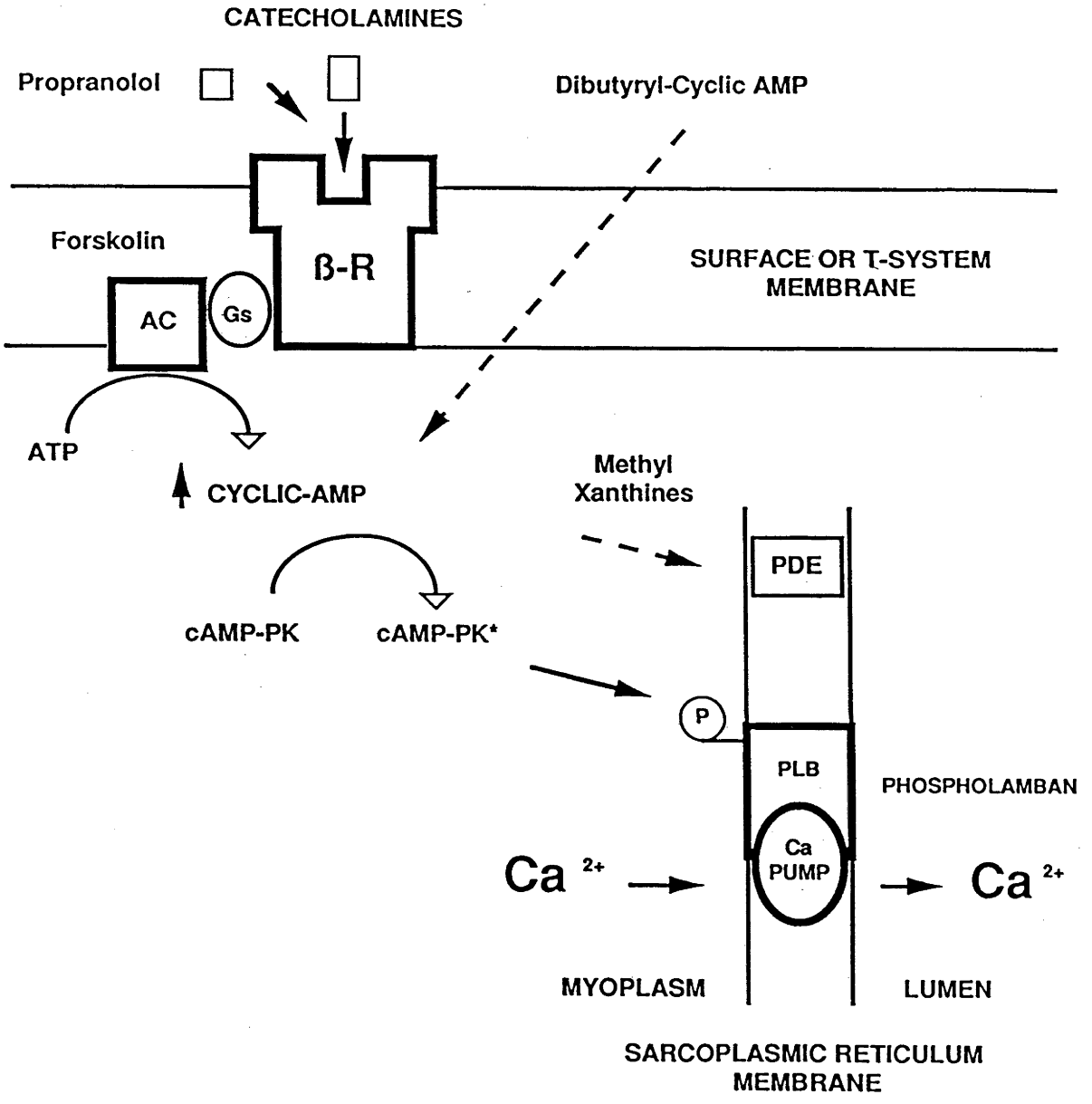


FIG. 3.13: Model for the β-adrenoceptor mediated activation of the cyclic-AMP dependent phospholamban system which is thought to be responsible for the acceleration of relaxation in cardiac muscle. β-R (β-adrenoceptor); Gs (G-protein); AC (adenylate cyclase); PDE (phosphodiesterase); PLB (phospholamban). Some of the pharmacological tools used to investigate this pathway are shown.

soleus fibres. This might be explained if the Ca^{2+}-calmodulin dependent mechanism for the phosphorylation of phospholamban in cardiac muscle (Lindemann & Watanabe 1985; Tada et al. 1988) also occurs during β-adrenergic activation under tetanic conditions in soleus fibres.

There are several conflicting studies in which cAMP-PK was found to either increase the rate of Ca^{2+} uptake into SR vesicles from fast-twitch muscle (Bornet et al. 1977; Schwartz et al. 1977; Kranias et al. 1980), or to produce relaxing effects in skinned fast-twitch fibres (Fabiato & Fabiato 1978). However, these effects were associated with the phosphorylation of a 100 kD protein, which might be the Ca^{2+}-ATPase and not the 22 kD phospholamban (Schwartz et al. 1977; Kranias et al. 1980). It remains to be determined whether catecholamines stimulate Ca^{2+} uptake in intact fast- or slow-twitch fibres. Measurement of the rate of decay of Ca^{2+} transients would help to establish the role of the SR in the effects of terbutaline on relaxation in the different types of muscle fibre.

Terbutaline and DBcAMP increased the rate of tetanic relaxation (1/80-20%RT) by about 30% in normal soleus fibres. In cardiac muscle, catecholamines can increase the rate of relaxation 2-3 fold (Nathan & Beeler 1975; Kurihara & Konishi 1987; Karczewski et al. 1990). Quantitative difference might arise because the density of phospholamban in slow-twitch muscle is only about 20% of that in cardiac muscle (Jorgensen & Jones 1986).

3.5 SUMMARY

(1) The effects of 10 μM terbutaline, a β_2-adrenergic agonist, were examined on isometric contractions in bundles of fibres isolated from soleus and sternomastoid muscles of the rat.

(2) In normal soleus, terbutaline induced a maximum potentiation of both peak twitch and tetanic force by about 15%. An acceleration of twitch and tetanic relaxation was associated with force potentiation.

(3) In both white- and red-sternomastoid preparations, terbutaline induced a maximum potentiation of peak twitch force of about 7%. This potentiation was significantly smaller than for the soleus twitch. A slowing of relaxation was associated with twitch potentiation.

(4) Terbutaline potentiated twitches and tetani in denervated soleus fibres. The potentiation was quantitatively similar to that for normal soleus fibres. Force potentiation was concentration- and time-dependent. In contrast to the normal soleus, terbutaline slowed twitch relaxation and had no effect on tetanic relaxation.

(5) Adrenaline (10 μM) also augmented isometric contractions. Peak tetanic force was increased by about 7% in normal and denervated soleus fibres.

(6) DL-Propranolol (0.1 μM) completely abolished the potentiation of peak tetanic force by terbutaline. This suggests that β-adrenoceptor activation is a prerequisite for force potentiation by terbutaline.

(7) DBcAMP (2 mM) quantitatively mimicked the effects of 10 μM terbutaline on the peak force and relaxation of tetani in both normal and denervated soleus fibres. These effects of DBcAMP were not mediated by butyrate. The similar effects of terbutaline and DBcAMP suggest that cAMP is the intracellular messenger responsible for the effects of terbutaline.

(8) DBcAMP induced a biphasic effect on peak twitch force in denervated soleus muscle: an initial depression that reversed into a potentiation. The final twitch potentiation by DBcAMP and terbutaline was quantitatively similar.

(9) It is suggested that the effects of sympathomimetic-amines on relaxation in all types of preparations are mediated by cAMP. The acceleration of twitch and tetanic relaxation in soleus may be due to the cAMP-dependent phosphorylation of phospholamban, resulting in increased activity of the SR Ca^{2+}-pump and a faster fall of $[Ca^{2+}]_i$.

(10) It is suggested that sympathomimetic-amines induce positive inotropic effects by the activation of β-adrenoceptors and is mediated by cAMP. The mechanism for force potentiation by sympathomimetics is independent of effects on relaxation and is examined in more detail in the next chapter.

CHAPTER 4

MECHANISM OF THE β-ADRENERGIC POTENTIATION OF ISOMETRIC FORCE

4.1 INTRODUCTION

The potentiation of peak tetanic force by terbutaline, adrenaline and dibutyryl cyclic-AMP (DBcAMP) in isolated rat skeletal muscle are new observations. Positive inotropic effects with β-adrenergic agonists have previously been demonstrated in mammalian skeletal muscle but only for submaximal contractions in fast-twitch muscle (Bowman 1980). The main aim in this chapter is, therefore, to establish the cellular mechanism/s that are responsible for the increase in peak tetanic force.

It is likely that β-adrenergic agonists and DBcAMP act by stimulating cyclic-AMP dependent protein kinases (cAMP-PK) (Tsien 1977; Drummond & Severson 1979), which then phosphorylate intracellular proteins (Seiler et al. 1984; Curtis & Catterall 1985; Hosey et al. 1986; Jorgensen & Jones 1986; Garvey et al. 1988). Any one or a combination of these phosphorylated proteins may directly or indirectly increase force. The ultimate site of action may be the myofilaments where force potentiation could result from (i) an increased maximum Ca^{2+}-activated force due to either an increased force production per crossbridge or by the participation of an increased number of crossbridges, or (ii) an increased sensitivity of troponin-C for Ca^{2+} so that, for a given myoplasmic $[Ca^{2+}]$, the Ca^{2+} occupancy of troponin-C is enhanced; assuming that troponin-C is not already saturated with Ca^{2+}. Alternatively, force may be augmented by increasing the $[Ca^{2+}]_i$ so that more Ca^{2+} would be available to activate the contractile proteins. In principle, this could result from (i) changes in the action potential so that a greater depolarization reaches the "voltage sensors" in the T-system or (ii) an increased efficacy of some process in excitation-contraction coupling so that the SR releases more Ca^{2+} for a given depolarization.

There have been numerous studies on the mechanism/s responsible for the positive inotropic effect of catecholamines in the heart (reviewed by Tsien 1977; Katz 1979; Tada & Katz 1982; Endoh 1986). The predominant factor in the β-adrenergic potentiation of cardiac twitch force is thought to be an increase in the amplitude of the Ca^{2+} transient (Allen & Blinks 1977; Fabiato 1981; Kurihara & Konishi 1987; Callerwert et al. 1988; Endoh & Blinks 1988; McIvor et al. 1988), which is intimately associated with an increase in the slow inward Ca^{2+} current (Reuter 1974; Nathan & Beeler 1975; Reuter & Scholz 1977; Tsien et al. 1986) and increased Ca^{2+} release from the SR (Fabiato & Fabiato 1975; Katz 1979; Fabiato 1981; Boller & Pott 1988). Involvement of the contractile proteins has also been suggested (McClellan & Winegrad

1980; Hoh et al. 1988). The phosphorylation sites and intracellular processes known to be affected by catecholamines in the heart may provide important clues as to the force potentiation mechanism in skeletal muscle.

4.2 METHODS

The muscle preparations and general methodology were the same as described in Chapters 2 and 3. Solution 1 (Table 2.1) was the bathing solution to which drugs were added. In a few experiments a solution with a high [Na^+] (Solution 2 - Table 2.1) was used, as indicated in the text. Experiments were performed at 24°C.

It was important to use the same preparation (hence paired data) to quantify the effect of terbutaline on force, in both control and test conditions, because of the considerable variation between preparations. Repeated applications of terbutaline produced force increases of similar magnitude in denervated soleus fibres, whereas the normal soleus displayed tachyphylaxis (Chapter 3.3c). It is reasonable to assume that the same force potentiating mechanism occurred in the denervated soleus as in the normal soleus fibres, since the increase in force was identical (Table 3.1). Thus, denervated soleus fibres were frequently used in paired experiments. Comparisons were made with normal soleus fibres in some experiments. In these paired experiments, the effect of terbutaline, under control or test conditions, was obtained in a randomized order. When the effect of the test drug failed to or only slowly washed out (e.g. with ouabain or iodoacetate), it was always added after the control response to terbutaline had been obtained. Values presented in the text are the mean value (± s.e.m.), where n = the number of preparations.

Additional drugs used and their sources were: (i) caffeine (AJAX); (ii) ouabain octahydrate (Sigma); (iii) nifedipine (Sigma); (iv) ethanol (AJAX); (v) iodoacetic acid (Sigma). Nifedipine was dissolved in 100% ethanol to make a stock solution of 10 mM. Ouabain and nifedipine solutions were stored in light-proof containers at 4°C.

4.3 RESULTS

(a) Effect of terbutaline and dibutyryl-cAMP on peak tetanic force at different stimulation frequencies

Fully fused tetani were evoked by direct stimulation at a frequency of 100 Hz. However, in Solution 1 at 24°C, maximum tetanic force was achieved at the lower frequency of 50 Hz in both normal and denervated soleus fibres. Peak tetanic force was depressed by 10-20% at 100 Hz relative to 50 Hz (Figure 4.1). A depression of peak tetanic force by indirect stimulation at high frequencies (greater than fusion frequency) is known as Wedensky inhibition (Lucas 1911; Cullingham et al. 1960; Truong et al.

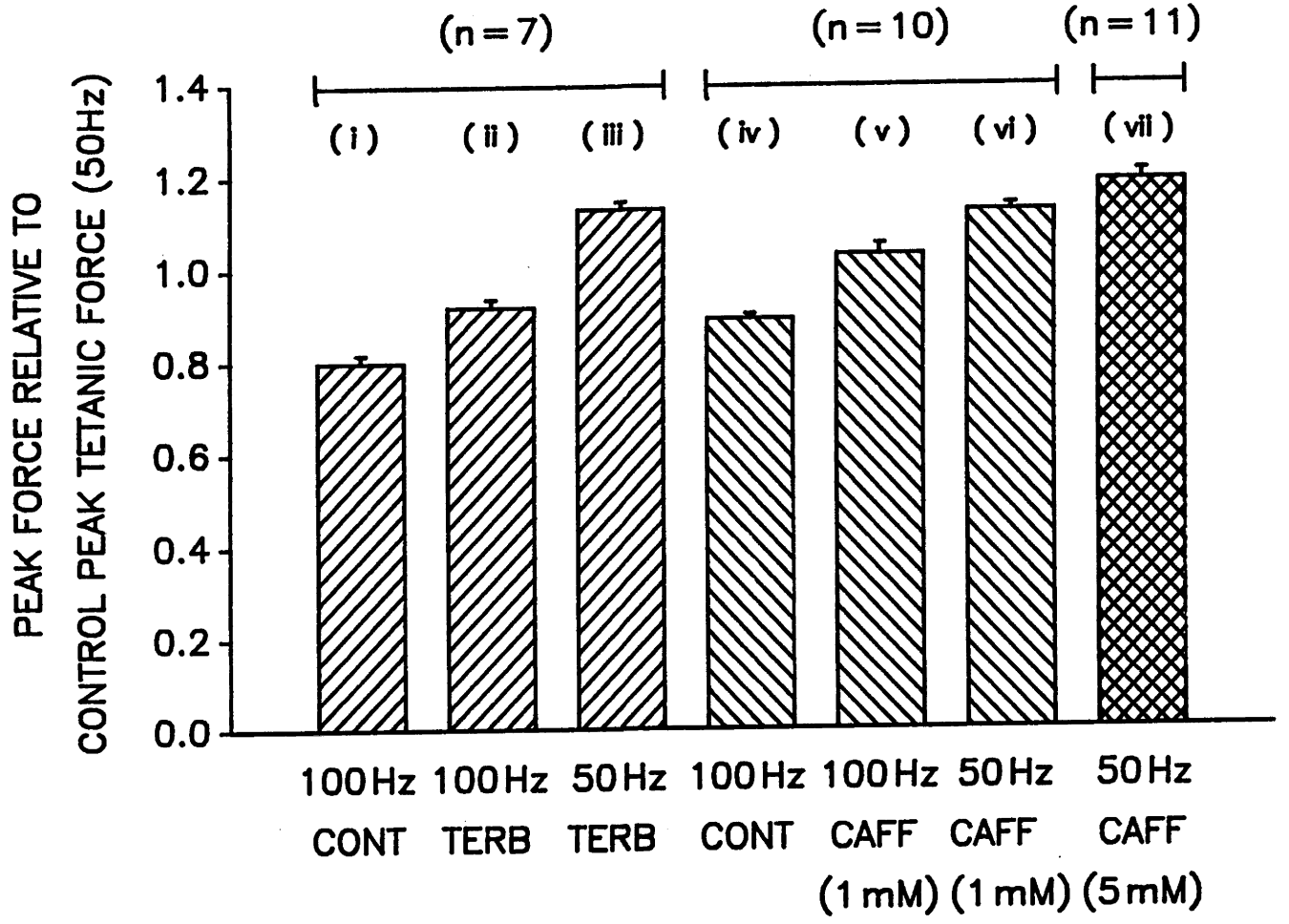


FIG. 4.1: Effects of terbutaline and caffeine on peak tetanic force at 100 or 50 Hz in denervated soleus fibres. Solution 1, 24°C. Peak tetanic force was normalized to the peak force of control tetani (50 Hz). Shown are the mean values ± s.e.m., n = number of preparations. Paired data are indicated by the horizontal lines above the bars. Stimulation frequency is shown beneath each bar. CONT and TERB are defined in the legends for Figs. 3.1 & 3.6. CAFF is the maximum force response to 1 or 5 mM caffeine. The 100 Hz control values (i & iv) were different ($P < 0.05$, ANOVA), which might reflect differences in the denervation duration (see Chapter 5.3e).

TABLE 4.1: Effect of terbutaline, caffeine, or dibutyryl-cAMP on peak tetanic force elicited by stimulation at 100 Hz or 50 Hz

MUSCLE PREPARATION	DRUG	PEAK TETANIC FORCE (% INCREASE)		
		100 Hz	50 Hz	
Normal Soleus	Terbutaline (10 μM)	14.6 ± 1.9 (n = 30)	8.6 ± 1.6 (n = 9)	$P > 0.1$
	Caffeine (1 mM)	20.7 ± 3.1 (n = 16)	16.6 ± 5.0 (n = 7)	$P > 0.1$
Denervated Soleus	Terbutaline (10 μM)	13.5 ± 1.1 (n = 44)	13.5 ± 1.2 (n = 46)	$P > 0.1$
	Caffeine (1 mM)	17.8 ± 2.6 (n = 18)	11.6 ± 1.2 (n = 30)	$P < 0.05$
	DBcAMP (2 mM)	17.5 ± 2.9 (n = 9)	13.1 ± 3.3 (n = 12)	$P > 0.1$

Shown are the mean values (± s.e.m.). n = number of preparations. The value for each preparation was that for the first application of each drug. Solution 1, at 24°C. The effect of each drug was significant for $P < 0.05$ (paired *t*-test). Differences in the effect of each drug at 100 and 50 Hz (shown) were tested by ANOVA.

1964) and has been attributed to a failure of neuromuscular transmission (Krnjevic & Miledi 1958a; Thesleff 1959; Cullingham et al. 1960). However, the depression of peak tetanic force was produced by direct stimulation in the present study. Hence, it cannot be the result of neuromuscular block. One possible explanation for the terbutaline-induced potentiation of peak tetanic force at 100 Hz was that terbutaline acted to reverse the frequency-dependent depression of force. Thus the effect of 10 μM terbutaline was examined on peak tetanic force at 100 and 50 Hz in normal and denervated soleus fibres.

In normal soleus fibres, stimulation at 100 Hz depressed peak tetanic force to 87.4 ± 2.0% (n = 9) of the control tetani at 50 Hz. Terbutaline increased peak tetanic force at 100 Hz by 14.6 ± 1.9% (n = 30) and augmented the 50 Hz tetani by 8.6 ± 1.6% (n = 9) (Table 4.1). The smaller potentiation at 50 Hz compared with 100 Hz, surprisingly, was not significantly different ($P > 0.1$, ANOVA). The results of paired experiments in seven denervated soleus preparations are illustrated in Figure 4.1. This shows that, following stimulation at 100 Hz, peak tetanic force was reduced to 79.8 ± 1.8% of the control 50 Hz tetani. Terbutaline potentiated the 100 and 50 Hz tetani by 15.0 ± 1.8% and 12.9 ± 1.6% respectively and these responses were not significantly different ($P >$ 0.1, ANOVA) from each other (Figure 4.1). There was a considerable variation in the terbutaline-induced force potentiation at 50 Hz (SD = 8.5%, range 2.8% - 38.7%). In addition, 2 mM DBcAMP potentiated peak tetanic force at 50 Hz by 13.1 ± 3.3% (n = 12). These results show that terbutaline and DBcAMP both potentiate peak tetanic force independently of any frequency-dependent depression of force.

(b) Effect of terbutaline on peak tetanic force in solutions with a high sodium concentration

In a later series of experiments it was observed that peak tetanic force was about 25% greater in Solution 2 ($[Na^+]$ = 196 mM) than in Solution 1 ($[Na^+]$ = 80.5 mM) (Chapter 5.3d). It is possible that the augmentation of peak tetanic force by terbutaline was an effect created by the low extracellular $[Na^+]$ in Solution 1, although tetanus potentiation was seen in normal Krebs solution ($[Na^+]$ = 120 mM) (Chapter 3.3c). Figure 4.2 shows that peak tetanic force was increased by terbutaline in both the low and high sodium solutions in a denervated soleus preparation. In three denervated preparations - all of which produced maximum force at 30 Hz (see Chapter 5.3e) - peak tetanic force was increased by 18.4 ± 5.0% when changing from the low to the high $[Na^+]$ solutions. Terbutaline increased peak tetanic force by 8.9 ± 0.9% in Solution 2 and by 18.5 ± 1.6% in Solution 1. These effects were not statistically different ($P > 0.1$, ANOVA) from each other.

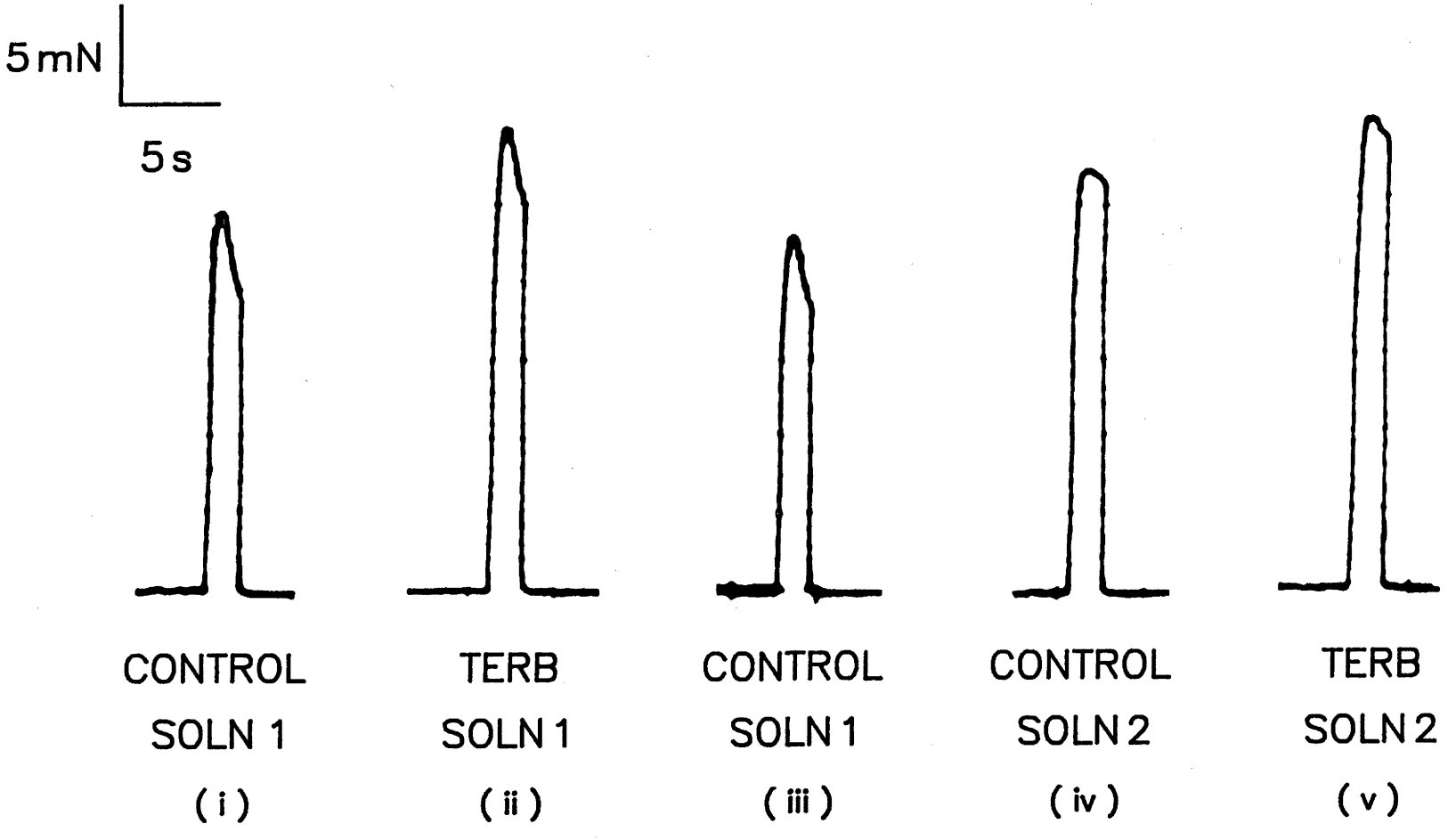


FIG. 4.2: Chart recordings showing the effect of 10 μM terbutaline on peak tetanic force in Solution 1 ($[Na^+]$ = 80.5 mM, $[Cl^-]$ = 16 mM) and Solution 2 ($[Na^+]$ = 196 mM, $[Cl^-]$ = 16 mM) in a denervated soleus preparation. Tetani were evoked at 30 Hz for 2 s at 24°C. In Solution 1 terbutaline increased peak tetanic force by 21.6% (ii), relative to the first control (i), and this effect washed out (iii). Changing from Solution 1 to Solution 2 resulted in an increase of peak tetanic force by 17.2% (iv). Terbutaline potentiated peak tetanic force by 10.0% in Solution 2 (v).

(c) Role of the sodium-potassium pump in the force response to terbutaline

Terbutaline and other sympathomimetic-amines increase the resting membrane potential of normal and denervated mammalian skeletal fibres by increasing the activity of the sarcolemmal Na-K pump (Tashiro 1973; Bray et al. 1976; Clausen & Flatman 1977; McArdle & D'Alonzo 1981; Juel 1988). Stimulation of the Na-K pump may increase the amplitude of the action potential (Smith & Thesleff 1976; McArdle & D'Alonzo 1981) by increasing the sodium equilibrium potential and hence the driving force for the Na^+ current. The possibility that this may lead to tetanus potentiation is consistent with the observation that peak tetanic force is augmented when extracellular [Na^+] is increased (Figure 4.2, Chapter 5.3d).

The effects of terbutaline and DBcAMP on resting membrane potential were measured to obtain an indirect assessment of Na-K pump activity (Sjodin 1989). Resting potentials were recorded 10-20 min after these drugs were first applied, corresponding to the time for maximum force potentiation. Results from these experiments are summarized in Table 4.2. In normal soleus fibres, terbutaline and DBcAMP both induced a small but significant hyperpolarization of 5.2 mV and 4.2 mV respectively. In white-sternomastoid fibres, terbutaline induced a smaller hyperpolarization of 2.3 mV. It was difficult to measure the resting potential in denervated soleus fibres bathed in Solution 1 because of "fibrillatory potentials" and action potentials in some fibres (see Bowman & Raper 1965; Dulhunty 1985). These terbutaline-induced membrane hyperpolarizations indirectly support the hypothesis that terbutaline and DBcAMP stimulate the Na-K pump in normally innervated fibres.

Ouabain, a specific inhibitor of the Na-K pump (Albers et al. 1968; Clausen 1986; Clausen & Everts 1989; Sjodin 1989), was used to test for the role of the Na-K pump in the force potentiation by terbutaline. Figure 4.3 shows a typical effect of 10 μM terbutaline on peak tetanic force (100 Hz) in the absence and presence of 1 mM ouabain, in a denervated soleus preparation. Clearly, force potentiation by terbutaline still occurred in the presence of ouabain. Addition of 1 mM ouabain for 20 min or longer - conditions known to maximally inhibit Na-K pump activity in isolated rat skeletal muscle (Clausen & Flatman 1977; Clausen et al. 1981; Everts pers. comm.) - depressed peak tetanic force. A ouabain-induced depression of force might have been expected if the amplitude of action potentials had been depressed during Na-K pump inhibition by ouabain (Smith & Thesleff 1976; see Chapter 6.4b for discussion). This depression of force indicates that ouabain worked effectively. In eight denervated soleus preparations, terbutaline potentiated peak tetanic force (100 or 50 Hz) by 12.1 ± 2.1% in control conditions. Ouabain, added for 20-60 min, reduced peak tetanic force to 48.0 ± 4.2% of its control value. Then terbutaline increased peak tetanic force by 41.8 ± 12.9%

TABLE 4.2: Effects of 10 μM terbutaline and 2 mM dibutyryl-cAMP on the resting membrane potential of surface fibres from soleus or white-sternomastoid muscles

MUSCLE PREPARATION	RESTING MEMBRANE POTENTIAL		
	Control (mV)	Terbutaline (mV)	DBcAMP (mV)
Normal Soleus	-85.4 ± 0.3 (229/18)	-90.6 ± 0.6 * (119/10)	-89.6 ± 0.5 * (117/8)
White-Sternomastoid	-83.8 ± 0.4 (119/9)	-86.1 ± 0.4 * (106/9)	_____

Shown are the mean values (± s.e.m.). Numbers in parentheses are the number of fibres / number of muscles used. The value recorded for each fibre was from one impalement. Preparations were bathed in Solution 1, at 22 ± 1°C. Effects of drugs were tested by unpaired *t*-test. * $P < 0.001$.

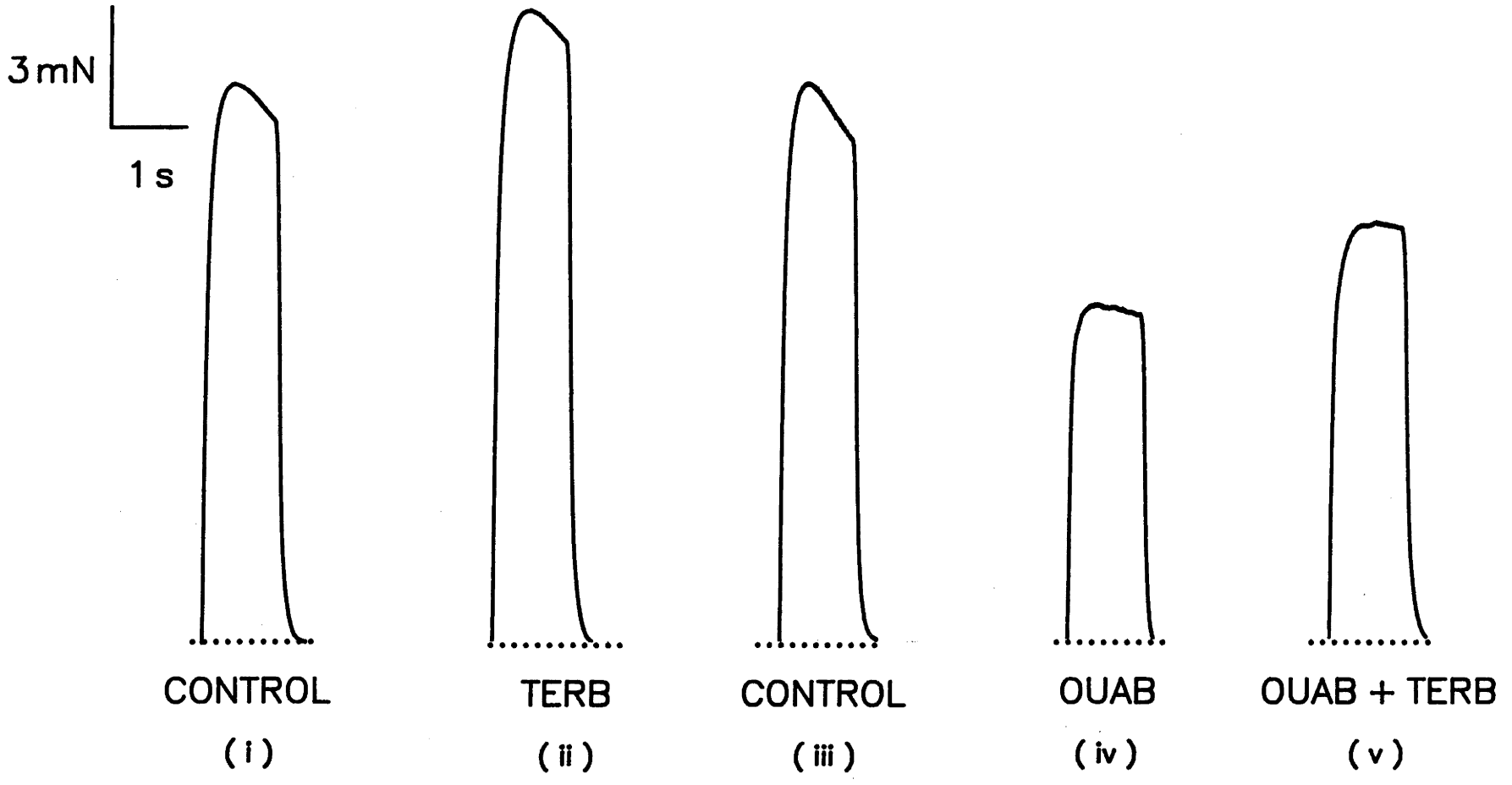


FIG. 4.3: Effect of 10 μM terbutaline on peak tetanic force (100 Hz) in the absence and presence of 1 mM ouabain in a denervated soleus preparation at 24°C. CONT and TERB are defined in the legends for Figs. 3.1 & 3.6. OUAB is the force response to 1 mM ouabain. OUAB + TERB is the maximum force response with both drugs. Terbutaline increased peak tetanic force by 13.1% (ii), relative to the first control (i). The second control was obtained 35 min after wash-out (iii). Ouabain depressed force to 60.2% of the second control in 40 min (iv). Terbutaline, in the presence ouabain, increased peak tetanic force by 23.9% (v), relative to the response with ouabain (iv).

during exposure to ouabain. Similarly, twitch potentiation by terbutaline occurred in the presence of ouabain. Ouabain was always added after control response to terbutaline since the effect of ouabain washed out only slowly (1-2 hours). These results confirm that stimulation of the Na-K pump is not required for the force potentiation by terbutaline.

(d) Effect of terbutaline and dibutyryl-cAMP on potassium contractures

β-adrenergic agonists modulate several trans-sarcolemmal ion currents, excluding Ca^{2+} currents, in cardiac muscle (Bennett & Begenisich 1987; Duchatelle-Gourdon et al. 1989; Matsuda et al. 1990). It is possible that terbutaline could also modulate ion currents in skeletal muscle to alter the action potential and increase force production. For example, terbutaline may (i) reduce Na^+ channel inactivation or (ii) reduce the voltage-dependent K^+ efflux. The influence of terbutaline on K-contractures, which do not depend on action potential activation, was investigated to exclude these possibilities. Contractures were produced by rapid application of solutions containing 60 mM K^+ (60K, Table 2.1). This concentration of K^+ was chosen because it evokes submaximal contractures; effects of terbutaline on maximal K-contracture might not be seen because of saturation of troponin-C with Ca^{2+}. The amplitude of each 60K-contracture was expressed as a percentage of the peak force of the control tetanus. Peak tetanic force usually recovered rapidly to a steady, although slightly diminished level after wash-out of 60K (Figure 4.4).

Figure 4.4 illustrates the effect of 10 μM terbutaline on K-contractures and tetani in a denervated soleus preparation. The amplitude of the first 60K-contracture was 61%. Following the recovery of peak tetanic force, terbutaline augmented peak tetanic force by 37%; one of the largest increases seen in these experiments. The amplitude of the 60K-contracture was potentiated by terbutaline to 88%. Following wash-out, the amplitude of the control 60K-contracture was again 61%. The average results of five such experiments are shown in Figure 4.5. Terbutaline increased peak tetanic force (i) by 22.4 ± 4.6%. The 60K-contracture amplitude was increased from 70.9 ± 6.3% (ii) to 93.6 ± 5.4% (iii) with terbutaline. Furthermore, the ratio of the peak force of the 60K-contracture to the tetanus was 76.7 ± 4.6% in terbutaline, which was not different to the control ($P > 0.1$, paired t-test). These results suggest that the potentiation of peak tetanic force by terbutaline was entirely independent of the action potential.

Figure 4.6 demonstrates that 2 mM DBcAMP increased the amplitude of the 60K-contracture in a denervated soleus preparation. In four experiments, DBcAMP increased the peak 60K-contracture force from 80.5 ± 12.1% (v) to 105.5 ± 13.5% (vi) (Figure 4.5). The ratio of peak 60K-contracture force to peak tetanic force in DBcAMP was 83.7 ± 8.5%, which was unchanged from the control ($P > 0.1$, paired t-test). Thus,

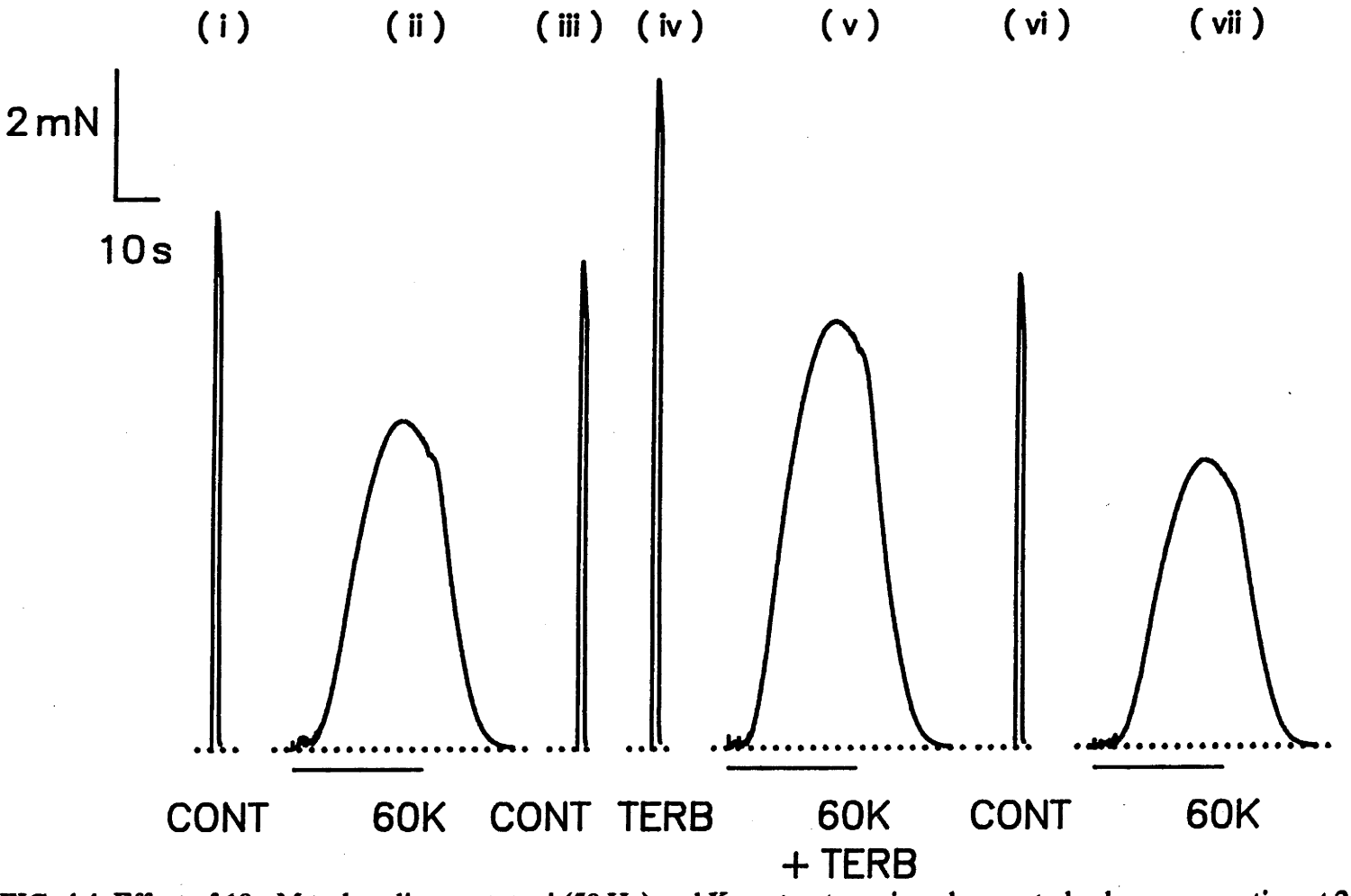


FIG. 4.4: Effect of 10 μM terbutaline on tetani (50 Hz) and K-contractures in a denervated soleus preparation at 24 °C. K-contractures were induced by rapid application of a solution containing 60 mM K^+, indicated by the horizontal lines. CONT is the control tetanic force record immediately prior to the application of terbutaline or high K^+ solutions. 60K is a contracture induced by 60 mM K^+. TERB is defined in the legend for Fig. 3.1. 60K + TERB is the 60K-contracture in the presence of terbutaline, recorded immediately after TERB. The initial 60K-contracture (ii), was 61.0% of peak tetanic force (i). Terbutaline potentiated peak tetanic force by 36.9% (iv), relative to the second control (iii). 60K + TERB (v) was 87.8% of control peak tetanic force (iii). Following wash-out, the 60K-contracture (vii) was 60.9% of peak tetanic force (vi).

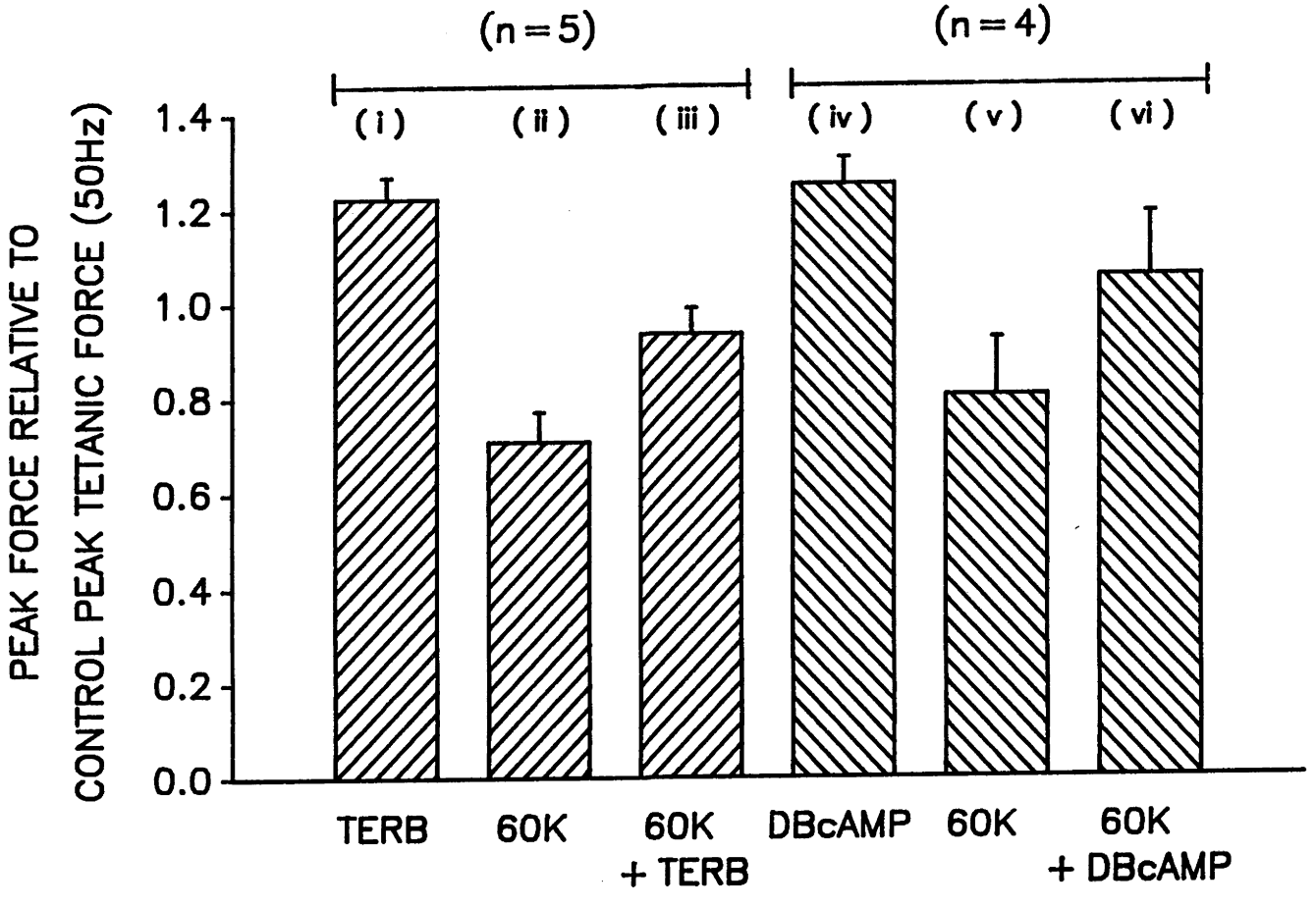


FIG. 4.5: Average effects of 10 μM terbutaline and 2 mM dibutyryl-cAMP on peak tetanic force (50 Hz) and the peak force of 60K-contractures. The peak force of K-contractures and tetani were normalized to control peak tetanic force (50 Hz). Shown are the mean values ± s.e.m. Paired data are indicated by the horizontal lines above the bars. n = number of preparations. TERB, DBcAMP, 60K, 60K + TERB, 60K + DBcAMP are defined in the legends for Figs. 3.1, 4.4 & 4.6. 60K + TERB (iii) was different from the control 60K (ii) ($P < 0.025$, paired *t*-test). 60K + DBcAMP (vi) was different from the control 60K (v) ($P < 0.001$, paired *t*-test).

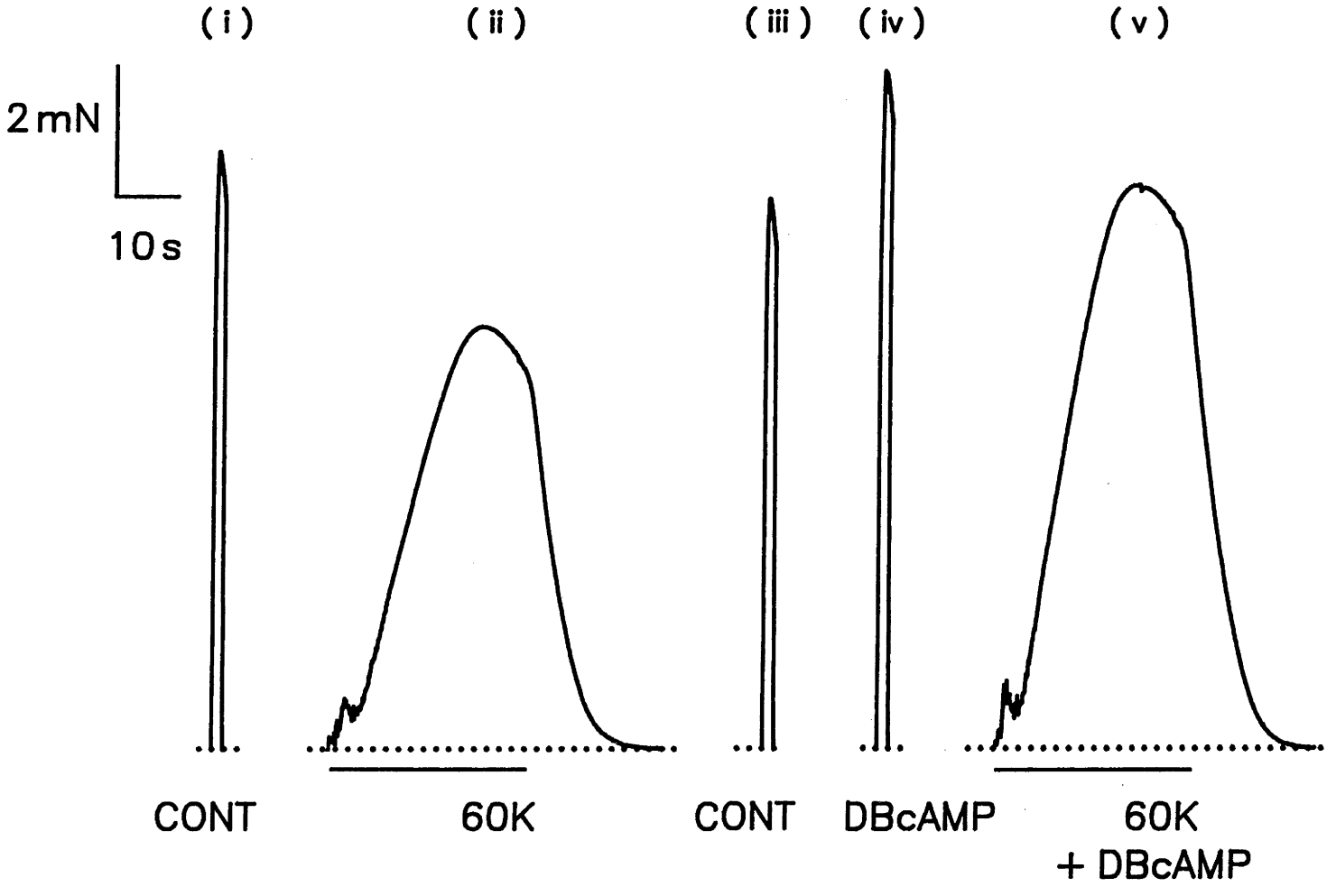


FIG. 4.6: Effect of 2 mM dibutyryl-cAMP on tetani (50 Hz) and K-contractures in a denervated soleus preparation at 24°C. CONT, 60K and DBcAMP are defined in the legends for Figs. 3.9 & 4.4. 60K + DBcAMP is the 60K-contracture in the presence of 2 mM dibutyry-cAMP, recorded immediately after DBcAMP. The first 60K-contracture (ii) was 70.9% of peak tetanic force (i). Dibutyryl-cAMP potentiated peak tetanic force by 23.3% (iv), relative to the second control (iii). 60K + DBcAMP (iv) was 102.4% of control peak tetanic force (iii).

as with terbutaline, the augmentation of peak tetanic force by DBcAMP was independent of the action potential.

These results suggest that terbutaline and DBcAMP both increase force by acting at a site beyond the action potential. That is, via either E-C coupling or the myofilaments.

(e) Effect of terbutaline on peak tetanic force in the presence of iodoacetate

Catecholamines are known to stimulate muscle carbohydrate metabolism (Ellis et al. 1955; Richter et al. 1982; Richter 1984; Chasiotis 1985; Spriet et al. 1988). It has been proposed that the increased glycolytic flux might augment force by increasing the supply of ATP (Ellis & Beckett 1954; Ellis 1959; Bowman & Raper 1964; Fellenius et al. 1980).

The effect of terbutaline on force production was examined in the absence and presence of iodoacetate - a glycolytic inhibitor - in denervated soleus fibres. Glycolysis is readily blocked with 0.5 mM iodoacetate in isolated muscle (Carlson & Siger 1959; Sahlin et al. 1981) due to the irreversible inhibition of glyceraldehyde phosphate dehydrogenase (Lehninger 1982). Following exposure to iodoacetate for 10-60 min, peak tetanic force was reduced to 81.6 ± 1.3% (n = 6) of the control. Force potentiation by terbutaline in the presence of iodoacetate was inconsistent. In three preparations, terbutaline increased peak tetanic force by 10.8 ± 2.4% in control conditions and then by 9.6 ± 3.7% in iodoacetate, suggesting that terbutaline potentiated force independently of a glycolytic flux. The other three preparations failed to show positive inotropic responses. The lack of response to terbutaline in these preparations was associated with small iodoacetate-induced increase in resting force, which presumably represents a high degree of iodoacetate poisoning and development of rigor contractures (Ellis & Beckett 1954; Bowman & Raper 1964).

(f) Effect of terbutaline on peak tetanic force in the presence of nifedipine

It is well documented that β-adrenoceptor activation leads to enhancement of a trans-sarcolemmal Ca^{2+} influx during the action potential in cardiac muscle (Reuter 1974; Reuter & Scholz 1977; Handa et al. 1982; Tsien et al. 1986; Callewaert et al. 1988). The increased Ca^{2+} current, via dihydropyridine (DHP) sensitive Ca^{2+} channels, is thought to play an important role in the β-adrenergic potentiation of force in the heart (Reuter 1974; Tsien 1977; Tsien et al. 1986; Callewaert et al. 1988). In mammalian cardiac cells the increased Ca^{2+} influx is thought to either trigger further Ca^{2+} release from the SR (Fabiato 1985; Callewaert et al. 1988) and/or more fully load the SR with Ca^{2+} for subsequent release (Katz 1979; Tada & Katz 1982; Fabiato 1985).

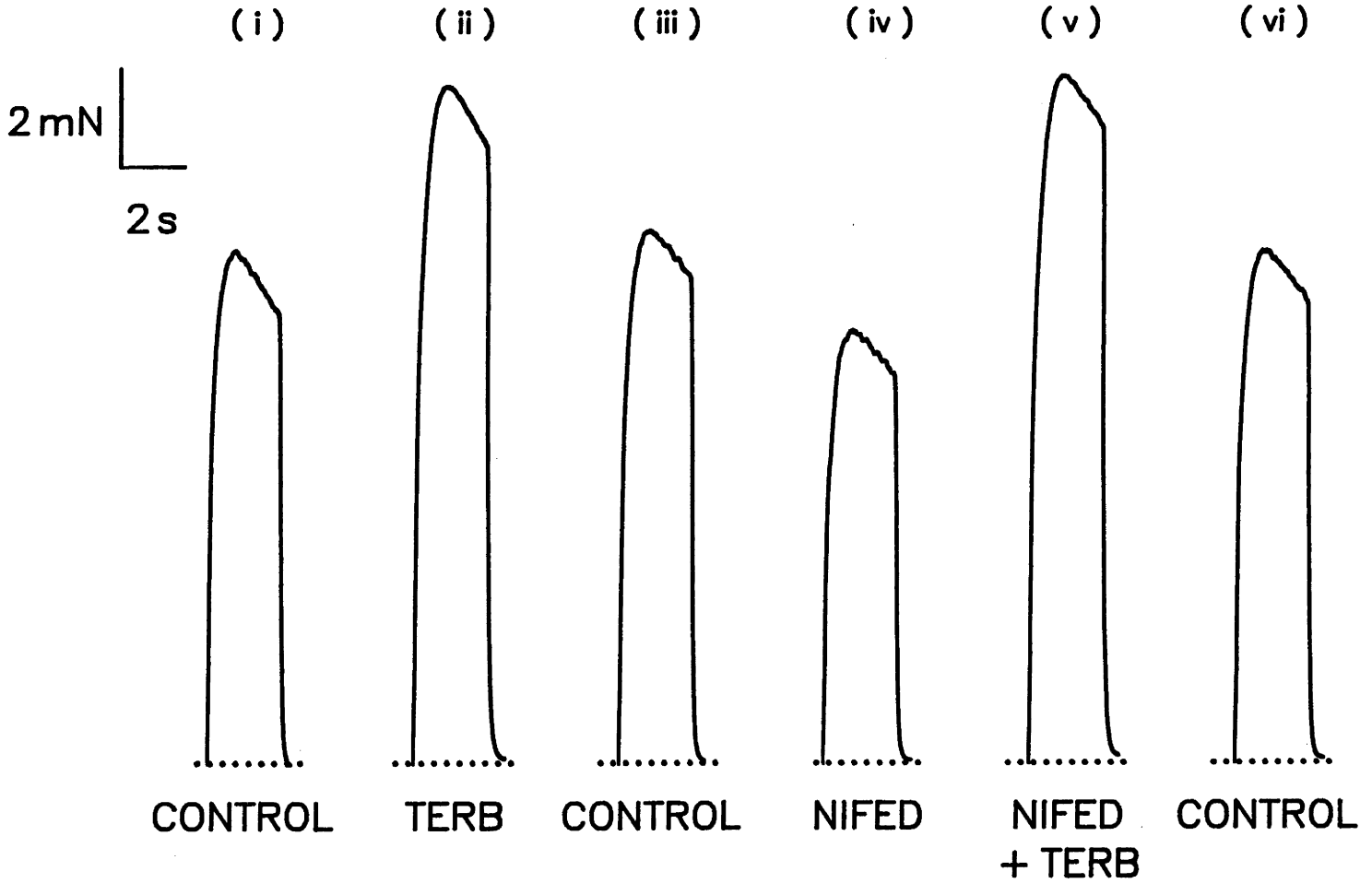


FIG. 4.7: Effect of 10 μM terbutaline on peak tetanic force (50 Hz) in the absence and presence of 10 μM nifedipine in a denervated soleus preparation at 24°C. All solutions contained 0.1% b.v. ethanol. CONT and TERB are defined in the legends for Fig. 3.1 & 3.6. NIFED is the force response to 10 μM nifedipine. NIFED + TERB is the maximum effect of terbutaline on peak tetanic force in the presence of nifedipine. Terbutaline increased peak tetanic force by 31.6% (ii), relative to the first control (i). The second control was obtained 63 min after wash-out (iii). Nifedipine, added for 20 min, depressed peak tetanic force (iv) to 81.2% of the control (iii). Terbutaline, added with nifedipine, increased peak tetanic force by 58.3% (v), relative to NIFED (iv), and the effects of both drugs washed out (vi).

The possiblity that terbutaline potentiated force in skeletal muscle by enhancement of a Ca^{2+} influx was tested with nifedipine, a Ca^{2+} channel blocker. Nifedipine (10 μM) completely abolishes slow Ca^{2+} currents (Chiarandini & Stefani 1983; Lamb 1986; Arreola et al. 1987; Lamb & Walsh 1987; Avila-Saka et al. 1990). Control experiments were performed to test the effect of ethanol - the agent in which nifedipine was dissolved - on contraction. Ethanol, at a concentration equivalent to that in the bath solution with 10 μM nifedipine (0.1% by volume), induced a small (2-3%) depression of peak tetanic force that was maintained for the duration of exposure to ethanol. Ethanol did not modify the potentiation of force by terbutaline.

Figure 4.7 shows typical effects of 10 μM terbutaline on peak tetanic force in the absence and presence of 10 μM nifedipine, in a denervated soleus preparation. Clearly, nifedipine did not prevent force potentiation by terbutaline. In five experiments, terbutaline increased peak tetanic force by 26.0 ± 4.5% in the absence of nifedipine and by 33.7 ± 11.7% in the presence of nifedipine. Nifedipine (10 μM) had direct effects on contraction. After exposure to nifedipine for 5 min, peak tetanic force was potentiated in four out of five preparations; the largest increase being 13%. Thereafter, a progressive decline of force was seen so that at 20 min peak tetanic force was 90.9 ± 4.4% (n = 5) of the control. These contractile responses to nifedipine were similar to those described previously (Ildefonse et al. 1985; Avila-Sakar et al. 1986; Dulhunty & Gage 1988; Frank et al. 1988) suggesting that nifedipine had been effective. To ensure that all Ca^{2+} channels were blocked, two experiments were performed with 50 μM nifedipine. Peak tetanic force was dramatically reduced to 55.4 ± 5.6% (n = 2) of the control, during a 15 min exposure to 50 μM nifedipine. Terbutaline augmented peak tetanic force by 45.0 ± 15.2% (n = 2) in 50 μM nifedipine. These results suggest that terbutaline does not potentiate force by increasing a slow Ca^{2+} current through dihydropyridine sensitive Ca^{2+} channels.

(g) Effect of caffeine on isometric force

It was necessary to discriminate between an increased myoplasmic [Ca^{2+}] and a direct involvement of the myofilaments in the force potentiation by terbutaline. Caffeine was used to resolve this question. Caffeine exerts two main effects on contraction, depending on the concentration used. High concentrations of caffeine, 5-20 mM, induce contractures, presumably by directly releasing Ca^{2+} from the SR (Weber & Herz 1968; Palade 1987; Rousseau et al. 1988; Allen et al. 1989) and by increasing the Ca^{2+}-sensitivity of the myofilaments (Wendt & Stephenson 1983). Low concentrations of caffeine, 0.5-2 mM, potentiate force by increasing the Ca^{2+} transient evoked by voltage activation (Kovács & Szucs 1983; Delay et al. 1986; Fryer & Neering 1989; Simon et al. 1989; Klein et al. 1990).

(i) Caffeine-induced contractures:

Application of caffeine evoked contractures in normal soleus fibres (n = 3). Figure 4.8 illustrates the generation of such contractures with increasing concentrations of caffeine (1-60 mM). Caffeine (1 mM) usually produced a small increase in resting force but this was not detected in every preparation. The amplitude of caffeine contractures increased in a concentration-dependent manner (5-60 mM). The largest contracture was recorded in 60 mM caffeine and reached about 30% of peak tetanic force. Peak contracture force was usually achieved 1-2 min after caffeine was first applied and force recovered when caffeine was washed out. Tetani could be evoked during the largest caffeine contractures (Figure 4.8). Hence, it is concluded that high caffeine concentrations alone did not induce maximal Ca^{2+} release from the SR in rat soleus. Denervated soleus fibres did not generate contractures with caffeine (1-5 mM), as seen previously in denervated slow-twitch muscle (Kotsias et al. 1987).

(ii) Caffeine-induced potentiation of contractions:

Figure 4.8 shows that caffeine also modulated isometric contractions. Maximum twitch potentiation was achieved with 15-20 mM caffeine. Tetani were potentiated in 1-5 mM caffeine. However, higher concentrations of caffeine, 20-60 mM, reduced peak tetanic force and this may have been related to the depression of maximum Ca^{2+}-activated force with high caffeine concentrations in skinned fibres (Wendt & Stephenson 1983). This depression may be a non-specific effect of caffeine (Wendt & Stephenson 1983).

Fryer and Neering (1989) showed that 1 mM caffeine increased peak tetanic force while simultaneously increasing resting $[Ca^{2+}]i$, and the amplitude and plateau phase of the tetanic Ca^{2+} transient in rat soleus fibres. Wendt and Stephenson (1983), using skinned fibres from rat soleus, demonstrated that 5-10 mM caffeine increased the Ca^{2+} sensitivity of the myofilaments and was without effect on maximum Ca^{2+}-activated force. Moreover, 1 mM caffeine had no detectable effect on Ca^{2+} sensitivity (Wendt, pers. comm.). Since caffeine acts independently of the action potential (Sandow et al. 1964; Lüttgau & Oetliker 1968; Kovács & Szucs 1983; Delay et al. 1986; Klein et al. 1990), it is likely that 1 mM caffeine potentiates force by increasing $[Ca^{2+}]i$; presumably by modulating the Ca^{2+} release channel (Rousseau et al. 1988).

Peak tetanic force (100 Hz) was increased with 1 mM caffeine by 20.7 ± 3.1% (n = 16, range 5.5-41.8%) in normal soleus and by 17.8 ± 2.6% (n = 18, range 0-54.6%) in denervated soleus fibres (Table 4.1). These responses were not different ($P > 0.1$, ANOVA). Caffeine usually exerted its maximal effect in the first 5 min following administration in both types of preparation. Caffeine (1 mM) had no significant effect

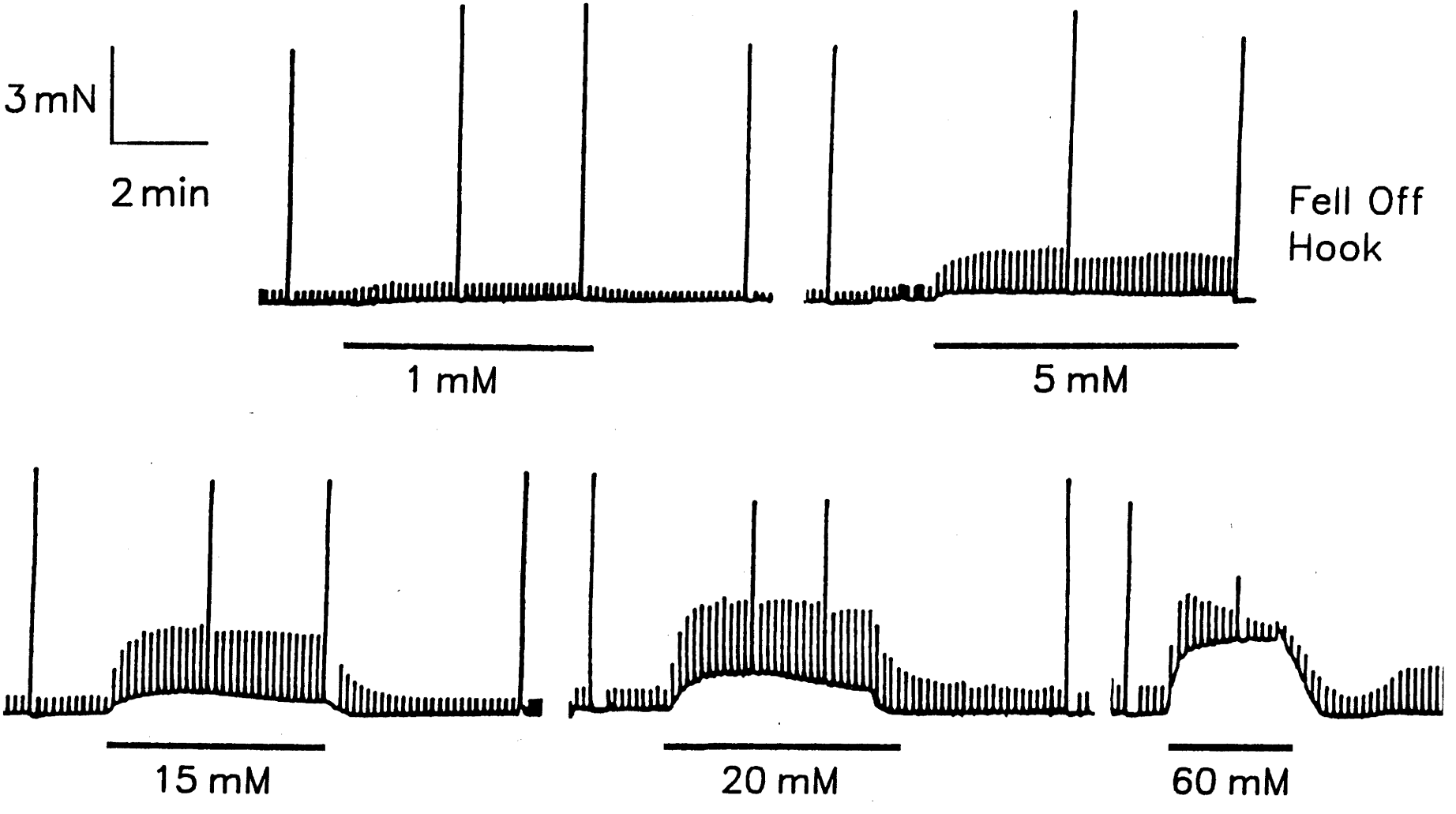


FIG. 4.8: Concentration-dependent effects of caffeine on resting force, twitch force (small responses) and tetanic force (large responses) in a single soleus preparation. Tetani were evoked at 100 Hz for 1 s, in Solution 1, at 22°C. The period of application of caffeine is indicated by the horizontal lines.

TABLE 4.3: Effect of caffeine concentration on peak tetanic force, rate of force development and rate of force relaxation in denervated soleus preparations

CONTRACTILE PROPERTY	EFFECT OF CAFFEINE (Ratio to control)		
	1 mM	2 mM	5 mM
Peak Tetanic Force #	1.116 ± 0.012 (n = 30)	1.191 ± 0.021 (n = 11)	1.187 ± 0.022 (n = 11)
Rate of Force Development	1.098 ± 0.040 * (n = 14)	1.079 ± 0.072 * (n = 8)	1.382 ± 0.172 (n = 6)
Rate of Force Relaxation	0.985 ± 0.019 * (n = 14)	0.922 ± 0.028 (n = 8)	0.840 ± 0.020 (n = 6)

The maximum effect of caffeine on each contractile property was expressed relative to the immediately preceeding control. The rate of force development 1/(20-60% rise time) and rate of force relaxation 1/(80-20% relaxation time) were calculated as described in Chapter 2. Shown are the mean values (± s.e.m.), n = number of preparations. Tetani were evoked at 50 Hz for 2 s. Solution 1, at 24°C. # Data is included from fatigue studies (50 Hz for 20 s). Effects of caffeine were significant for $P < 0.05$ except * where $P > 0.05$ (paired t-test).

on the rates of tetanic force development or relaxation in either normal or denervated soleus fibres (Table 4.3).

Table 4.1 shows the potentiation of peak tetanic force by caffeine at 100 and 50 Hz was not significantly different in normal soleus fibres although a small difference was seen in denervated soleus fibres. Paired data showing the effect of caffeine on peak tetanic force at 100 and 50 Hz in denervated soleus fibres are shown in Figure 4.1. This figure also shows a striking similarity between the effects of 1 mM caffeine and 10 μM terbutaline on peak tetanic force at either 100 or 50 Hz.

In order to assess whether possible interactions between caffeine and terbutaline could occur, it was necessary to (i) establish whether maximal tetanus potentiation was produced with 1 mM caffeine and (ii) to estimate how close control peak tetanic force was to the maximum Ca^{2+}-activated force. Thus, the effects of 1-5 mM caffeine on peak tetanic force (50 Hz) were examined. Results of these experiments are presented in Table 4.3. Increasing the caffeine concentration from 1 to 2 mM caused a further increase in peak tetanic force ($P < 0.05$, ANOVA). Peak tetanic force in 2 or 5 mM caffeine was not significantly different ($P > 0.1$, ANOVA) suggesting that the caffeine-induced potentiation of peak tetanic force was saturated. Both 2 and 5 mM caffeine slowed relaxation (Table 4.3) which is consistent with a caffeine-induced increase in the myofilament Ca^{2+} sensitivity (Wendt & Stephenson 1983) and/or a slowing of Ca^{2+} removal by the SR (Weber & Herz 1968; Palade 1987).

These results suggest that: (i) potentiation of peak tetanic force with 1 mM caffeine was submaximal; (ii) 2-5 mM caffeine increased peak tetanic force above that with 1 mM caffeine which may have been due to mechanisms additional to an enhancement of SR Ca^{2+} release; (iii) maximal Ca^{2+}-activated force was at least 20% greater than control peak tetanic force.

(h) Effect of terbutaline on isometric force in the presence of caffeine

The ability of terbutaline to potentiate peak tetanic force was tested in the absence and presence of 1 mM caffeine. There were two reasons for using 1 mM caffeine. Firstly, it was postulated that 1mM caffeine augmented peak tetanic force solely by increasing $[Ca^{2+}]_i$ through a modulation of Ca^{2+} release from the SR. Secondly, since 1 mM caffeine produced a notable but submaximal increase of peak tetanic force, additive but attenuated effects due to further Ca^{2+} activation might be seen. Therefore it was predicted that (i) if terbutaline acted by increasing the maximum Ca^{2+}-activated force then tetanus potentiation by terbutaline would be unaffected by the presence of caffeine and (ii) if terbutaline acted by increasing the Ca^{2+} occupancy of troponin-C then caffeine would attenuate the force potentiation by terbutaline.

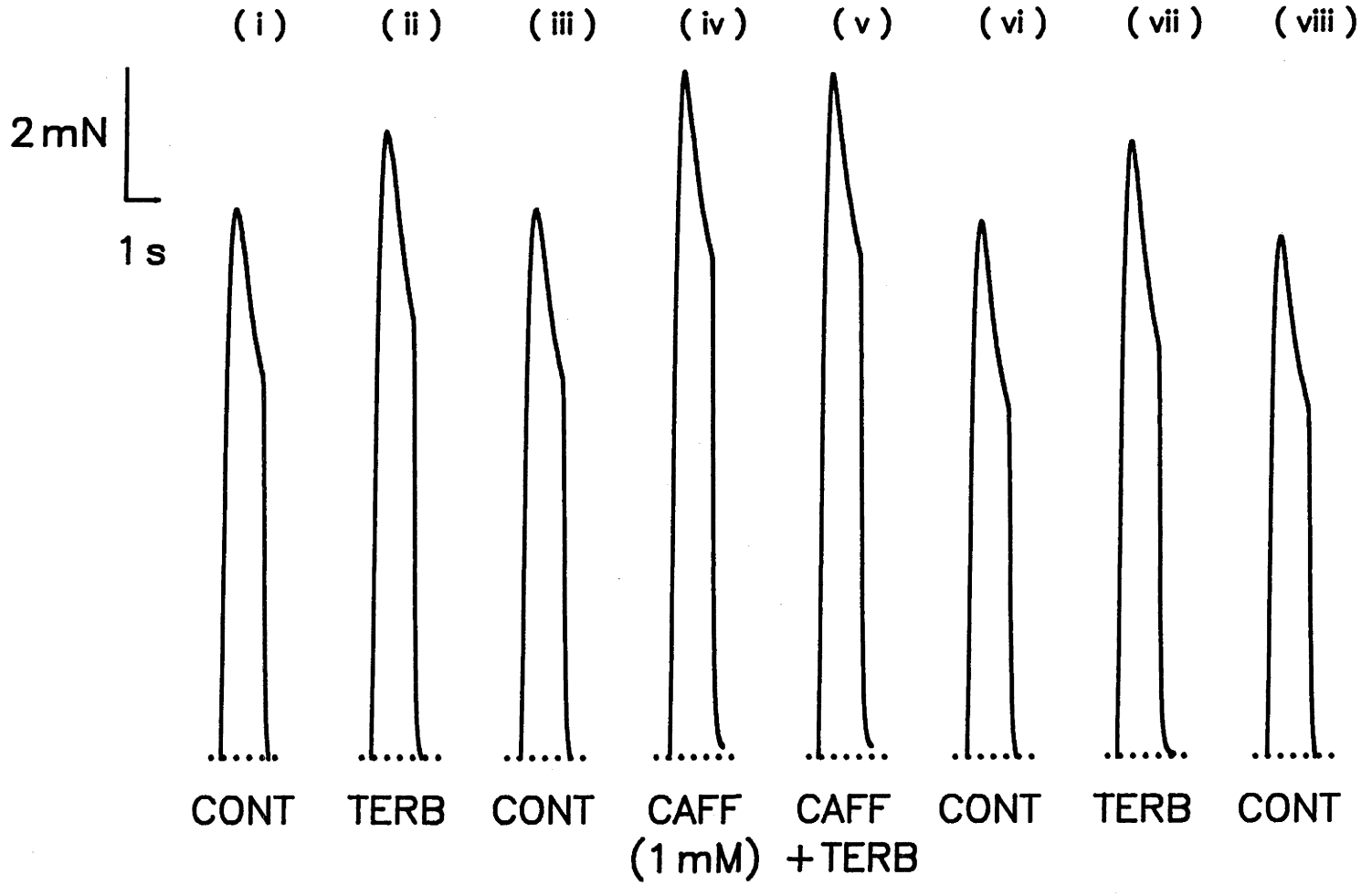


FIG. 4.9: Effect of 10 μM terbutaline on peak tetanic force (100 Hz) in the absence and presence of 1 mM caffeine in a denervated soleus preparation at 24°C. CONT and TERB are defined in the legends for Figs. 3.1. & 3.6. CAFF is the maximum effect of 1 mM caffeine on peak tetanic force in this preparation. CAFF + TERB is the maximum force response with both drugs. The increases in peak tetanic force with the drugs were: TERB (ii) 13.6%, relative to (i); CAFF (iv) 23.8%, relative to (iii); CAFF + TERB (v) 24.2%, relative to (iii); and TERB (vii) 15.0%, relative to (vi). At least 25 min was allowed after wash-out of terbutaline between drug applications.

TABLE 4.4: Effect of 10 μM terbutaline on isometric contractions in the absence and presence of 1 mM caffeine in normal and denervated soleus preparations

MUSCLE PREPARATION			PEAK FORCE (% INCREASE)		
		n	TERB	CAFF	CAFF + TERB
Normal Soleus	Tetanus (100 Hz)	5	12.2 ± 2.7	14.9 ± 4.3	16.4 ± 2.9
Denervated Soleus	Tetanus (100 Hz)	10	15.4 ± 1.7	21.3 ± 4.2	23.2 ± 4.6 *
	Tetanus (50 Hz)	8	11.4 ± 2.6	12.2 ± 1.8	14.8 ± 3.9
	Twitch	5	16.4 ± 7.0	40.3 ± 8.2	43.1 ± 10.3

Shown are the mean values (± s.e.m.), n = number of preparations. TERB, CAFF, and CAFF + TERB are described in the legend for Fig. 4.9. Solution 1, at 24°C. Differences between CAFF and CAFF + TERB were tested by paired *t*-test. $P > 0.1$ for all contractions except for * where $P < 0.05$.

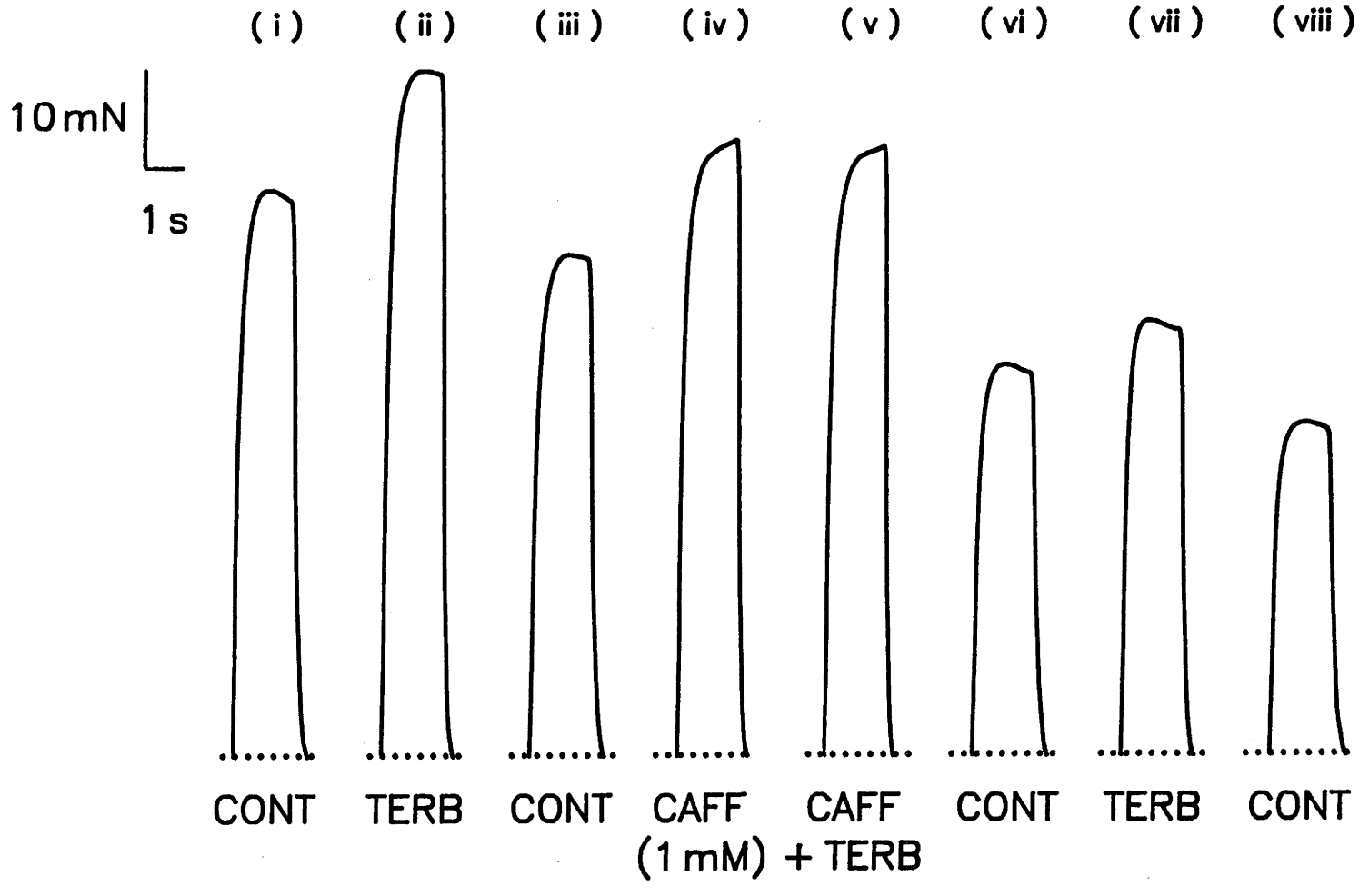


FIG. 4.10: Effect of 10 μM terbutaline on peak tetanic force (100 Hz) in the absence and presence of 1 mM caffeine in a normal soleus preparation at 24 °C. CONT, TERB, CAFF and CAFF + TERB are defined in the legends for Figs. 3.1, 3.6 & 4.9. The increases in peak tetanic force with the drugs were: TERB (ii) 20.9%, relative to (i); CAFF (iv) 22.6%, relative to (iii); CAFF + TERB (v) 21.1%, relative to (iii); and TERB (vii) 11.1%, relative to (vi). A minimum time of 30 min was allowed following wash-out of terbutaline between drug applications.

Figure 4.9 shows a representative effect of 1 mM caffeine on the ability of 10 μM terbutaline to potentiate peak tetanic force in a denervated soleus preparation. Tetani were evoked at 100 Hz to ensure that the response with 1 mM caffeine was submaximal (see Figure 4.1). Clearly, caffeine pretreatment prevented further tetanus potentiation by terbutaline. The average results are summarized in Table 4.4. In denervated soleus fibres, terbutaline produced an augmentation of 1.9 ± 0.9% over that with caffeine. The increase was significant but dramatically attenuated when compared with the control response to terbutaline. The effect of terbutaline was completely prevented by caffeine in five of the ten preparations. In the other five preparations, increases of up to 6% were seen but it was noted that these preparations usually displayed a small potentiation in caffeine alone and/or a large potentiation in terbutaline alone. Similarly, exposure to caffeine abolished the effect of terbutaline on the peak tetanic force at 50 Hz. Remarkably, caffeine also abolished the effect of terbutaline on twitches in the denervated preparation (Table 4.4).

Figure 4.10 shows the same experiment as in Figure 4.9 in a normal soleus preparation. Again, terbutaline failed to potentiate tetani during exposure to caffeine (see Table 4.4). The terbutaline-induced acceleration of tetanic relaxation still persisted in the presence of caffeine.

The ability of caffeine to dramatically attenuate or completely abolish the positive inotropic effect of terbutaline suggests that the action of caffeine, to increase the myoplasmic [Ca^{2+}], almost or completely masks the intracellular effect of terbutaline. Thus, terbutaline must act independently of the myofilaments to increase the Ca^{2+} transient and thereby makes more Ca^{2+} available to further activate the contractile proteins.

4.4 DISCUSSION

(a) Role of the action potential in the positive inotropic effect of terbutaline

(i) Reversal of the frequency-dependent depression of peak tetanic force:

High frequency stimulation depressed peak tetanic force in a manner similar to Wedensky inhibition (Lucas 1911; Cullingham et al. 1960; Truong et al. 1964). It was speculated that this depression of force was due to action potential failure and that terbutaline might potentiate force by restoring the action potential. However this was not the case as both terbutaline and DBcAMP augmented peak tetanic force at the optimal frequency where there was no frequency-dependent depression of force.

Furthermore, in conditions when the high frequency depression was not seen, i.e. in the high sodium solution, peak tetanic force was still potentiated by terbutaline.

Conceivably action potentials could be modulated by terbutaline so that the voltage sensors are further activated during stimulation thereby increasing force. Sympathomimetics do not significantly alter the amplitude or shape of intracellularly recorded action potentials in muscle fibres of the frog (Oota & Nagai 1977; Gonzalez-Serratos et al. 1981; Arreola et al. 1987) or anaesthetised cat (Lewis et al. 1977). In contrast, Smith and Thesleff (1976) reported that isoprenaline increased the overshoot of the action potential in the denervated mouse diaphragm *in vitro*, although it was without effect on the innervated diaphragm. Furthermore, terbutaline increased the amplitude and prolonged the duration of the action potential in rat muscle fibres *in vitro* (McArdle & D'Alonzo 1981). These changes in the action potential may be secondary to a either a stimulation of the Na-K pump or modulation of sarcolemmal ion channels.

(ii) Role of the sodium-potassium pump:

Several lines of evidence suggest that β-adrenergic agonists and DBcAMP increase the activity of the Na-K pump. These include: (i) an increased rate of Na^+ efflux and K^+ influx (Clausen & Flatman 1977; Pfliegler et al. 1983); (ii) an increased $[K^+]_i$ and reduced $[Na^+]_i$ (Clausen & Flatman 1977; Molnár et al. 1986; Ballanyi & Grafe 1988); (iii) an increased resting membrane potential (Bray et al. 1976; Clausen & Flatman 1977; McArdle & D'Alonzo 1981; Juel 1988); (iv) all three effects are blocked by Na-K pump inhibitors such as ouabain (Clausen & Flatman 1977; McArdle & D'Alonzo 1981; Pfliegler et al. 1983; Molnár et al. 1986). However, increases in the resting potential have not always been seen (Bray et al. 1976; Krnjevic & Miledi 1959b). In the present study, terbutaline increased the resting potential in normal soleus and sternomastoid fibres, as did DBcAMP in normal soleus fibres. The magnitude of the membrane hyperpolarization and the greater effect in slow- than in fast-twitch fibres were similar to previous studies (Tashiro 1973; Bray et al. 1976; Clausen & Flatman 1977; McArdle & D'Alonzo 1981; Juel 1988). These results support the suggestion that β-adrenergic agonists stimulate the Na-K pump via cAMP.

Terbutaline potentiated peak tetanic force in the presence of ouabain. The negative inotropic effect of ouabain suggested that the drug was effective in blocking the Na-K pump. Hence, it is concluded that force potentiation by terbutaline occurs independently of the Na-K pump. Moreover, Holmberg and Waldeck (1980) demonstrated that terbutaline potentiated twitches and unfused tetani in the presence of ouabain. They also noted that the initial terbutaline-induced depression of force was eliminated with ouabain.

(iii) Effects on potassium contractures:

Terbutaline and DBcAMP both increased the amplitude of submaximal K-contractures. Peak tetanic force and peak K-contracure force were potentiated by the same extent in terbutaline or DBcAMP. Furthermore, Oota and Nagai (1977) reported that isoprenaline and DBcAMP increased the amplitude of submaximal K-contractures (40 mM K^+) in frog twitch fibres. Neither drug influenced the membrane depolarization by high K^+ (Oota & Nagai 1977). These results unequivocally demonstrate that all of the force potentiation by β-adrenergic agonists occur at a site beyond the action potential.

(b) Mechanism of action of caffeine in rat skeletal muscle

A similarity between some of the effects of caffeine and terbutaline was observed (Figure 4.1 & Table 4.1) and, as caffeine was used in experiments with terbutaline, the postulated mechanisms of action of caffeine are discussed.

Caffeine (1-60 mM) evoked contractures in soleus fibres, the largest of which was 30% of peak tetanic force. These results were similar to those obtained previously in slow-twitch muscles at room temperature (Frank & Buss 1967; Isaacson et al. 1970; Fryer & Neering 1989). These caffeine contractures were considerably smaller than those in amphibian twitch fibres which reach 80-90% of peak tetanic force (Ludin et al. 1966; Koshita & Oba 1989; Lännergren & Westerblad 1989). Notably, the species' difference in the caffeine response disappears at higher temperatures (Frank & Buss 1967; Isaacson et al. 1970). The difference at room temperature is not due to the inability of caffeine to cross the sarcolemma (Isaacson & Sandow 1967) but could be due to a lower caffeine sensitivity of the SR and/or myofilaments in mammalian muscle. However, a discrepancy exists since 10 mM caffeine evokes force transients equivalent to maximum Ca^{2+}-activated force in skinned rabbit soleus fibres (Donaldson 1985; Donaldson, pers. comm.) yet produced only small contractures in the present study.

Caffeine contractures may be ascribed to an increase of $[Ca^{2+}]_i$ (Allen et al. 1989; Fryer & Neering 1989) due to Ca^{2+} released from the SR (Yoshioka & Somlyo 1984). The possibility that caffeine, at these high concentrations, acts by directly releasing Ca^{2+} from the SR is supported by the observations that caffeine increases the rate of Ca^{2+} release from isolated vesicles of junctional SR (Weber & Herz 1968; Palade 1987; Rousseau et al. 1988) and triggers Ca^{2+} release in skinned fibres (Donaldson 1985; Lamb & Stephenson 1990a, 1990b). These effects can be explained by a caffeine-induced activation of Ca^{2+} release channels (Rousseau et al. 1988). In

addition, a caffeine-induced sensitization of the myofilaments for Ca^{2+} may contribute to contracture force (Wendt & Stephenson 1983).

Low concentrations of caffeine have been shown to potentiate tetani in isolated mammalian skeletal muscle (Isaacson et al. 1970; Fryer & Neering 1989).This was also demonstrated in the present study where 1-5 mM caffeine increased peak tetanic force by 10-20% in both normal and denervated soleus fibres. Denervation caused no significant modification of the caffeine-induced potentiation of peak tetanic force (Table 4.1).

The mechanism by which low concentrations of caffeine potentiate tetani can be pinpointed to Ca^{2+} release from the SR. It has been shown that 1 mM caffeine increases resting $[Ca^{2+}]i$ and the peak and integral of the tetanic Ca^{2+} transient (Fryer & Neering 1989), and is without effect on the myofilaments (Wendt & Stephenson 1983; Wendt, pers. comm.). Furthermore, in amphibian fibres, low concentrations of caffeine increase the amplitude of K-contractures (Sandow et al. 1964; Lüttgau & Oetliker 1968) and the amplitude of Ca^{2+} transients evoked by voltage clamp depolarization (Kovács & Szucs 1983; Delay et al. 1986; Simon et al. 1989; Klein et al. 1990). Therefore, the potentiation of both force and $[Ca^{2+}]_i$ by caffeine does not involve the action potential. In addition, the effects of caffeine on force and $[Ca^{2+}]_i$ are independent of resting membrane potential (Axelsson & Thesleff 1958; Sandow et al. 1964; Lüttgau & Oetliker 1968), the amount of charge movement (Kovács & Szucs 1983; Klein et al. 1990) or the extracellular $[Ca^{2+}]$ (Delay et al. 1986). Furthermore, force potentiation by caffeine does not depend on phosphodiesterase inhibition (Kramer & Wells 1980; Fryer & Neering 1989). These observations support the notion that low caffeine concentrations increase force by modulating E-C coupling so as to increase the myoplasmic $[Ca^{2+}]$.

When comparing the effects of terbutaline and caffeine, several features were noted. Firstly, the potentiation of peak tetanic force by 10 μM terbutaline or 1 mM caffeine was of a similar magnitude, although the effects of caffeine occurred more rapidly. Secondly, both drugs acted independently of the action potential as K-contractures were augmented by terbutaline (present study) and low concentrations of caffeine (Sandow et al. 1964; Lüttgau & Oetliker 1968). Thirdly, the effects of both drugs on the rate of development of tetanic force and relaxation were similar in denervated soleus fibres. However, in normal soleus fibres, relaxation occurred more rapidly in terbutaline whereas caffeine was without effect. Fourthly, terbutaline did not evoke contractures as seen with high concentrations of caffeine. This is consistent with an inability of cAMP to directly release Ca^{2+} from isolated SR (Weber 1968).

Since caffeine can potentiate tetani, and maximum K-contractures can generate about 20% more force than the control peak tetanic force (Chapter 5.3c), it must therefore be possible to increase peak tetanic force by either increasing the myoplasmic $[Ca^{2+}]$ or increasing the Ca^{2+} sensitivity of the myofilaments.

(c) Role of the myofilaments in the positive inotropic effect of terbutaline

To test the hypothesis that terbutaline increased force by acting on the myofilaments, terbutaline was added with 1 mM caffeine. Under these conditions the terbutaline-induced potentiation of force was almost or completely abolished. This result suggests that terbutaline increases force by a mechanism independent of the myofilaments since an increase in either crossbridge activity or Ca^{2+} sensitivity would have been expected to potentiate submaximal tetani.

Since caffeine is a weak phosphodiesterase inhibitor, and consequently increases $[cAMP]_i$ (Butcher & Sutherland 1962; Kramer & Wells 1980), caffeine might have attenuated further increases in $[cAMP]_i$ with terbutaline, and thus limited force potentiation. However, in normal soleus fibres caffeine did not accelerate relaxation, but terbutaline with caffeine did accelerate relaxation. Since the acceleration of relaxation by terbutaline depends on cAMP, it is likely that terbutaline could further increase $[cAMP]_i$ in the presence of caffeine. Furthermore, biochemical studies have shown that sympathomimetics increase $[cAMP]_i$ by 100-200% (Al-Jeboory & Marshall 1978; Fellenius et al. 1980; Bowman et al. 1985; Chasiotis 1985) which is in excess of the 50% increase seen with 3 mM caffeine (Kramer & Wells 1980). The change of $[cAMP]_i$ with 1 mM caffeine, 10 μM terbutaline, or both together, remains to be determined.

Other studies also suggest that the myofilaments are not involved in the positive inotropic effect of catecholamines. Oota and Nagai (1977) found that isoprenaline did not potentiate maximal K-contractures nor caffeine contractures. In a more direct approach, Fabiato and Fabiato (1978), were unable to show any influence of cAMP on the myofilament Ca^{2+} sensitivity in skinned fast-twitch fibres. Similarly, Gonzalez-Serratos et al. (1981), using split fibres from frog twitch muscle, demonstrated that cAMP had no effect on maximum Ca^{2+}-activated force but reduced the Ca^{2+} sensitivity and hence could not account for the force potentiation.

In cardiac muscle, two myofilament proteins are phosphorylated during β-adrenoceptor activation; namely troponin-I (Solaro et al. 1976; England et al. 1984; Garvey et al. 1988) and C-protein (England et al. 1984; Hartzell 1984; Garvey et al. 1988). Both of these proteins are present in skeletal muscle but as different isozymes to their cardiac counterparts (Stull & High 1977; Moos & Feng 1980; Yamamoto & Moos 1983).

Neither troponin-I nor C-protein are phosphorylated during β-adrenergic activation in intact skeletal muscle (Stull & High 1977; Stull et al. 1980; England et al. 1984). Furthermore, phosphorylation of troponin-I causes a decrease in Ca^{2+} sensitivity in cardiac muscle (Endoh & Blinks 1988; McIvor et al. 1988); an effect opposite to that required for an increase of force.

(d) Role of excitation-contraction coupling in the positive inotropic effect of terbutaline

Catecholamines are known to increase the amplitude of the Ca^{2+} transient in cardiac muscle (Allen & Blinks 1977; Fabiato 1981; Kurihara & Konishi 1987; Callewaerts et al. 1988; Endoh & Blinks 1988). Recently, Brum et al. (1990) reported that adrenaline increases the amplitude of the Ca^{2+} transient during voltage clamp depolarization in amphibian twitch fibres. No such studies have been performed on mammalian skeletal muscle. Certainly the simultaneous measurement of force and $[Ca^{2+}]_i$ in the presence of terbutaline or DBcAMP would provide invaluable information as to their mechanism of action. The possibility that terbutaline modulates processes involved in E-C coupling resulting in increased Ca^{2+} release are discussed.

(i) Stimulation of glycogenolysis:

It has been postulated that a catecholamine-induced stimulation of carbohydrate metabolism might increase force production (Ellis & Beckett 1954; Ellis 1959; Bowman & Raper 1964; Fellenius et al. 1980). An increased supply of ATP via the glycolytic pathway might affect E-C coupling by directly modulating the Ca^{2+} release channel (Meissner 1984; Smith et al. 1985, 19866) or possibly by influencing inositol trisphosphate metabolism (Vergara & Asotra 1987). A high [ATP] does not increase force via an effect on the myofilaments (Ashley & Moisescu 1977).

Terbutaline potentiated tetani in the presence of iodoacetate, thus eliminating a role of the glycolytic pathway in the positive inotropic response. Twitch potentiation by adrenaline in the presence of iodoacetate has also been observed (Ellis & Beckett 1954; Bowman & Raper 1964).

(ii) Role of a trans-sarcolemmal Ca^{2+} current:

An enhanced Ca^{2+} influx might be important in the catecholamine-induced potentiation of force in skeletal muscle for the following reasons. Firstly, DHP-sensitive Ca^{2+} channels which are present in the sarcolemma of mammalian skeletal muscle are phosphorylated by cAMP-PK when reconstituted into lipid bilayers (Curtis & Catterall 1985; Hosey et al. 1986; Nastainczyk et al. 1987; Hymel et al. 1988; Nunoki et al. 1989) and following β-adrenoceptor activation in intact fibres (Weilenmann et al.

1990). Secondly, sympathomimetic agonists and activators of the cAMP pathway increase the amplitude of Ca^{2+} currents recorded in single fibres (Arreola et al. 1987; Stefani et al. 1987; Gamboa-Aldeco et al. 1989) and increase the activity of purified Ca^{2+} channels reincorporated into artificial membranes (Flockerzi et al. 1986; Trautwein et al. 1987; Yatani et al. 1988; Nunoki et al. 1989). Thirdly, sympathomimetics stimulate Ca^{2+} movements into cultured muscle cells (Schmid et al. 1985; Mills & Ng 1990).

In previous studies, twitch potentiation by sympathomimetic agonists has been shown to be antagonized by: (i) removal of external Ca^{2+} (Varagic & Kentera 1978; Arreola et al. 1987; Prostan & Varagic 1986; Olin 1987; Williams & Barnes 1989a); (ii) exposure to Ca^{2+} channel blockers D-600 and diltiazem (Williams & Barnes 1989a); (iii) a lack of action potential stimulation as Ca^{2+} channels are mainly closed at rest (Arreola et al. 1987; Williams & Barnes 1989a). All of these results support the hypothesis that an increased Ca^{2+} influx contributes to twitch potentiation. The Ca^{2+} channel blocker nifedipine was used in the present study, rather than lowering extracellular [Ca^{2+}], as the voltage sensors of E-C coupling require Ca^{2+} to function normally (Brum et al. 1988a, 1988b; Dulhunty & Gage 1988). Exposure to nifedipine did not prevent tetanus potentiation by terbutaline, a similar observation to that of Arreola et al. (1987). One criticism might be that nifedipine had not blocked the Ca^{2+} current (Walsh et al. 1986). However, the concentration of nifedipine was well in excess of that needed to abolish slow Ca^{2+} currents (Chiarandini & Stefani 1983; Lamb 1986; Arreola et al. 1987; Lamb & Walsh 1987; Avila-Sakar et al. 1989). Furthermore, the contractile effects of nifedipine *per se* suggest it was active, i.e. effects attributed to either a blockade of slow Ca^{2+} currents or to direct effects on the voltage sensor (Ildefonse et al. 1985; Avila-Sakar et al. 1986; Rios & Brum 1987; Dulhunty & Gage 1988; Frank et al. 1988). Therefore, the present results suggest that terbutaline potentiates force independently of the slow Ca^{2+} current through DHP-sensitive Ca^{2+} channels.

Sympathomimetic agonists also enhance a fast Ca^{2+} current through nifedipine-insensitive Ca^{2+} channels in frog twitch fibres (Stefani et al. 1977; Arreola et al. 1978). However, such fast Ca^{2+} currents have not been recorded in adult mammalian skeletal muscle (Beam & Knudson 1988; Lamb & Walsh 1987) although this mechanism remains a possibility.

(iii) Role of asymetric charge movement:

The DHP-receptor-Ca^{2+} channel of the T-system is thought to have an additional role as the voltage sensor of E-C coupling (Lamb & Walsh 1987; Rios & Brum 1987; Ríos & Pizarró 1988; Tanabe et al. 1988). Since this protein is phosphorylated by cAMP-PK (Curtis & Catterall 1985; Hosey et al. 1986; Nastainczyk et al. 1987; Hymel et al. 1988;

Nunoki et al. 1989; Weilenmann et al. 1990), it is possible that charge movement is increased by sympathomimetic agonists. However, in two studies to date, neither DBcAMP nor adrenaline had any effect on charge movement (Gamboa-Aldeco et al. 1989; Brum et al. 1990).

(iv) Increased Ca^{2+} loading of the SR:

In cardiac muscle, some of the β-adrenoceptor-mediated increase in Ca^{2+} release occurs independently of a trans-sarcolemmal Ca^{2+} influx (Fabiato 1981; Boller & Potts 1988; Calleweart et al. 1988) and has been attributed to the cAMP-dependent phosphorylation of phospholamban, resulting in enhanced Ca^{2+} loading of the SR (Katz 1979; Boller & Potts 1988; Callewaert et al. 1988). A similar mechanism has been proposed for twitch potentiation by catecholamines in skeletal muscle (Gonzalez-Serratos et al. 1981). This proposal is supported by the observations that adrenaline caused a reduction in resting $[Ca^{2+}]_i$ as measured with microelectrodes (Ballanyi & Grafe 1988), and cAMP caused force relaxation in skinned fibres (Fabiato & Fabiato 1978). In contrast, cAMP-PK has no effect on the SR Ca^{2+} storage capacity in skinned fibres (Salviati et al. 1982). Moreover, phospholamban is present in slow- but not fast-twitch fibres (Inui et al. 1985; Jorgensen & Jones 1986; Tada et al. 1988) but positive inotropic responses to terbutaline occur in both types of muscle. Therefore phospholamban does not have a force potentiating role, at least not in fast-twitch fibres.

(v) Role of the Ca^{2+} release channel:

Meissner (1984) demonstrated that cAMP enhanced the rate of Ca^{2+}-induced Ca^{2+} release from isolated SR. Although cAMP does not directly release Ca^{2+} from the SR (Weber 1968), cAMP could well have a modulatory role in Ca^{2+} release. In support of this hypothesis, Seiler et al. (1984) demonstrated that cAMP-PK phosphorylates high molecular weight proteins in junctional SR which they postulated were junctional foot proteins - now thought to be the Ca^{2+} release channel (Fleischer et al. 1985; Inui et al. 1987). Indeed, cAMP-PK can phosphorylate the isolated Ca^{2+} release channel (Timerman et al. 1990) and stimulate channel activity (Hymel et al. 1989).

Caffeine activates the Ca^{2+}-induced Ca^{2+} release mechanism (Endo 1977; Rousseau et al. 1988), enhances Ca^{2+} release channel activity (Rousseau et al. 1988), and abolishes the effect of terbutaline on force. Since caffeine-induced force potentiation was submaximal, force could be further increased by elevating myoplasmic $[Ca^{2+}]$. The abolishment of the terbutaline response in caffeine may therefore be interpreted as if: (i) caffeine saturated the Ca^{2+}-induced Ca^{2+} release mechanism to prevent the additional effects of cAMP or (ii) caffeine desensitized the Ca^{2+}-induced Ca^{2+} release mechanism to cAMP (Koshita & Oba 1989). Hence, it is hypothesized that the positive

inotropic effects of terbutaline are due to a cAMP-dependent modulation of the Ca^{2+} release channel.

(e) Model for the force potentiation by terbutaline

(i) Binding of terbutaline to β-adrenoceptors activates adenylate cyclase, resulting in an increased myoplasmic [cAMP]. Subsequently, cAMP activates a number of protein kinases, one of which phosphorylates the Ca^{2+} release channels of the SR.

(ii) During twitch or tetanic stimulation the normal voltage-dependent processes are initiated to activate the Ca^{2+} release channels of the SR. Subsequently, Ca^{2+} is released into the myoplasm.

(iii) Those Ca^{2+} release channels phosphorylated by cAMP-PK display a greater degree of activation during action potential stimulation, resulting in a greater amount of Ca^{2+} being released, and thereby increasing the amplitude of the Ca^{2+} transient.

(iv) More Ca^{2+} is now available to bind to troponin-C resulting in an increased force production.

4.5 SUMMARY

(1) The mechanism/s responsible for the terbutaline-induced potentiation of isometric force were investigated in bundles of intact fibres isolated from normal and denervated rat soleus muscles at 24°C.

(2) Maximum tetanic force was generated at 50 Hz; force was depressed at higher stimulation frequencies. The potentiation of peak tetanic force at 100 Hz was not simply a reversal of this force depression since terbutaline potentiated tetani evoked at 50 Hz.

(3) Addition of ouabain failed to prevent the terbutaline-induced potentiation of force, suggesting that the Na-K pump was not involved.

(4) Terbutaline (10 μM) and DBcAMP (2 mM) both increased the amplitude of submaximal K-contractures. The ratio of peak K-contracture force to peak tetanic force was unchanged in the presence of each drug. This suggests that both terbutaline and DBcAMP act to increase force at a site beyond the action potential.

(5) Terbutaline augmented peak tetanic force in the presence of 0.5 mM iodoacetate, a glycolytic inhibitor, suggesting that a stimulation of carbohydrate metabolism was not responsible for the increase of force.

(6) Exposure to 10 μM nifedipine, which is known to abolish the slow Ca^{2+} current, failed to prevent the positive inotropic effect of terbutaline. This suggests that a Ca^{2+} influx through DHP-sensitive Ca^{2+} channels was not necessary for the force potentiation by terbutaline.

(7) Caffeine (1-60 mM) evoked contractures in normal soleus fibres. The caffeine contracture amplitude increased in a concentration-dependent manner with the largest increase being about 30% of peak tetanic force.

(8) Low concentrations of caffeine (1-5 mM) potentiated twitches and tetani. The potentiation of peak tetanic force by 1 mM caffeine at both 100 and 50 Hz was quantitatively similar to that of 10 μM terbutaline in both normal and denervated soleus fibres. In denervated soleus fibres, 2-5 mM caffeine induced a maximum tetanus potentiation of about 20%.

(9) Prior exposure to 1 mM caffeine prevented the terbutaline-induced potentiation of peak tetanic force, although troponin-C was not saturated with Ca^{2+}. This suggests that terbutaline does not increase force via the myofilaments.

(10) These results support the notion that terbutaline potentiates force by increasing the efficacy of a process involved in E-C coupling, i.e. somewhere from charge movement to Ca^{2+} release from the SR. A model is proposed whereby terbutaline, via the cAMP-dependent phosphorylation of the Ca^{2+} release channel, triggers additional Ca^{2+} release from the SR during tetanic stimulation, resulting in the further activation of the contractile proteins.

CHAPTER 5

MECHANISMS OF HIGH-FREQUENCY FATIGUE

5.1 INTRODUCTION

Muscle fatigue has been defined as "the inability to sustain the required force or power output" (Edwards 1981, 1986). This definition is useful for describing whole animal performance, however, it implies that fatigue occurs suddenly after a delay. Subsequently, Bigland-Ritchie and Woods (1984) proposed that fatigue is "any reduction in the force generating capacity of the total neuromuscular system regardless of the force required" which accounts for the reduction of force in a maximum voluntary contraction occurring soon after the onset of contractile activity.

Fatigue may be due to an impairment of any process in the chain of events linking activation of motor cortical neurons in the central nervous system (CNS) to the interaction between actin and myosin proteins in the myofibril. A component of fatigue can originate in the CNS since peripheral nerve or percutaneous stimulation can sometimes generate more force than maximal voluntary activation (Bigland-Ritchie et al. 1978, 1986; Gandevia 1990). Most human studies indicate that peripheral factors account for most, if not all, of the fatigue (Merton 1954; Merton et al. 1981; Bigland-Ritchie et al. 1986; Vollestad et al. 1988; Thomas et al. 1989). Neuromuscular transmission failure is not normally a cause of fatigue in human voluntary contractions since there is no change in the amplitude of the muscle compound action potential (Merton 1954; Merton et al. 1981; Bigland-Ritchie et al. 1982). Furthermore, in studies on isolated nerve-muscle preparations, there is usually no difference in the force generated by either muscle or nerve stimulation during fatigue (Fitts & Holloszy 1977; Roed 1988). However, there is some evidence for impaired neuromuscular transmission during continuous high-frequency stimulation (Brown & Burns 1949; Krnjevic & Miledi 1958a, 1959; Thesleff 1959). Thus most of the fatigue is thought to occur in the muscle fibres. In fact, direct muscle stimulation can produce all the characteristic features of fatigue that are seen during voluntary contractions (Eberstein & Sandow 1963; Jones et al. 1979; Jones 1981; Allen et al. 1989; Lännergren & Westerblad 1989).

Fatigue may be due to impaired function at a number of sites in the muscle fibres. (i) The muscle action potential has been implicated in some studies because of a reduction in the amplitude of the action potential and electromyogram (EMG) (Bigland-Ritchie et al. 1979; Jones et al. 1979; Hultman & Sjöholm 1983; Clamann & Robinson 1985; Sandercock et al. 1985; Lännergren & Westerblad 1986). (ii) A failure of E-C coupling was proposed by Eberstein and Sandow (1963) on the basis that rapid application of high $[K^+]$ or caffeine solutions could restore force in fatigued muscle. Similar findings

have since been obtained many times (Grabowski et al. 1972; Gonzalez-Serratos et al. 1978; Kanaya et al. 1983; Allen et al. 1989; Lännergren & Westerblad 1989). Recent direct measurement of tetanic $[Ca^{2+}]_i$ has confirmed a reduction of SR Ca^{2+} release during fatigue (Allen et al. 1989; Allen & Westerblad 1990; Westerblad et al. 1990). (iii) Impaired myofilament function has also been implicated in skinned fibre studies, since solutions composed to mimic the metabolic changes during fatigue (acidosis, increased inorganic phosphate) depress force (Donaldson et al. 1978; Cooke et al. 1988; Godt & Nosek 1989). Measurements of $[Ca^{2+}]_i$ and force in intact, single fibres have confirmed that a reduction of maximum Ca^{2+}-activated force and myofibrillar Ca^{2+} sensitivity occurs during fatigue (Allen et al. 1989; Allen & Westerblad 1990).

The cellular mechanisms responsible for impaired function at these different sites are not well understood. Several putative factors have been linked with the decline of force and these include: (i) a depletion of fuel stores such as muscle glycogen (Bergström et al. 1967; Costill 1973; Saltin 1981); (ii) changes in metabolite levels - in particular an increased concentration of lactic acid (Fitts & Holloszy 1976; Hermansen 1981; Sahlin et al. 1981) and an associated acidosis (Dawson et al. 1978; Westerblad & Lännergren 1988), or increased concentrations of inorganic phosphate (Dawson et al. 1978; Cady et al. 1989a); (iii) changes in the trans-sarcolemmal ionic distribution - especially for K^+ and Na^+ (Jones et al. 1979; Juel 1986; Sjogaard 1986). The site and relative contribution of metabolic, ionic, and/or other factors during fatigue presumably depend on the extent of fatigue and the stimulation protocol used. Two types of fatigue have frequently been described based largely on the contractile responses to two markedly different patterns of stimulation. (i) Fatigue induced by intermittent short duration tetanic stimulation, called intermittent tetanic fatigue, is characterised by a relatively slow decline of tetanic force (several min.) and slow recovery (min. to hours). This protocol has been used to mimic repetitive activation as in locomotion. (ii) Fatigue produced by continuous high frequency tetanic stimulation (e.g. > 30 Hz) is called high-frequency fatigue (HFF), and typically shows a rapid decline of peak tetanic force (< 30 s) and rapid recovery (few seconds) (Jones et al. 1979; Jones 1981; Lännergren & Westerblad 1986). This protocol has been used to represent the type of fatigue in a sustained maximum voluntary contraction. However, there are some important differences. In a maximum voluntary contraction, the motoneuron firing frequency slowly declines in order to optimise force production (Bigland-Ritchie et al. 1979; Jones et al. 1979; Gandevia 1990) and the muscle fibres are ischaemic (Bigland-Ritchie & Woods 1984; Sjogaard et al. 1988). These features are not usually reproduced in studies on HFF in isolated muscle preparations.

In this thesis fatigue is defined as "any decline in maximum force generation resulting from direct electrical stimulation", i.e. any decline of peak tetanic force. The objectives

of this study were to (i) establish the site and cellular mechanism/s involved in HFF and (ii) to investigate differences in the rate of HFF following denervation and in different fibre-types.

5.2 METHODS

Muscle preparations, methodology and the measurement of contractile properties are described in Chapter 2. The composition of solutions used is shown in Table 2.1. Preparations were usually bathed in Solution 1 at 24°C. Contractile properties measured were: peak twitch and tetanic force; 80-20% relaxation time (80-20%RT); 20-60% rise time (20-60%AT); 95-50% fatigue time (95-50%FT); plateau time (PT); and fatigue indices at 1, 2, 5, 10, 20 s where the fatigue index is defined as the force generated at a given stimulation duration expressed as a percentage of peak tetanic force (see Figure 2.3). Values in the text are the mean value (± s.e.m.) and n is the number of preparations.

In preliminary experiments it was observed that the rate of HFF was not altered by the absence or presence of 100% O_2, although solutions were bubbled with 100% O_2 to help slow run-down. To allow full recovery of peak tetanic force sternomastoid or soleus preparations (normal and denervated) were allowed to rest for 3-4 min or 10 min respectively between prolonged tetani.

5.3 RESULTS

(a) High-frequency fatigue: Role of fibre-type

Differences in the rate of fatigue, i.e. fatigue resistance during intermittent tetanic nerve stimulation have been described for different types of motor unit in mammalian muscle (Burke et al. 1973; Clamann & Robinson 1985; Sandercock et al. 1985). However, much less is known about differences in fatigue resistance between different fibre-types during prolonged continuous tetanic stimulation.

(i) Effects on force:

HFF was produced by continuous stimulation at 100 Hz until peak tetanic force had fallen by more than 50%. This usually took 1-3 s in sternomastoid and 10-20 s in soleus preparations. A common stimulation frequency of 100 Hz was used to compare the rate of fatigue in preparations composed of different fibre-types. This frequency induced fused tetani in all preparations. However, maximum force was slightly depressed at 100 Hz compared to 50 Hz in soleus fibres (see Chapter 5.3e), while frequencies lower than 100 Hz failed to evoke maximum force in sternomastoid fibres. Consecutive fatigue curves - force records in prolonged tetani when expressed as a percentage of peak

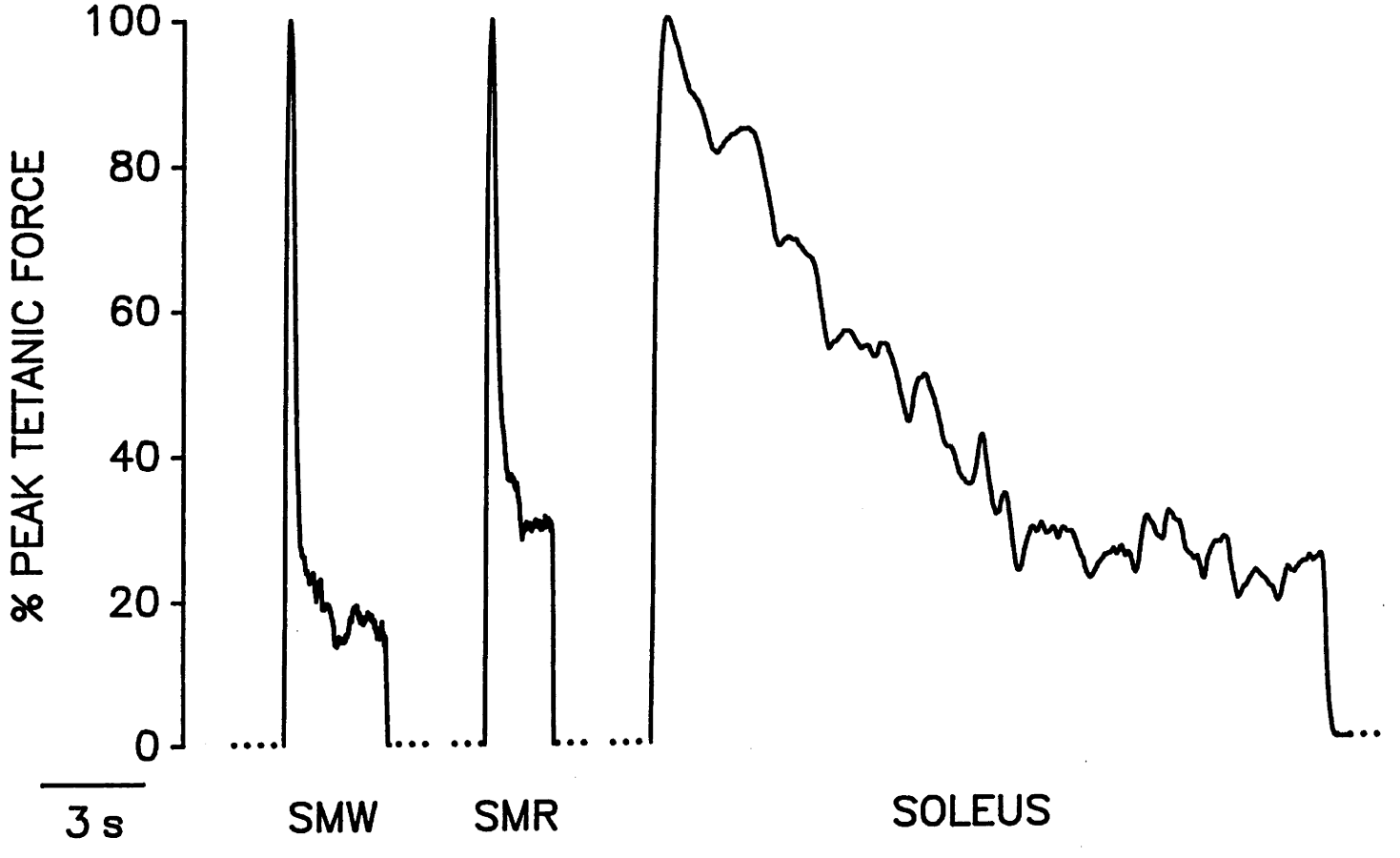


FIG.5.1: High-frequency fatigue induced by continuous tetanic stimulation at 100 Hz in preparations composed of different fibre-types. These fatigue curves were produced by normalizing the absolute force records to peak tetanic force and show representative differences in the rate of fatigue between the white-sternomastoid (SMW), red-sternomastoid (SMR) and soleus fibres. Solution 1, 24°C.

TABLE 5.1: High-frequency fatigue properties in preparations composed of different fibre-types

FATIGUE PROPERTY	White-Sternomastoid	Red-Sternomastoid	Normal Soleus
95-50% FT (ms)	319 ± 54 (n = 12)	838 ± 198 (n = 11)	4627 ± 744 (n = 9)
FI_1	40.6 ± 5.1% (n = 19)	60.4 ± 4.7% (n = 19)	———
FI_2	32.0 ± 7.3% (n = 11)	50.8 ± 5.4% (n = 14)	———
FI_5	———	———	59.6 ± 5.2% (n = 10)

Shown are the mean values (± s.e.m.). n = number of preparations. Tetani were evoked at 100 Hz. Solution 1, at 24°C. Measurement of the fatigue properties (95-50% fatigue time (95-50%FT) and fatigue indices at 1, 2 and 5 s (FI_1, FI_2, FI_5) are described in Chapter 2. Fatigue properties were different between the different types of preparation. $P < 0.05$ (unpaired t-test).

tetanic force - were reproducible to within 5%. The rate of HFF increased with the duration of the experiment and consequently, the fatigue parameters for a given preparation were calculated as the average from the first two fatigue curves produced following equilibration.

Figure 5.1 shows representative fatigue curves (100 Hz) from individual white- and red-sternomastoid, and soleus preparations. This figure illustrates the relative differences in fatigue resistance between these preparations. Clearly, HFF occurred more rapidly in fast-twitch than in slow-twitch fibres. These preparations did not produce the prolonged plateau of force seen in amphibian fibres (Nassar-Gentina et al. 1981; Lännergren & Westerblad 1986). Instead, peak tetanic force declined rapidly and this was followed by a slower and often more irregular reduction in force that could sometimes be maintained for several seconds.

Average fatigue parameters are presented in Table 5.1. Red-sternomastoid fibres were slightly more resistant to HFF than white-sternomastoid fibres. The 95-50% fatigue time (95-50%FT) which describes the fast phase of fatigue, was 319 ± 54 ms (n = 12) and 838 ± 198 ms (n = 11) in the white- and red-sternomastoid preparations respectively ($P < 0.02$, unpaired *t*-test). The slower phase of fatigue, indicated by the 2 s fatigue index (FI_2), was also more marked in the white-sternomastoid: 32.0 ± 7.2% (n = 11) in white- and 50.8 ± 5.4% (n = 14) in the red-sternomastoid preparations ($P < 0.05$, unpaired *t*-test).

The greater resistance to HFF in soleus than in sternomastoid fibres is demonstrated by the 95-50%FT of 4627 ± 744 ms (n = 9) in soleus ($P < 0.001$, unpaired *t*-test). A slower rise of tetanic force in soleus (Table 2.2) meant that peak tetanic force was achieved after stimulation for about 1 s so that fatigue indices at 1 and 2 s were not calculated. In soleus, the fast and slow phases of HFF were not as distinct as in sternomastoid fibres and the force decline tended to be irregular as in Figure 5.1.

Also associated with HFF in white-sternomastoid fibres (100 Hz for 4 s) was the presence of post-tetanic potentiation. Peak twitch force was increased when evoked a few seconds after the cessation of stimulation at a time when peak tetanic force had only partially recovered. Thus, it is clear that different mechanisms are involved in the regulation of twitch and tetanic force after HFF.

(ii) Effects on relaxation:

A slowing of relaxation is a characteristic feature associated with fatiguing activity in human muscle (Hultman et al. 1981, Hultman & Sjöholm 1983; Cady et al. 1989b) and intermittent tetanic fatigue in isolated muscle preparations (Dawson et al. 1980; Jones

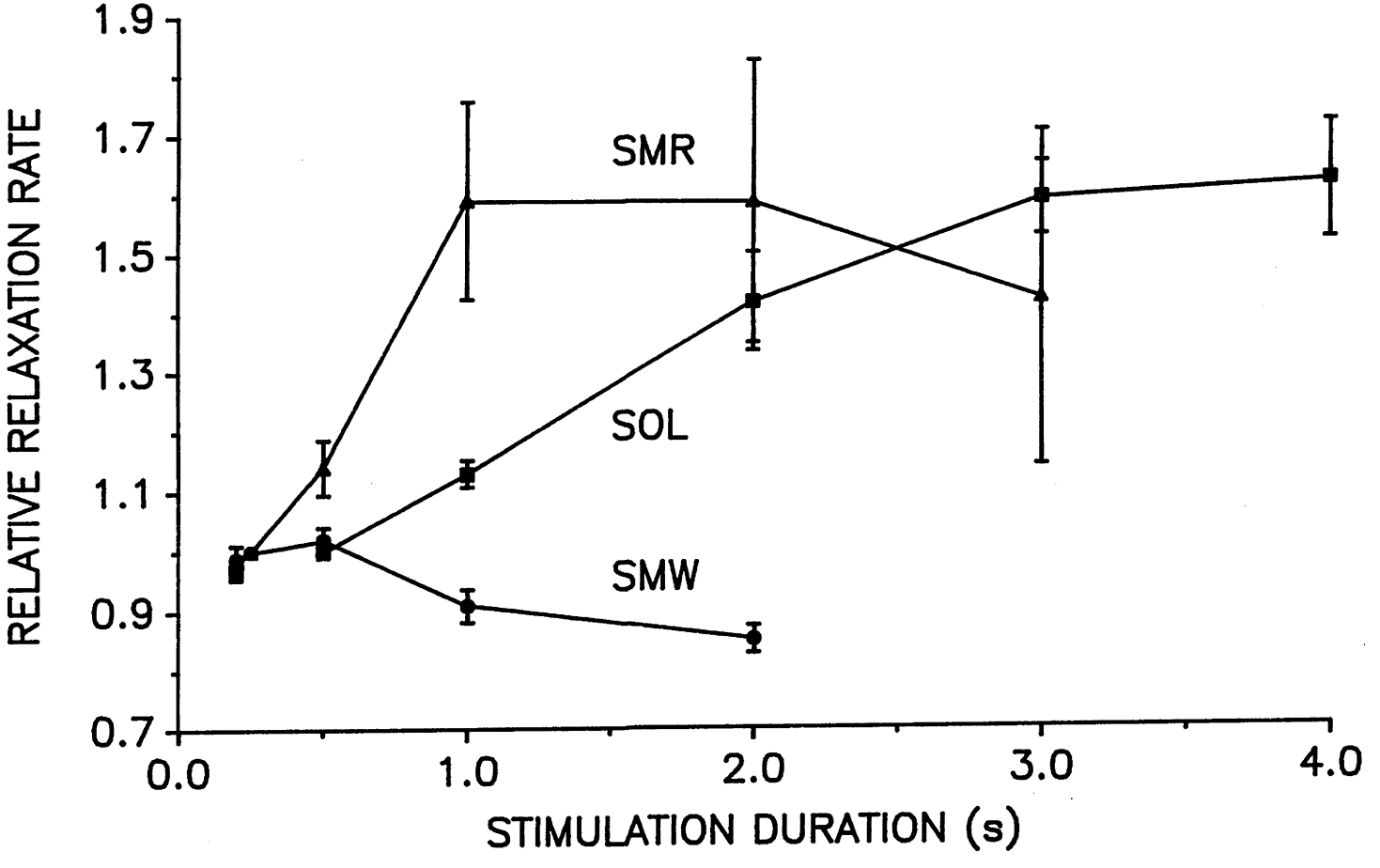


FIG.5.2: Effect of stimulation duration on the rate of relaxation in white-sternomastoid (SMW), red-sternomastoid (SMR) and soleus (SOL) fibres. Tetani were evoked at 100 Hz. Solution 1, 24°C. The relaxation rate (1/(80-20%RT)) at test stimulation durations was expressed relative to bracketting controls of 250 ms and 500 ms duration for sternomastoid and soleus fibres respectively. Each data point shows the mean value ± s.e.m., where the number of preparations used was 5-10 (SMW), 6-7 (SMR) and 8-12 (SOL).

1981; Allen et al. 1989). This is thought to be a consequence of changes in metabolite levels (Dawson et al. 1980; Hultman et al. 1981; Sahlin et al. 1981; Cady et al. 1989b). The aim of the present experiments was to examine the relationship between HFF and the rate of relaxation in different fibre-types.

The extent of HFF was varied by altering the duration of tetanic stimulation. However, changes in the rate of relaxation produced using this protocol may be due to either processes that cause fatigue or processes altered simply by increasing the tetanus duration, for example, due to saturation of myoplasmic Ca^{2+} buffers. Preliminary experiments revealed a slowing of relaxation with the duration of the experiment. Consequently, tetani of various test durations were expressed relative to bracketting tetani of 250 ms in sternomastoid and 500 ms in soleus fibres. Test tetani were evoked randomly for durations from 200 ms to 4 s.

Figure 5.2 shows the relationship between stimulation duration and relaxation rate (1/80-20%RT) in different fibre-types. A slowing of relaxation with increasing stimulation duration was only seen in white-sternomastoid fibres. Here the 80-20%RT increased from 46.0 ± 4.5 ms to 56.5 ± 7.0 ms ($n = 5$, $P < 0.01$, paired t-test) when the stimulation duration was increased from 250 ms to 2 s. Whenever relaxation was slowed there was also some fatigue and in several preparations a good linear correlation was seen between the slowing of relaxation and the extent of fatigue. However, some preparations showed considerable fatigue with very little slowing of relaxation.

There was no slowing of relaxation in red-sternomastoid and soleus fibres despite severe fatigue. This result shows that a slowing of relaxation is not a concurrent characteristic of HFF. In red-sternomastoid fibres, the relaxation rate increased over those stimulation durations in which there was rapid fatigue (compare Figures 5.1 & 5.2). Thus, increasing stimulation duration produced markedly different effects on relaxation in the two types of sternomastoid even though there were only small differences in the rate of fatigue. If the same processes cause HFF in white- and red-sternomastoid fibres, then either the slowing of relaxation in the white-sternomastoid is independent of fatigue processes or this fatigue process does not affect relaxation in the red-sternomastoid.

The relaxation rate increased with stimulation duration to a plateau in about 1-2 s or 3-4 s in red-sternomastoid and soleus fibres respectively (Figure 5.2). This acceleration of relaxation is possibly determined by the same mechanism in soleus and red-sternonastoid fibres and might be due to a delayed activation of the SR Ca^{2+}-pump. This effect could possibly mask or counteract the process which slows relaxation in the white-sternomastoid.

(b) Effect of denervation on the rate of high-frequency fatigue

Several changes in muscle fibres resulting from denervation may alter the rate of HFF. These include: (i) a reduced number of oxidative enzymes (Gundersen et al. 1988; Ohira 1989); (ii) a reduced number of Na-K pumps (Clausen et al. 1981, 1983); (iii) a reduction in the rate and extent of mechanical inactivation (Dulhunty 1985; Patterson 1988) and (iv) a shift in the curve for slow inactivation of Na^+ currents to more positive potentials (Kirsch & Anderson 1986). HFF may become faster due to the first two mechanisms or slower because of the last two mechanisms.

HFF was produced at 50 Hz because this frequency evokes maximum force in normal soleus fibres (Chapter 5.3e). Representative fatigue curves for normal and denervated soleus fibres are presented in Figure 5.3. The rate of HFF in denervated soleus fibres depends on the duration of denervation (Figure 5.4). This shows that following denervation for 2-3.5 weeks, the rate of HFF was increased and then as the denervation duration was prolonged (up to 14.5 weeks) the fatigue parameters were restored towards those in the normal soleus. There was no significant difference in the rate of HFF between the normal soleus and in preparations from soleus muscles denervated for more than 8 weeks. To illustrate this point, the fatigue indices at 10 s (FI_{10}) were 64.5 ± 6.8% (n = 8) in normal soleus and 36.5 ± 2.6% (n = 7) at 2 weeks, 52.5 ± 3.5% (n = 6) at 6 weeks, 64.6 ± 3.0% (n = 12) at 9 weeks and 70.7 ± 5.4% (n = 4) at 14.5 weeks of denervation. This recovery of fatigue parameters was not due to reinnervation since atrophy, fibrillations and other contractile properties typical of denervated muscle were present in long term denervated muscles (Table 2.3).

In about 30% (17 out of 58) of the denervated preparations studied, oscillations occurred during HFF which appear distinct from the irregular force decline in innervated fibres. The record in Figure 5.3 (iv) shows the largest oscillations seen at 50 Hz in a denervated soleus preparation. Oscillations (i) were larger in amplitude and more regularly observed at 100 Hz than at 50 or 30 Hz, (ii) became larger in amplitude towards the end of the tetanus and (iii) were independent of denervation duration, the relative force level or the presence of fibrillations. These oscillations might be related to similar fluctuations in EMG amplitude thought to be caused by action potential failure and then recovery in different fibres (Clamann & Robinson 1985). More often, however, the fatigue curves were considerably smoother in denervated than in normal soleus fibres (Figures 5.3, 5.9) and were extremely reproducible in individual preparations with variations of less than 1-2% (Figure 5.5A). Furthermore, the rate of HFF was often unaltered over several hours. Consequently, the denervated soleus preparation was used in most of the following studies to best quantify experimentally induced changes.

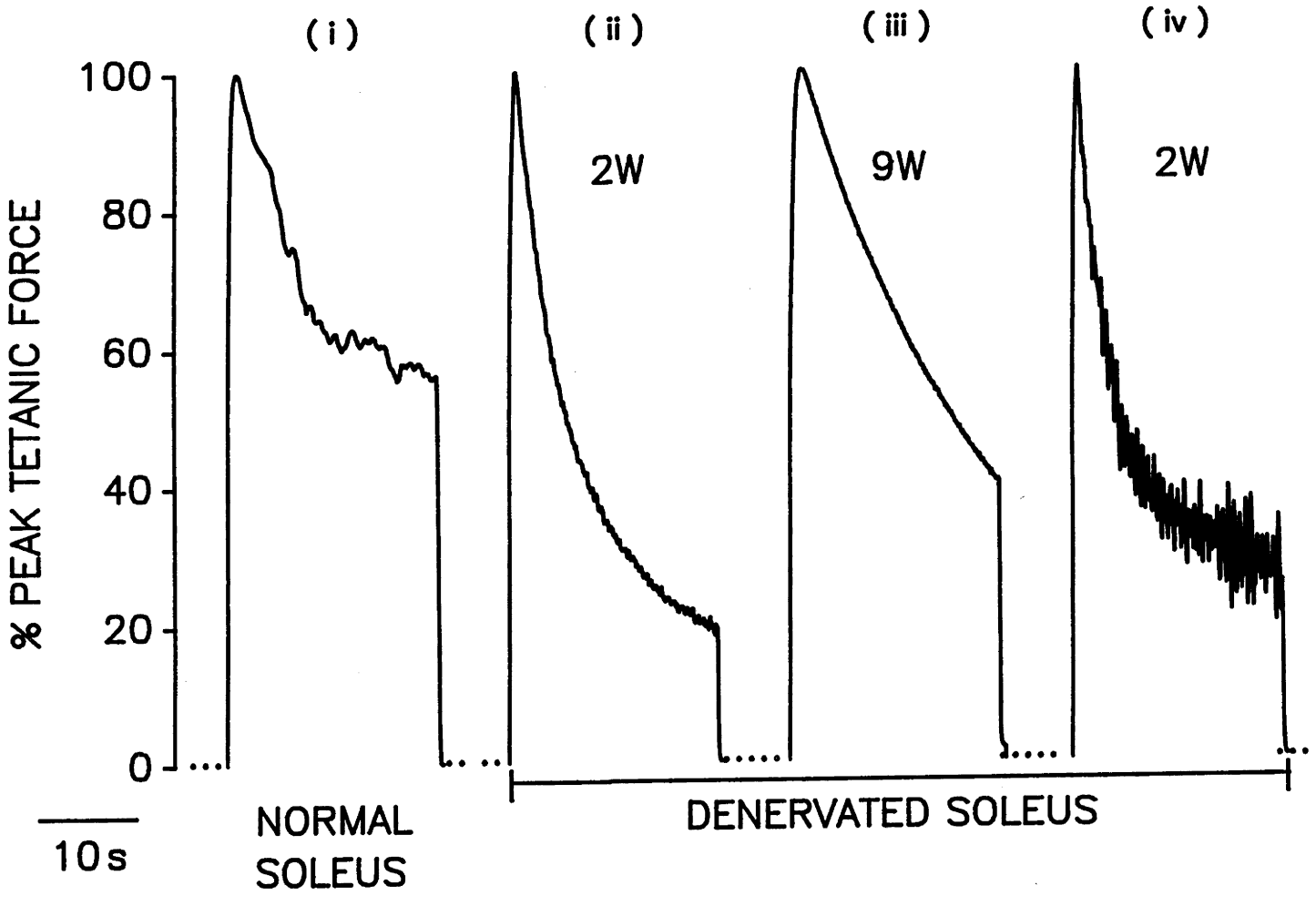


FIG. 5.3: High-frequency fatigue induced by continuous tetanic stimulation at 50 Hz in normal and denervated soleus preparations. Solution 1, 24 °C. Preparations from muscles denervated for 2 or 9 weeks are indicated by 2W and 9W respectively. The first three fatigue curves (i, ii, iii) were typical. The forth curve (iv) shows the oscillations that were sometimes seen during fatigue in denervated preparations. These were the largest oscillations seen at 50 Hz.

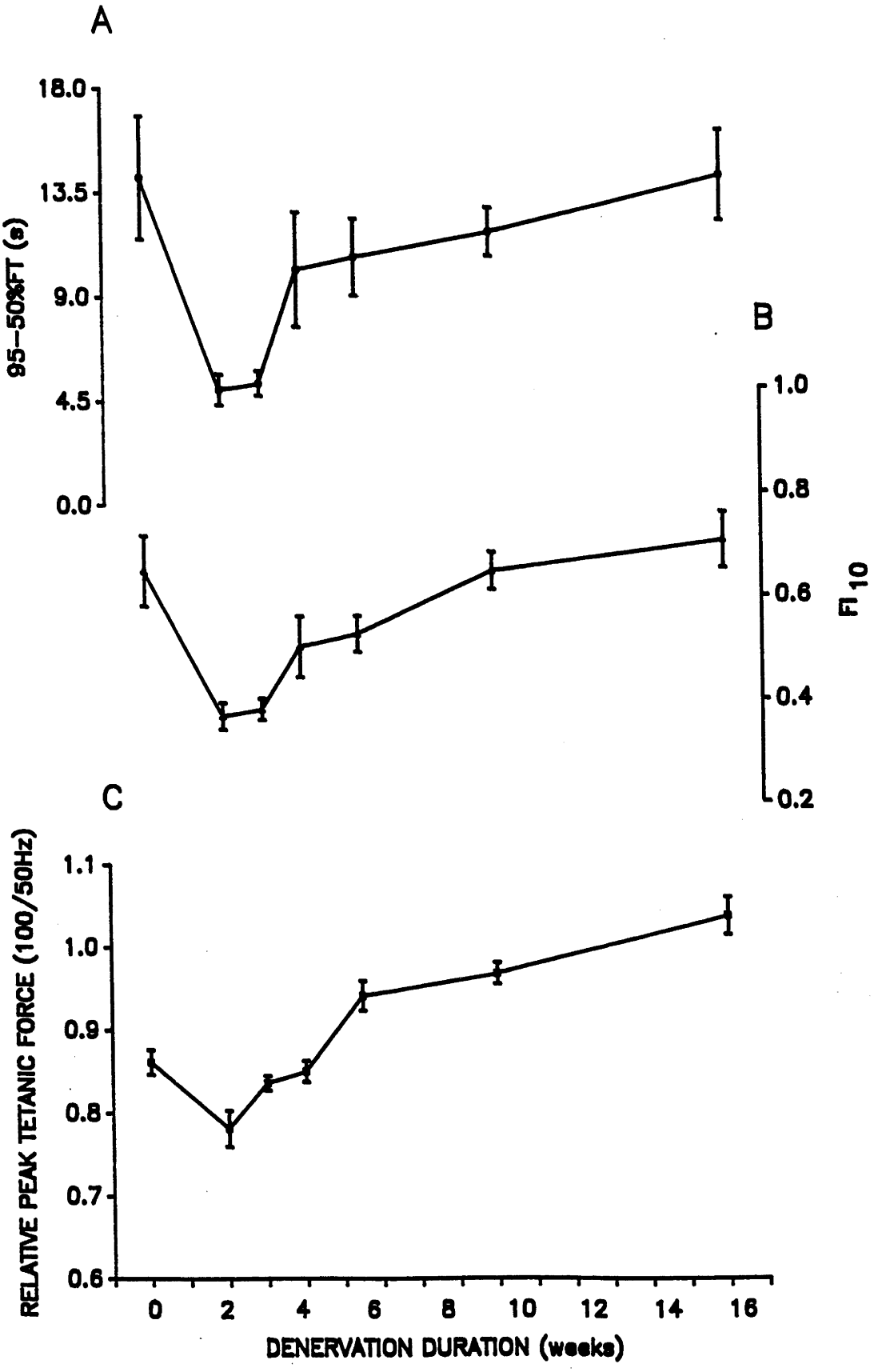


FIG. 5.4: Effect of the duration of denervation on (A) 95-50% fatigue time (95-50%FT), (B) 10 s fatigue index (FI_{10}) and (C) the ratio of peak tetanic force at 100 to 50 Hz. Solution 1, 24°C. Each data point shows the mean value ± s.e.m. The number of preparations used in A and B are shown in Table 5.2, and in C was 4-28.

(c) Potassium contractures during high-frequency fatigue

A popular hypothesis is that HFF is largely caused by a failure of the muscle action potential (Edwards 1981; Jones 1981; Lännergren & Westerblad 1986; Westerblad et al. 1990). In single amphibian twitch fibres there is a progressive decrease in amplitude and increase in duration of intracellularly recorded action potentials during HFF (Lännergren & Westerblad 1986, 1987). However, Lüttgau (1965) showed that, during continuous high frequency stimulation, a progressive decline in action potential amplitude and the drop out of every second action potential, i.e. "action potential fatigue", had little effect on tetanic force. Therefore, "action potential fatigue" does not necessarily cause HFF. Alternatively, HFF may be caused by the processes responsible for mechanical inactivation - the spontaneous decay of force that occurs during maintained depolarization (Hodgkin & Horowicz 1960; Lüttgau 1963; Caputo 1976; Chua & Dulhunty 1988). This hypothesis was suggested by Chua and Dulhunty (1988) on the basis of a similarity in time courses of mechanical inactivation and HFF in fast-twitch fibres.

The purpose of the present experiments was to test for a role of either mechanical inactivation and/or action potential failure in HFF. Action potential failure is defined here as "any reduction in the ability of tetanic action potentials to produce the normal activation of the voltage sensors for E-C coupling". To answer this question, a high $[K^+]$ solution was rapidly applied during severe HFF, usually after 10-12 s of stimulation. It was predicted that the direct activation of the voltage sensors with high $[K^+]$ would have restored force if action potential failure had occurred. If mechanical inactivation was important, on the other hand, then peak K-contracture force would be depressed during fatigue. 200 mM K^+ was used because it evokes maximal contractures (maximum voltage-activated force) in normal and denervated soleus (Dulhunty & Gage 1985; Chua & Dulhunty 1988), and produces a more rapid depolarization than submaximal $[K^+]$ (Dulhunty 1985).

Figure 5.5A illustrates the effect of rapid application of 200K on force during continuous fatiguing stimulation at 30 Hz in a denervated soleus preparation. The HFF force record, produced 10 min earlier in the absence of high $[K^+]$, was superimposed on the record with 200K. A small dip in the force trace occurred soon after application of 200K which was possibly due to a depolarization-induced inactivation of Na^+ channels. Subsequently, tetanic force was completely restored with bonus force and this was presumably due to the depolarization-induced activation of the voltage sensors. 200K increased force, expressed as a percentage of peak tetanic force, from $31.8 \pm 3.1\%$, immediately prior to K^+ application, to $101.0 \pm 4.7\%$ ($n = 7$) in denervated soleus preparations. It might be thought from this result that mechanical inactivation had no

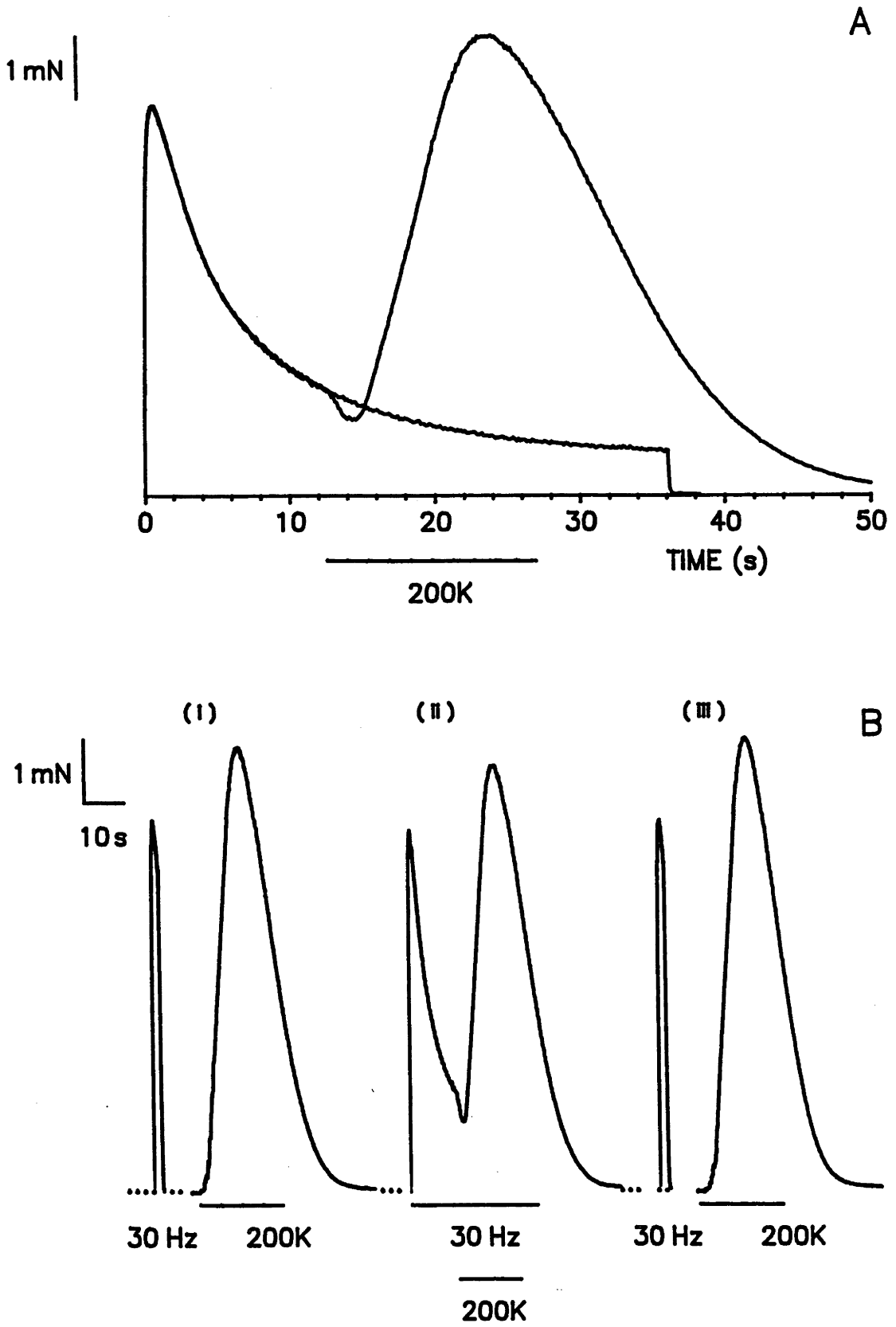


FIG. 5.5: (A) Effect of rapid application of a solution containing 200 mM K^+ (200K) during high-frequency fatigue at 30 Hz in a denervated soleus preparation. The preceeding fatigue record was superimposed on the record with 200K. The period of application of 200K is represented by the horizontal line beneath the record. (B) The same experiment with control tetani and 200K-contractures (i & iii) included. The ratio of peak 200K-contracture force relative to peak tetanic force was 1.21 prior to (i), 1.18 during (ii), and 1.22 after (iii) fatiguing stimulation.

role in HFF but since 200K can partially restore force during mechanical inactivation with submaximal [K^+] (Dulhunty 1990), it was necessary to compare 200K-contractures during fatigue with control contractures. Figure 5.5B shows the same experiment as in Figure 5.5A with control non-fatigued tetani and 200K-contractures included. Clearly the amplitude of 200K-contractures were similar in control conditions and during fatigue. Using the same preparations the amplitude of 200K-contractures was 102.09 ± 5.8% in control conditions which was the same as during fatigue ($P > 0.1$, ANOVA). Peak K-contracture force was reproducible to within 5% in a given preparation. In two of these experiments control peak K-contracture force was 82.8% and 89.4% of peak tetanic force. The smaller size of these K-contractures was because the bundles were slightly thicker than usual, which presumably resulted in diffusion problems, so that surface fibres were undergoing mechanical inactivation prior to the full activation of deeper fibres. In all denervated soleus preparations, excluding the above two, the amplitude of control 200K-contractures was 19.3 ± 6.1% (n = 9) greater than peak tetanic force.

Several important conclusions can be made from these results: (i) Ca^{2+} release from the SR is impaired during HFF; (ii) processes responsible for mechanical inactivation are not involved in HFF; (iii) the voltage sensors and voltage-dependent activation of E-C coupling can still operate maximally during HFF i.e. maximum voltage-activated force is unaffected.

(d) Effect of extracellular ions on the rate of high-frequency fatigue

An alternative method that can be used to test for action potential failure in HFF is to manipulate the amplitude of action potentials by changing the extracellular ion concentrations.

(i) Effect of sodium ions:

Changing the extracellular Na^+ concentration ($[Na^+]_o$) alters the overshoot of the action potential (Nastuk & Hodgkin 1950; Grabowski et al. 1972) but does not directly affect either E-C coupling or myofilament function (Miledi et al. 1984). Increasing $[Na^+]_o$ might, therefore, slow HFF if a decrease in the amplitude of the action potential causes fatigue. There is also some evidence that a depletion of Na^+ in the T-system and/or interfibre space may be a cause of HFF (Bezanilla et al. 1972, Jones et al. 1979). Thus, it was postulated that increasing $[Na^+]_o$ would produce a smaller depletion of $[Na^+]_o$ and less action potential failure, and therefore a slowing of HFF.

Figure 5.6 shows that increasing [Na^+]o from 80.5 mM (Solution 1) to 196.5 mM (Solution 2) potentiated peak force (A) and slowed the decay of force in prolonged

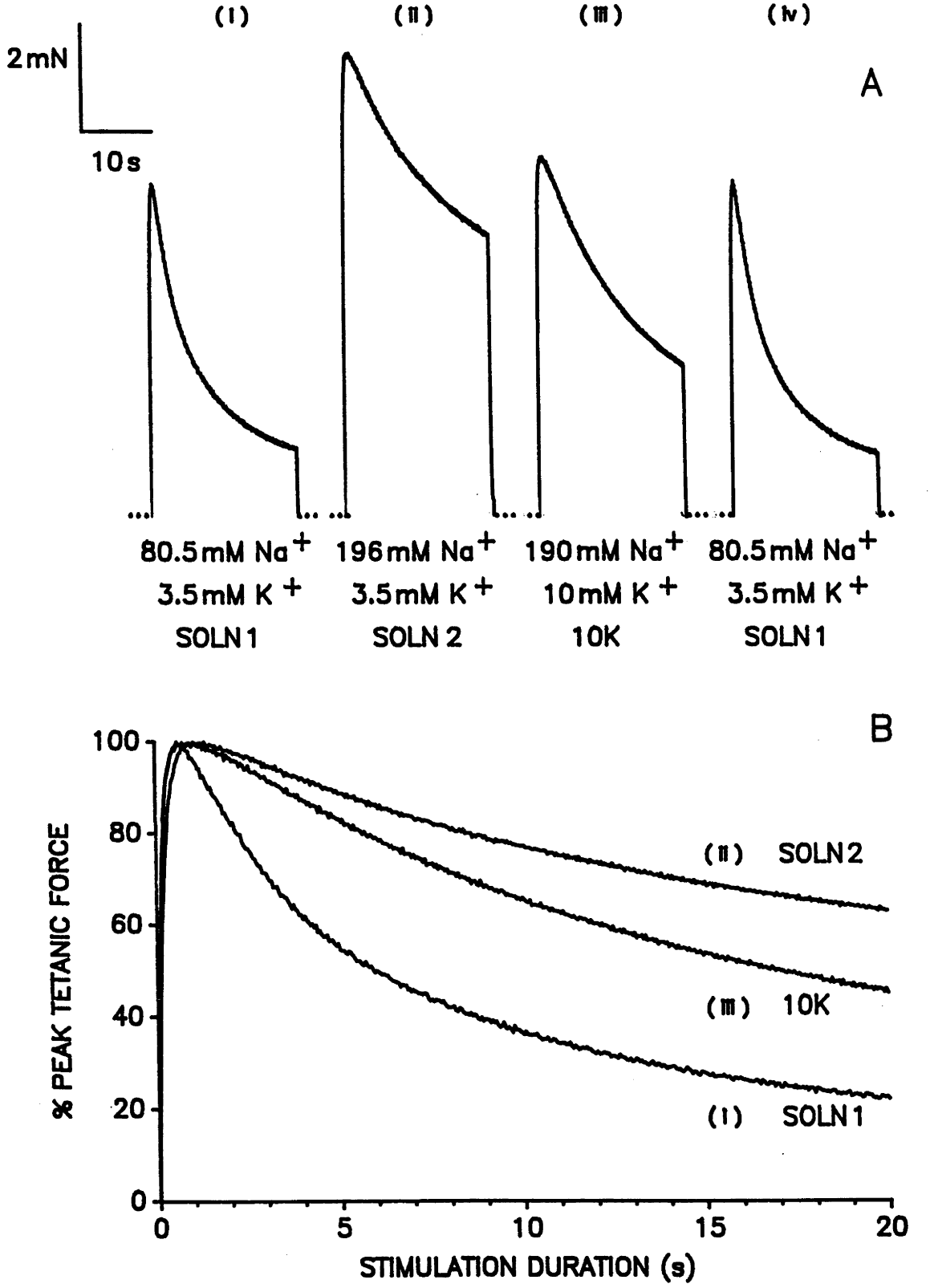


FIG. 5.6: (A) Effects of extracellular [Na^+] and [K^+] on prolonged tetani evoked at 30 Hz in a denervated soleus preparation at 24°C. When extracellular [Na^+] was increased from 80.5 mM (i) to 196.5 mM (ii) (changing from SOLN 1 to SOLN 2), peak tetanic force increased by 38.5% and the rate of fatigue was reduced. When extracellular [K^+] was increased from 3.5 mM (ii) to 10 mM (iii) (changing from SOLN 2 to 10K) peak tetanic force was reduced by 22.2% and the rate of fatigue was increased. Force recovered (iv) after wash-out of 10K (iii). (B) Fatigue curves produced from the records in A show that both increasing [K^+] (iii) and reducing [Na^+] (i) accelerated fatigue relative to the control (ii).

tetani (B). Peak tetanic force was increased on average by 27.6 ± 3.3% (n = 14), 5 min after exposure to the high [Na^+] solution. This effect was reproducible and independent of the incubation duration, although some preparations showed a small increase with time (Figure 5.8). Peak twitch force was unaltered in Solution 2 i.e. the ratio of peak twitch force in Solution 2 to 1 was 1.000 ± 0.022 (n = 4). The rate of fatigue was also considerably reduced in the high $[Na^+]_o$ solution. In ten denervated soleus preparations, where HFF was produced at 30 Hz, the FI_{10} were 37.6 ± 2.8% and 75.2 ± 2.6% in Solutions 1 and 2 respectively. These effects on peak tetanic force and fatigue rapidly reversed on wash-out. In addition to the higher [Na^+], Solution 2 also had a higher ionic strength (but the same osmolarity) compared to Solution 1. To control for an effect of ionic strength, some of the sodium sulphate in Solution 2 was replaced with equimolar guanidium sulphate. After 5 min in the guanidium sulphate solution ([Na^+] = 80.5 mM), the peak tetanic force was reduced to 78.0 ± 4.8% (n = 3) of the control (in Solution 1) and the FI_{10} had shown a relatively moderate increase from 39.3 ± 4.3% to 46.6 ± 5.8% (n = 3), suggesting that the effects of Solution 2 were due to the increased [Na^+]. Thus, the protective effect of high [Na^+] against HFF suggests that action potential fatigue is important in HFF and supports the Na^+ depletion hypothesis (Bezanilla et al. 1972) as a possible mechanism for causing HFF.

(ii) Effect of potassium ions:

It has been suggested that there may be an accumulation of K^+ in the T-system and interfibre space during fatigue (Jones et al. 1979; Juel 1986). A small increase in the extracellular K^+ concentration ($[K^+]_o$) depresses and prolongs the action potential (Jones 1981; Lännergren & Westerblad 1986), and decreases the resting membrane potential (Chua & Dulhunty 1988; Juel 1988), thus mimics the effect of HFF on membrane potential (Lüttgau 1965; Lännergren & Westerblad 1986, 1987). It was anticipated that increasing $[K^+]_o$ to 10 mM - comparable to plasma levels in fatiguing exercise (Saltin et al. 1981; Juel et al. 1990; Medbo & Sejersted 1990) - would increase the rate of action potential failure and consequently HFF.

Figure 5.6 shows that increasing $[K^+]_o$ from 3.5 mM (Solution 2) to 10 mM (10K) depressed peak tetanic force and increased the rate of HFF. Shortly after application of 10K, peak tetanic force was reduced to 80.1 ± 1.5% (n = 10) of the control (Figure 5.6A iii verse ii). The extent of force depression did not change when 10K was added for up to 20 min. In seven denervated soleus preparations fatigued at 30 Hz, the FI_{10} decreased from 76.9 ± 2.7% to 68.7 ± 2.9% when changing from 3.5 to 10 mM K^+. These results suggest that an increase of $[K^+]_o$ may contribute to HFF.

(e) Effect of stimulation frequency on the rate of high-frequency fatigue

During continuous high frequency stimulation there may be insufficient time between action potentials for the complete restoration of the trans-sarcolemmal ionic distribution. The consequent decline of the Na^+ and K^+ gradients may be responsible for HFF. The hypothesis that HFF was caused by trans-sarcolemmal ionic shifts (hereafter abbreviated to ionic shift - see definitions) associated with action potential activity was tested by varying the stimulation frequency. Effects were seen on both peak tetanic force and the rate of HFF.

(i) Effect of stimulation frequency on peak tetanic force:

In normal soleus fibres, maximum tetanic force was evoked at 50 Hz in Solution 1 at 24°C (Figure 5.7). Small ripples were seen in the early rise of tetanic force prior to fusion suggesting that 50 Hz was close to the fusion frequency. Further increases in the stimulation frequency resulted in a progressive depression of peak tetanic force. This effect was similar to Wedensky inhibition - attributed to a failure of neuromuscular transmission (Lucas 1911; Krnjevic & Miledi 1958a; Thesleff 1959; Cullingham et al. 1960; Truong et al. 1964) - but in this case, force depression occurred with direct stimulation and thus must have been due to processes in the muscle fibres. A similar frequency-dependent depression of force was seen in denervated soleus fibres (Figure 5.7). The frequency required to evoke maximum force, and the extent of force depression, depended on the denervation duration (Figures 5.4 & 5.7). Maximum tetanic force was produced at 30 Hz during 2-3.5 weeks of denervation but at 50 Hz thereafter (Figures 5.7). The depression of maximum force at 100 Hz was greater in 2 week denervated soleus than in normal soleus fibres. The extent of this force depression recovered and reversed relative to the normal soleus as the denervation duration was prolonged (up to 14.5 weeks) (Figures 5.4, 5.7).

The frequency-dependent depression of maximum tetanic force was a consequence of the composition of Solution 1 as it was not seen in normal Krebs solution. The ratio of peak tetanic force at 100 Hz relative to 50 Hz was 0.990 ± 0.010 in normal Krebs solution and 0.876 ± 0.012 in Solution 1 in four denervated soleus preparations. Two possible causes of the force depression are the reduced $[Na^+]$ and/or the low $[Cl^-]$ in Solution 1 (Table 2.1). Figure 5.8 shows that the depression of force at 100 Hz in Solution 1 ($[Na^+] = 80.5$ mM) was not seen in Solution 2 ($[Na^+] = 196.5$ mM). In seven denervated soleus preparations, the ratio of peak tetanic force at 100 Hz, relative to 30 Hz, was 0.756 ± 0.019 in Solution 1 and 1.003 ± 0.014 in Solution 2. The frequency-dependent depression of maximum tetanic force could thus be attributed to the low $[Na^+]$ in Solution 1.

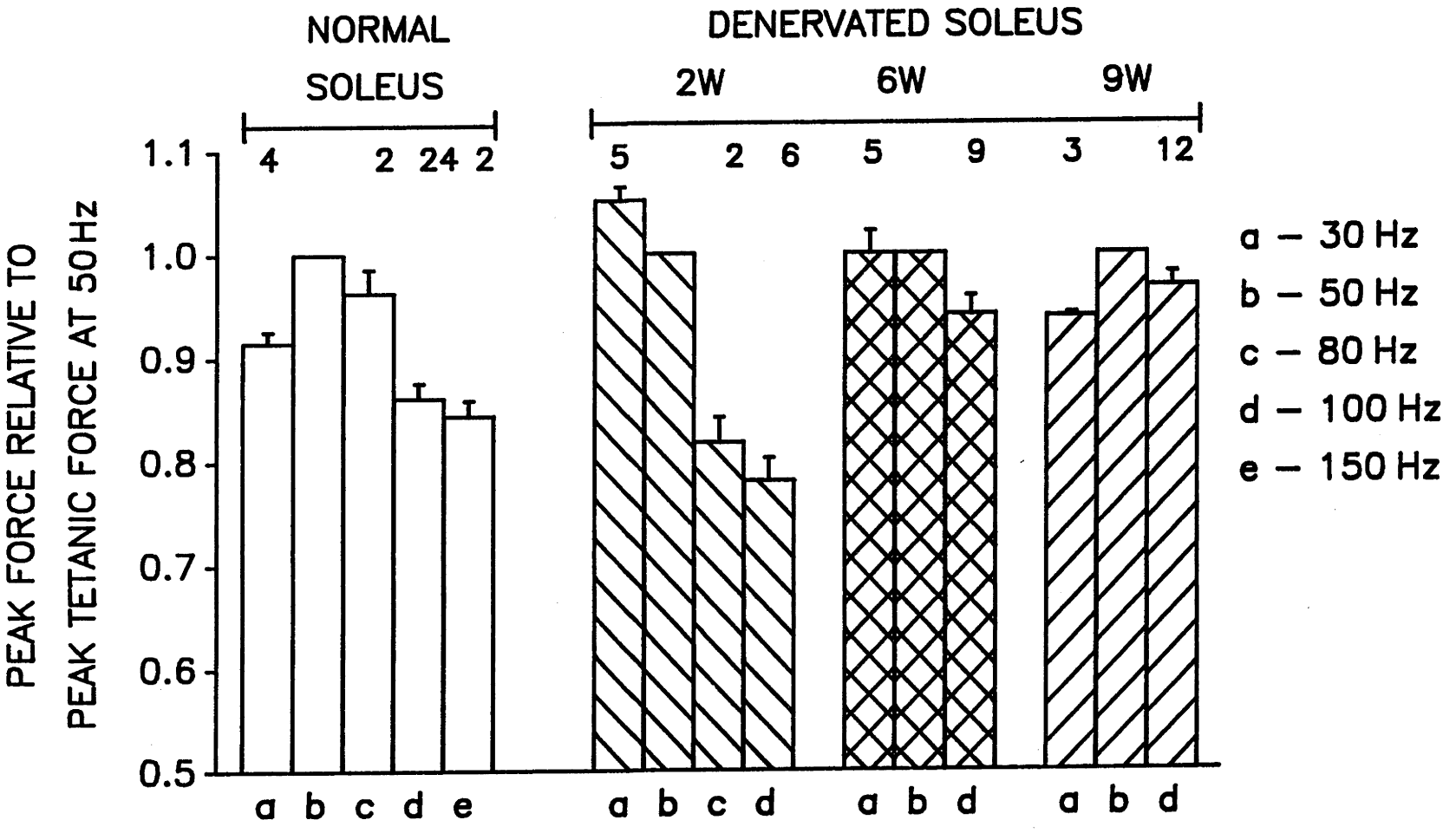


FIG. 5.7: Effect of stimulation frequency on peak tetanic force in normal and denervated soleus preparations. Solution 1, 24°C. The peak force at each frequency was expressed as a ratio relative to the peak force of adjacent tetani evoked at 50 Hz. Each bar shows the mean value ± s.e.m. The number of preparations is shown above each bar. Preparations denervated for 2, 6 and 9W are used to show how the force-frequency relation changes with denervation duration.

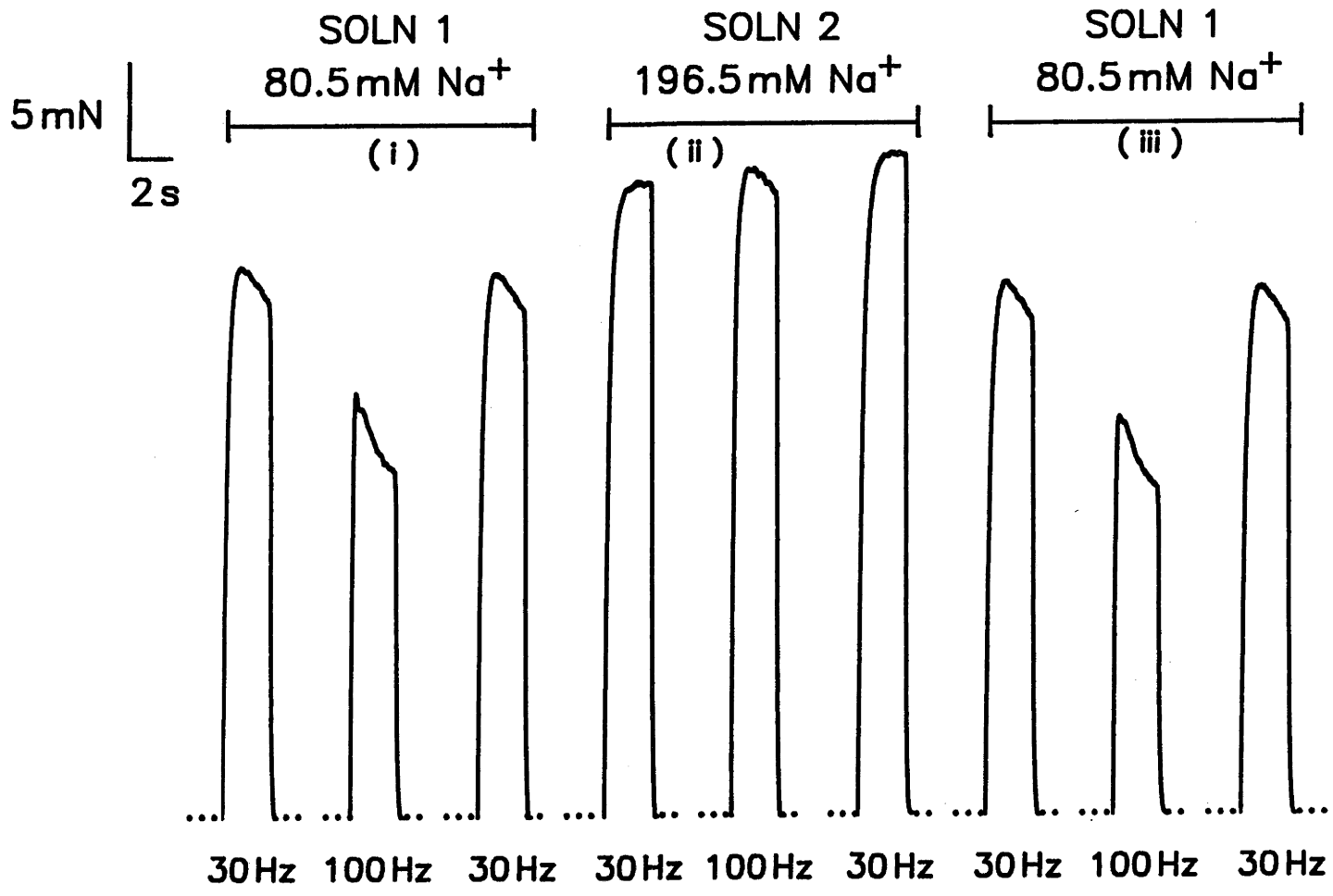


FIG. 5.8: Representative effects of high-frequency stimulation on peak tetanic force in SOLN 1 ($[Na^+]$ = 80.5 mM) and SOLN 2 ($[Na^+]$ = 196.5 mM) in a denervated soleus preparation at 24°C. Stimulation at 100 Hz depressed peak tetanic force in low $[Na^+]$ but not in high $[Na^+]$. The ratio of peak tetanic force at 100 to 30 Hz was 0.77 in SOLN 1 (i), 0.99 in SOLN 2 (ii), and 0.75 after wash-out in SOLN 1 (iii). Tetani were evoked every 5 min. Change over from SOLN 1 to SOLN 2 increased the peak tetanic force at 30 Hz by 16.8%.

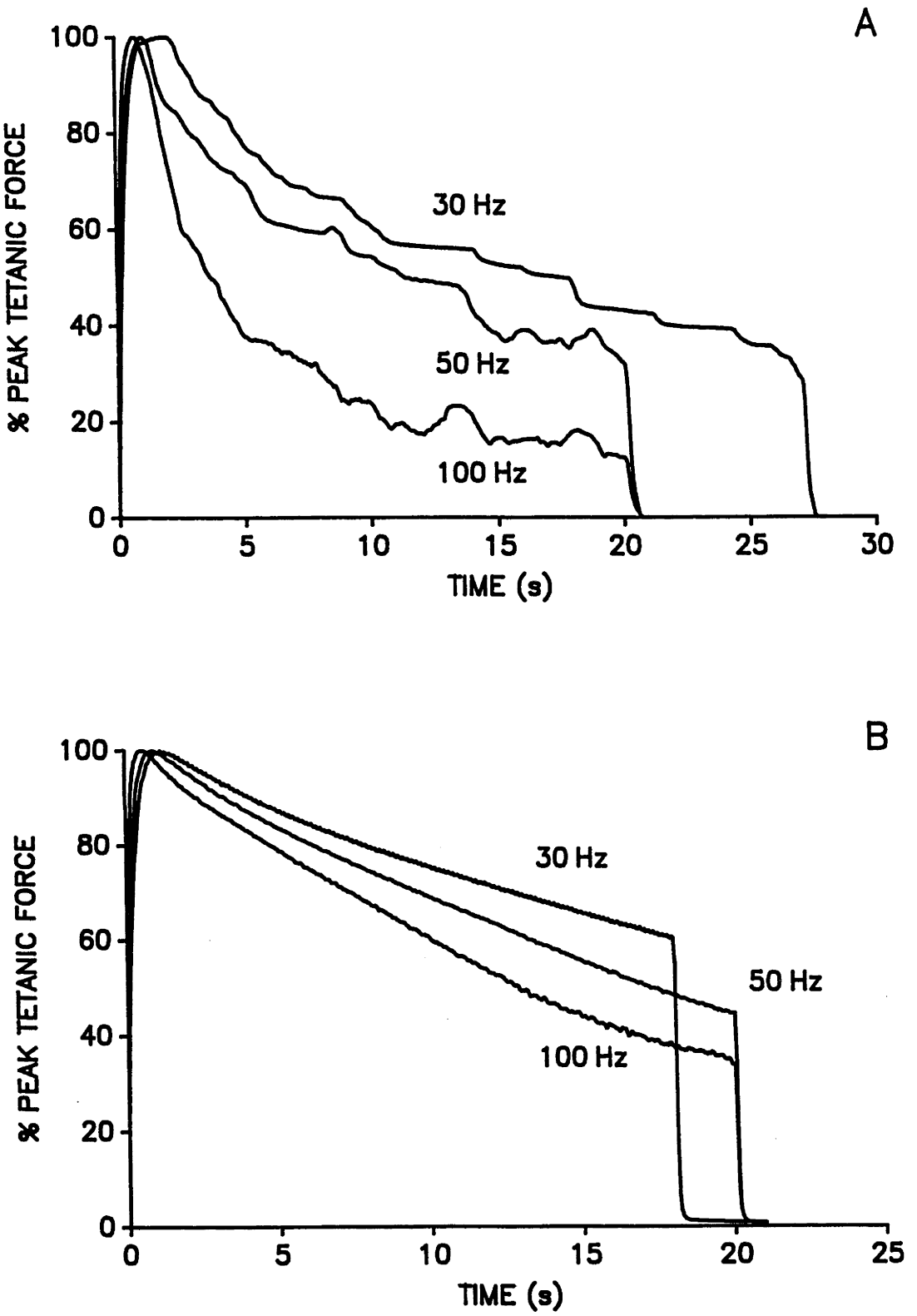


FIG. 5.9: Representative fatigue curves showing the effect of different stimulation frequencies on the rate of fatigue in (A) a normal soleus and (B) a denervated soleus preparation (9W). Solution 1, 24°C. The superimposed curves were from adjacent tetani.

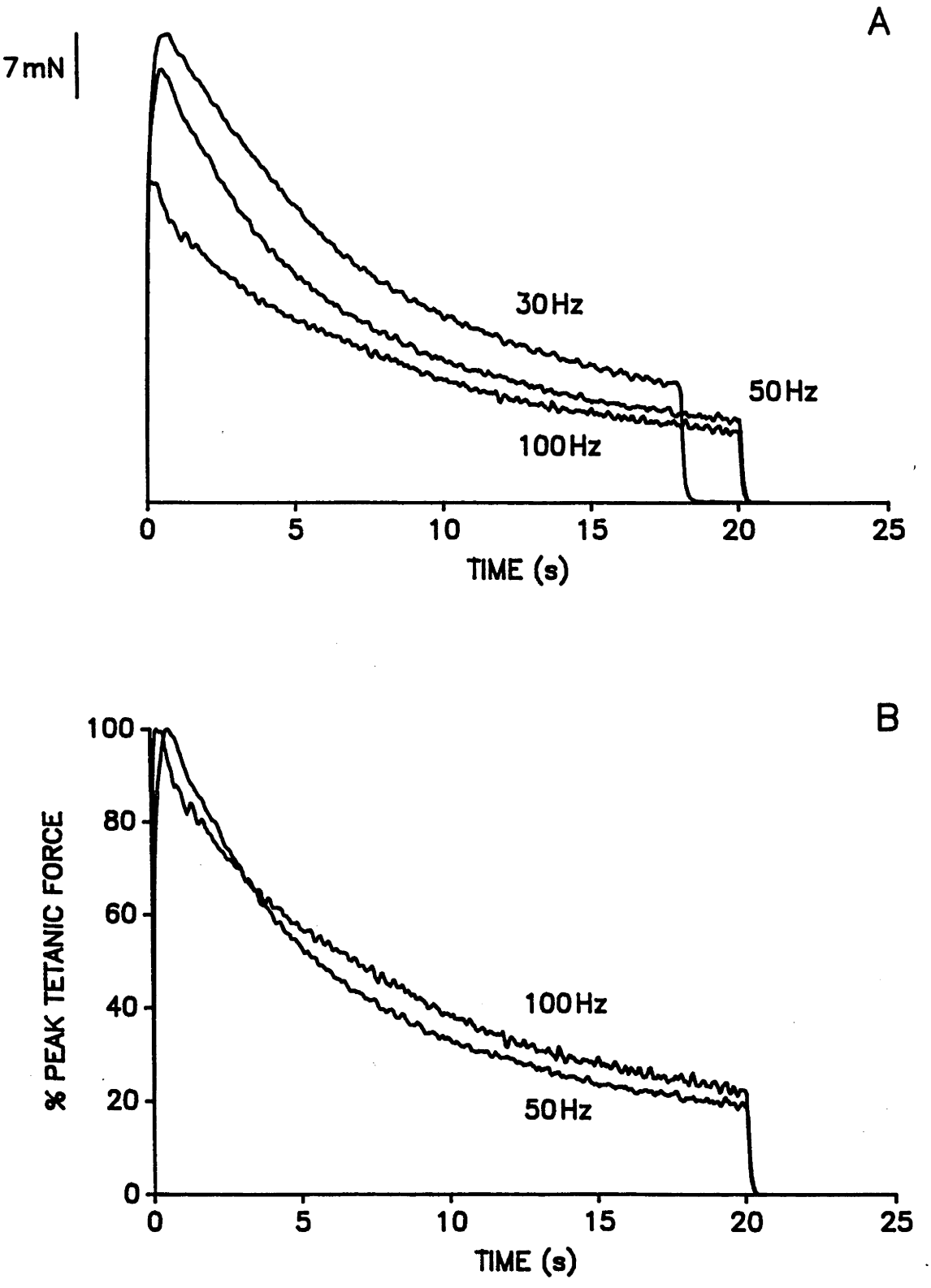


FIG. 5.10: Effect of continuous tetanic stimulation at different frequencies in a denervated soleus preparation (2W). (A) absolute force records and (B) fatigue curves produced from the 100 and 50 Hz records. The 30 Hz tetanus in A is left out in B to show the difference between 50 and 100 Hz fatigue curves more clearly. Solution 1, 24°C. The superimposed curves were from adjacent tetani.

(ii) Effect of stimulation frequency on the rate of force decline:

The rate of HFF increased with stimulation frequency in normal soleus fibres. Figure 5.9A shows fatigue curves at 100, 50 and 30 Hz from adjacent tetani in a normal soleus preparation. There were clear differences at 100 and 50 Hz but only small differences at 50 and 30 Hz. On average, the FI_{10} was 64.5 ± 6.8% (n = 8) at 50 Hz and 41.3 ± 4.7% (n = 9) at 100 Hz. In two preparations the FI_{10} was 67.7 ± 6.6% at 30 Hz and 51.1 ± 5.4% at 50 Hz for adjacent tetani. The more rapid fatigue at 100 Hz compared to 50 Hz was due to a shortening of the plateau time and a steeper slope for the force decline. In soleus muscles denervated for 5-6 weeks, or longer, the rate of HFF clearly increased with stimulation frequency (Figure 5.9B). In 8-9W denervated soleus fibres the FI_{10} were 53.9 ± 3.4% (n = 12) at 100 Hz, 64.9 ± 3.6% (n = 12) at 50 Hz and 73.9 ± 0.6% (n = 3) at 30 Hz. In soleus muscles denervated for 2-3.5W a direct relation between stimulation frequency and the rate of HFF was not so clear at all frequencies. Figure 5.10 shows the absolute force records (A) and corresponding fatigue curves (B) at 100, 50, and 30 Hz in a 2W denervated soleus preparation. The absolute tetanic force at each duration was depressed progressively with increases in the frequency. However, the fatigue curves at 100 and 50 Hz indicate a different relation. On average, the FI_{10} were 41.3 ± 3.1% at 100 Hz and 38.1 ± 2.5% at 50 Hz (n = 11), which were not different ($P > 0.1$, paired *t*-test). Most of the force lost at the very high frequencies in the 2W denervated fibres seem to be manifested as a depression of peak force rather than as a faster force decline.

In summary, increasing the stimulation frequency resulted in a progressive loss of force (depression of peak tetanic force and faster decline of force) which suggests that the mechanisms responsible for both effects are directly related to the generation of action potentials.

(f) Rate of force recovery from high-frequency fatigue

HFF may be due to changes in the trans-sarcolemmal ion distribution resulting from the inability of diffusional processes and/or Na-K pump proteins to restore ionic gradients (Sejersted 1988). Alternatively, changes in the ionic distribution may result from the effect of metabolites acting on ion channels (Grabowski et al. 1972; Renaud & Mainwood 1985). Thus the rate of tetanic force recovery should help to discriminate between ionic or metabolic factors. A rapid force recovery would be expected if restoration of trans-sarcolemmal ionic gradients is involved (Nakajima et al. 1973; Kirsch et al. 1977) and a slower recovery may reflect the slower recovery of the intracellular metabolic milieu (Kushmerick & Meyer 1985).

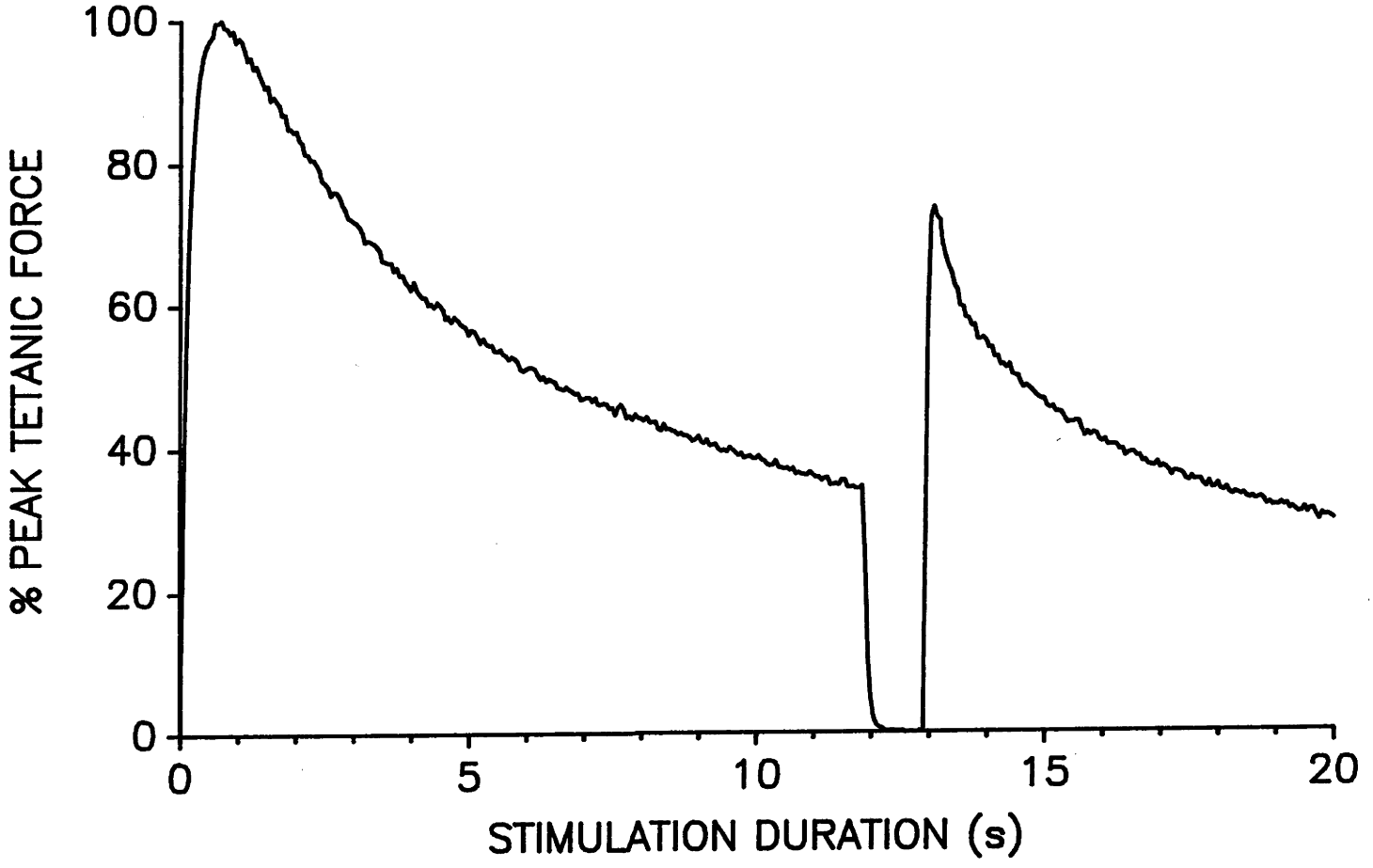


FIG. 5.11: A rapid recovery of tetanic force occurs following a 1 s cessation during continuous tetanic stimulation at 30 Hz in denervated soleus fibres. Solution 1, 24°C.

Figure 5.11 shows the extremely rapid recovery of tetanic force that occurs following the cessation of stimulation in a denervated soleus preparation. In seven experiments stimulation was stopped for 1 s after 12 s of stimulation at 30 Hz and this allowed the recovery of force from 34.8 ± 3.3% to 62.7 ± 3.0% of peak tetanic force, i.e. the recovery of 42.7 ± 3.7% of the force lost. A qualitatively similar force recovery was seen in normal soleus preparations (n = 2). In three denervated soleus preparations, the tetanic force recovery following stimulation stoppages of 1 and 4 s were 41.3 ± 4.9% and 63.5 ± 1.8% of the force lost respectively. Thus, the additional 3 s allowed the recovery of 22.2 ± 2.8% of the force lost. These results show that a large portion of the force lost during HFF is recovered in the first few seconds after the cessation of stimulation and is consistent with the hypothesis that HFF results largely from ionic shifts across the T-tubule membrane.

5.4 DISCUSSION

(a) Possible site/s of failure during high-frequency fatigue

There were two major findings in the present study which help to establish the cellular site/s of impairment during HFF. Firstly, rapid application of high $[K^+]$ solutions could completely reverse the force lost during extensive fatigue and the amplitude of these K-contractures was unaffected by fatigue processes. From this result several conclusions can be made about the site/s of HFF. (i) The contractile proteins can still generate maximum force if, during these maximal K-contractures, troponin is saturated with Ca^{2+}. (ii) There is reduced SR Ca^{2+} release during fatigue. This interpretation is consistent with the reduced tetanic $[Ca^{2+}]_i$ seen during HFF in frog fibres (Westerblad et al. 1990). (iii) Impaired Ca^{2+} release due to processes that cause mechanical inactivation - possibly the inactivation of voltage sensors (Chandler et al. 1976b; Rakowski 1981; Chua & Dulhunty 1988) - is not responsible for HFF. (iv) The voltage sensors of the T-system and voltage-dependent activation of E-C coupling can still operate maximally during HFF. However, it is uncertain whether HFF is entirely due to action potential failure. Because peak tetanic force was less than maximum K-activated force, i.e. in 200K, the possibility exists that there was a shift in the force-$[K^+]$ relation towards higher $[K^+]$ during fatigue, i.e. a decreased voltage-sensitivity that was not detected by 200K. In this scenario, action potential failure and impaired E-C coupling might both contribute to the impaired Ca^{2+} release, and a reduced Ca^{2+} sensitivity may also contribute to fatigue. The generation of submaximal K-contractures during HFF may help to discriminate between these possibilities. Certainly, if the entire force-$[K^+]$ relation is unaltered during HFF, one could conclude that HFF is entirely due to action potential failure.

The second important result in the present study was that the rate of HFF depended on the ionic composition of the bathing solutions. Fatigue occurred more rapidly when there was either a reduction in $[Na^+]_o$ or a small increase in $[K^+]_o$. Since these ionic changes are known to depress the amplitude of the action potential (Nastuk & Hodgkin 1950; Grabowski et al. 1972; Jones 1981; Lännergren & Westerblad 1986), but are without direct effects on either E-C coupling or myofilament function, it must be concluded that action potential failure can account for at least some HFF.

In support of the hypothesis that action potential failure has an important role in HFF, several studies have shown that a reduced amplitude of the EMG, muscle compound action potential and intracellularly recorded action potentials correlate well with the reduction of force during prolonged tetanic stimulation (Bigland-Ritchie et al. 1979; Clamann & Robinson 1985; Hultman & Sjöholm 1983; Sandercock et al. 1985; Lännergren & Westerblad 1986, 1987). The apparently contradictory observation by Lüttgau (1965) where "action potential fatigue" was not associated with a reduction of force may be due to the very high stimulation frequency of 200 Hz used. In this case, the remaining action potentials after the loss of every second action potential were possibly sufficient to fully activate the voltage sensors.

Action potential failure might arise from the inability to initiate or propagate action potentials along the surface and/or T-tubular membranes. However, another possibility is that a reduced amplitude of tetanic action potentials could present the voltage sensors with a smaller depolarization and hence less activation. Whatever the mechanism, several pieces of evidence suggest that the T-system is an important site for action potential failure. (i) Tetanic force recovers rapidly after the cessation of stimulation (Nassar-Gentina et al. 1981; Lännergren & Westerblad 1986; present study) or by lowering the frequency (Jones et al. 1979; Jones 1981; Lännergren & Westerblad 1986; Westerblad et al. 1990), and these results are consistent with a rapid process such as diffusion of ions into out of the T-system (Nakajima et al. 1973). (ii) The decline of action potential amplitude during prolonged tetanic stimulation occurs more slowly following disconnection of the T-tubules from the surface membrane (Lännergren & Westerblad 1987). (iii) A reduction of $[Na^+]_o$ increases the rate of fatigue while simultaneously producing wavy (or non-contracting) myofibrils which originate in the centre of the fibre and then spread towards the periphery suggesting a failure of action potential propagation down the T-tubules (Bezanilla et al. 1972). (iv) Radial gradients of tetanic $[Ca^{2+}]_i$ occur during HFF which indicates less Ca^{2+} release in the centre of the fibre than at the periphery (Westerblad et al. 1990).

(b) Role of trans-sarcolemmal ion shifts in high-frequency fatigue

During each action potential there is an influx of Na^+ and an efflux of K^+ across the sarcolemma (Hodgkin & Horowicz 1959a; Adrian et al. 1970; Clausen & Everts 1989). These ion fluxes would be expected to produce trans-sarcolemmal ionic shifts during high intensity exercise or with prolonged high frequency stimulation. If the capacity of the processes that act to restore the ion gradients - diffusional fluxes or transport by Na-K pumps - is exceeded, then large changes in the trans-sarcolemmal ionic distribution would occur (Clausen & Everts 1988; Sejersted 1988). The possible contribution of Na^+, K^+, and Cl^- shifts across the sarcolemma to HFF is discussed below.

(i) Sodium ions:

It has been suggested that during prolonged tetanic stimulation there is a depletion of Na^+ in the lumen of the T-system (Bezanilla et al. 1972; Adrian & Peachey 1973). In fact, it was predicted in the modelling work of Bezanilla et al. (1972) that when $[Na^+]_o$ was 120 mM, the tubular $[Na^+]$ would fall to 60-70 mM in 1-2 s of stimulation at 60 Hz. This model did not allow for tubular $[Na^+]$ gradients, so it is likely that Na^+ depletion would be even greater in the depths of the T-system. In support of a Na^+ shift, $[Na^+]_i$ is increased during continuous high frequency stimulation (Stréter 1963). The effect of a reduced Na^+ gradient would be a smaller action potential overshoot due to the reduced sodium equilibrium potential (Nastuk & Hodgkin 1950; Grabowski et al. 1972). Certainly, support for this mechanism comes from contractile studies where experimental reductions of $[Na^+]_o$ increase the rate of HFF (Bezanilla et al. 1972; Jones et al. 1979; present study).

(ii) Potassium ions:

During continuous tetanic stimulation, interstitial $[K^+]$ can increase from about 5 to 9 mM (Hník et al. 1976) and $[K^+]i$ falls (Stréter 1963). It is likely that the increase in tubular $[K^+]$ is even greater than the increase in interstitial $[K^+]$ because of the small volume in the T-tubule lumen and because a $[K^+]$ gradient is likely to exist from the depths of the T-system to the interstitial fluid. Further support for tubular K^+ accumulation comes from indirect evidence. The resting membrane potential can fall by as much as 35 mV during continuous high frequency stimulation (Freygang et al. 1964; Gage & Eisenberg 1969; Kirsch et al. 1977; Lännergren & Westerblad 1986, 1987) and this depolarization is abolished or attenuated by de-tubulation (Gage & Eisenberg 1969; Kirsch et al. 1977; Lännergren & Westerblad 1987). Furthermore, increasing $[K^+]_o$ to 10-14 mM depolarizes the sarcolemma by 15-30 mV (Kirsch et al. 1977; Lännergren & Westerblad 1986; Chua & Dulhunty 1988). The membrane repolarization on reducing

$[K^+]_o$ is prolonged, as is also seen following trains of action potentials, i.e. the "late after-depolarization", and both effects can be attributed to a delayed K^+ efflux from the T-system (Kirsch et al. 1977).

The effect of K^+ accumulation is probably due to the depolarization which, although insufficient to directly activate the voltage sensors, would presumably inactivate some Na^+ channels (Ruff et al. 1987, 1988). Certainly, a maintained depolarization of 15-30 mV from the normal resting potential is in the range which produces a large increase in the slow inactivation of Na^+ channels (Ruff et al. 1987, 1988). Subsequently, the maximum trans-sarcolemmal Na^+ current associated with tetanic action potentials would be reduced resulting in a smaller depolarization and hence activation of the voltage sensors would be diminished. In fact, the K^+ accumulation hypothesis is supported by studies where elevated $[K^+]_o$ depresses action potential amplitude (Jones 1981; Lännergren & Westerblad 1986) and also causes a reduction in peak tetanic force (Juel 1988; present study) and a faster tetanic force decline (present study).

(iii) Chloride ions:

Another possibility is that a depletion of tubular Cl^- contributes to HFF. Some results indicating a role for Cl^- have been obtained in the present study. When denervated soleus fibres were fatigued at 50 Hz in normal Krebs solution ($[Na^+]$ = 150 mM, $[Cl^-]$ = 159.5 mM) the reduction in tetanic force occurred more slowly than in fibres fatigued at 30 Hz in Solution 2 ($[Na^+]$ = 196.5 mM, $[Cl^-]$ = 16 mM). A possible explanation for this effect is that the Cl^- in normal Krebs solution increases the resistance to HFF. However, controlled experiments have to be done to test this hypothesis. A role for Cl^- has also been implicated in recent experiments in which the developmental increase in Cl^- conductance was blocked, producing muscles that were more susceptible to HFF (De Luca et al. 1990).

Mammalian skeletal muscle normally has a high Cl^- conductance in T-tubular membranes (Palade & Barchi 1977; Dulhunty 1979) and this may be important to prevent an excessive depolarization by extracellular K^+ accumulation (Almers 1980). In fact, when the Cl^- conductance is absent or reduced, such as in myotonia congenita, excessive depolarizations, repetitive firing and prolonged late after-depolarizations are seen following short trains of action potentials (Adrian & Bryant 1974; Almers 1980). The Cl^- might provide some protection against fatigue by its ability to reduce the depolarization by K^+. This could be due to a Cl^- influx (Lipicky & Bryant 1966; Vaughan-Jones 1982) which increases the extent of membrane repolarization during or between action potentials (Kirsch et al. 1977; Almers 1980; Heiny et al. 1990), or to a reduced space constant which may attenuate the passive spread of depolarization (Bryant et al. 1969; Dulhunty et al. 1984).

To date, there is evidence in support of trans-sarcolemmal K^+, Na^+ and Cl^- shifts during intense activity or intermittent tetanic stimulation (Stréter 1963; Gonzalez-Serratos et al. 1978; Hirche et al. 1980; Saltin et al. 1981; Juel 1986, 1988; Sjogaard 1986; Lindinger & Heigenhauser 1988; Medbo & Sejersted 1990), but there are few studies on ion movements during continuous high-frequency stimulation (Stréter 1963; Hník et al. 1976). Further evaluation of ionic involvement in HFF requires quantification of intracellular, interstitial and perhaps more importantly T-tubular ion concentrations.

The rate of HFF increases with stimulation frequency (Bezanilla et al. 1972; Bigland-Ritchie et al. 1979; Jones et al. 1979; Nassar-Gentina et al. 1981; Sandercock et al. 1985; present study) and this effect is likely to be caused by increased Na^+ and K^+ fluxes through voltage-activated Na^+ and delayed rectifier K^+ channels during each action potential. This effect of stimulation frequency on the rate of fatigue cannot be accounted for by differential metabolic changes (Nassar-Gentina et al. 1981; Chasiotis et al. 1987). However, there may be additional ion shifts due to other mechanisms, for example, K^+ efflux through Ca^{2+}-activated K^+ channels (Fink et al. 1983; Lüttgau & Wettwer 1983) or effects of metabolites on ionic conductances (Renaud & Mainwood 1985).

A fast component of tetanic force recovery (Vergara et al. 1977; Nassar-Gentina et al. 1981; Lännergren & Westerblad 1986; present study) possibly correlates with recovery of the resting membrane potential (Gage & Eisenberg 1969; Kirsch et al. 1977; Lännergren & Westerblad 1986). This may reflect the time-course of K^+ diffusion from the T-system (Nakajima et al. 1973; Kirsch et al. 1977) or the maximum rates of Na^+ and K^+ transport by Na-K pumps (Clausen & Everts 1988). However, complete recovery of tetanic force in soleus fibres took about 10 min and this might reflect a component due to a slower recovery of metabolites (Kushmerick & Meyer 1985). The extremely rapid fatigue in sternomastoid fibres (1-2 s), however, was probably too fast for large metabolic changes and could be caused solely by trans-sarcolemmal ionic shifts.

Increasing the stimulation frequency produced similar effects on contraction to those caused by small increases in $[K^+]_o$ or reductions in $[Na^+]_o$, i.e. depression of peak tetanic force and increased rate of force decline. Since the frequency-dependent depression of peak tetanic force was caused by the same ion shifts that caused the decline in tetanic force, it might also be regarded as fatigue. Furthermore, the lack of a distinct plateau in peak tetanic force also indicates that fatigue processes have an early onset. This means that normalizing to peak tetanic force at stimulation frequencies

greater than fusion frequency would underestimate the loss of force due to fatigue processes.

(c) Differences in the rate of HFF with fibre-type and denervation

Fibre-type differences in the resistance to fatigue induced by repeated tetani are so distinct that they have been used to define different motor unit or fibre-types (Burke et al. 1973; Clamann & Robinson 1985; Westerblad & Lännergren 1986). In this case, fatigue resistance is well correlated with the oxidative capacity of the fibres (Burke et al. 1973; Kugelberg & Lindegren 1979) indicating that metabolic factors are involved. Results in the present study confirm that slow-twitch fibres are more resistant to high-frequency fatigue than fast-twitch fibres (Jones 1981; Crow & Kushmerick 1983; Clamann & Robinson 1985; Sandercock et al. 1985). The two different types of fast-sternomastoid preparations, although differing in their resistance to intermittent tetanic fatigue (Luff 1985) and oxidative capacities (Dulhunty & Dlutowski 1979), revealed only small differences in the rate of HFF. Other studies have also shown that fast motor units or fibre-types have considerable overlap in the rate of force decline in prolonged tetani (Clamann & Robinson 1985; Lännergren & Westerblad 1986). This suggests that resistance to HFF may not be linked to the oxidative capacity and this agrees with the idea that HFF is mainly caused by trans-sarcolemmal ionic shifts.

Several factors may be responsible for the greater resistance to HFF in slow-twitch than fast-twitch fibres. This difference could be explained by a greater resistance to changes in the action potential (Hanson 1973). If this is the case, the slower HFF in slow-twitch fibres might be attributed to: (i) smaller trans-sarcolemmal ionic shifts, as seen with repeated tetani (Juel 1986; Lindinger & Heigenhauser 1988); (ii) a greater ability to increase Na-K pump activity (Everts et al. 1989); (iii) dimensions of the T-tubule lumen which are greater in slow-twitch fibres and allow greater diffusional fluxes (Dulhunty et al. 1984); (iv) fast and slow inactivation of Na^+ channels occurring at more positive membrane potentials (Ruff et al. 1987, 1988). The relative importance of these processes remains speculative.

Similarly, the denervation-induced decrease in fatigue resistance might be attributed to a smaller action potential overshoot (McArdle et al. 1981; Kirsch & Anderson 1986) or a reduced number of Na-K pumps (Clausen et al. 1981, 1983), although the second finding has been disputed (Festoff et al. 1977). Recovery of the early changes in fatigue resistance at long denervation durations may be linked to a recovery of the overshoot of the action potential (Kirsch & Anderson 1986). The reason for the more regular fatigue curves in denervated than in normal soleus bundles is uncertain but it may reflect a more homogeneous fibre-type distribution in the denervated preparation (Dulhunty 1985).

(d) Effect of high-frequency fatigue on the rate of relaxation

A slowing of relaxation during fatigue is thought to be a consequence of changes in metabolite levels (Edwards et al. 1975; Dawson et al. 1980; Hultman et al. 1981; Sahlin et al. 1981; Cady et al. 1989b). The absence of any slowing of relaxation in red-sternomastoid and soleus fibres would not be unexpected if HFF is solely caused by action potential failure due to ionic shifts. The slowing of relaxation in white-sternomastoid fibres may be caused by the fatigue processes that cause a slower decline of tetanic $[Ca^{2+}]_i$ (Allen et al. 1989) or slower crossbridge detachment (Edwards et al. 1975). However, an alternative explanation is that parvalbumin, a myoplasmic Ca^{2+} buffer, becomes saturated with Ca^{2+} at the longer tetanus durations and thereafter does not contribute to Ca^{2+} removal. Cannell and Allen (1984) predicted that at long tetanus durations parvalbumin would become saturated with Ca^{2+} and thus no longer contributes to the decline of $[Ca^{2+}]_i$ or to relaxation. This hypothesis is supported in studies using prolonged tetani in frog twitch fibres (Blinks et al. 1978; Cannell 1986). In mammalian skeletal muscle, parvalbumin is present only in fast-twitch fibres (Celio & Heizmann 1982), and specifically in the type IIb fibres (Leberer & Pette 1986), that predominate in the white-sternomastoid (Dulhunty & Dlutowski 1979). It is therefore possible that the slowing of relaxation with stimulation duration in white-sternomastoid fibres is caused by maximum loading of parvalbumin with Ca^{2+} rather than a metabolism related fatigue process.

5.5 SUMMARY

(1) High-frequency fatigue - the decline of peak tetanic force induced by prolonged continuous tetanic stimulation - was studied in bundles of fibres isolated from rat skeletal muscles.

(2) HFF induced at 100 Hz was markedly slower in the slow-twitch soleus that in fast-twitch sternomastoid fibres. Only small differences in the rate of fatigue were seen between white- and red-sternomastoid fibres.

(3) A slowing of relaxation during HFF was seen in white-sternomastoid fibres but not in red-sternomastoid or soleus fibres. It is suggested that the slowing of relaxation in white-sternomastoid fibres is a consequence of a saturation of parvalbumin with Ca^{2+} due to increasing the stimulation duration.

(4) HFF induced at 50 Hz was compared in normal and denervated soleus fibres. The rate of fatigue was increased following denervation for 2-3 weeks, then as the duration

of denervation was increased, the rate of fatigue was restored towards that in the normal soleus.

(5) Rapid application of high K^+ (200K) during HFF completely restored force in denervated soleus fibres. The amplitude of 200K-contractures were the same in control and fatigued conditions. This suggests that during HFF: (i) there is impaired SR Ca^{2+} release; (ii) mechanical inactivation is not involved; (iii) the voltage sensors can still operate maximally.

(6) The rate of HFF depended on the composition of the bathing solution. Increasing $[K^+]$ from 3.5 to 10 mM and decreasing $[Na^+]$ from 196.5 to 80.5 mM both depressed peak tetanic force and increased the rate of fatigue. This suggests that the reduction in force during prolonged tetani was due, at least in part, to action potential failure resulting in impaired Ca^{2+} release.

(7) Increasing the stimulation frequency resulted in a greater reduction of force during prolonged tetani in both normal and denervated soleus fibres. This suggests that changes in the trans-sarcolemmal ionic distribution, consequent to action potential activity, are likely to be an important cause of HFF.

(8) A rapid recovery of tetanic force occured following a brief cessation of stimulation which is consistent with a rapid recovery process such as for ion diffusion. This indicates that the T-system may be an important site of fatigue.

CHAPTER 6

EFFECTS OF β-ADRENOCEPTOR ACTIVATION ON CONTRACTION IN FATIGUED SKELETAL MUSCLE

6.1 INTRODUCTION

Early studies on the action of catecholamines on skeletal muscle were mainly performed on fatigued muscle. In these experiments, it was observed that catecholamine administration, stimulation of the splanchic nerve to the adrenal medulla, or sympathetic nerve stimulation could partially restore force in muscles fatigued by continuous twitch or prolonged tetanic stimulation via their motor nerves (Cannon & Nice 1913; Gruber 1922a; 1922b; Orbeli 1923; Corkill & Tiegs 1933; Bülbring & Burn 1939, 1940; Brown et al. 1948). In amphibian muscle, these defatiguing effects have been entirely attributed to the recovery of neuromuscular transmission (Corkill & Tiegs 1933; Bowman & Nott 1969). In mammalian muscle, an adrenaline-induced restoration of force in prolonged tetani can be explained, in part, by an effect on neuromuscular transmission which is mediated by α-adrenoceptors (Bülbring & Burn 1940; Bowman & Nott 1969). However, there are also defatiguing effects in the muscle fibres due to activation of β-adrenoceptors (Bülbring & Burn 1939, 1940; Jami et al. 1984).

This chapter deals with the effect of catecholamines on fatigue processes in the muscle fibres. The primary aim of this project was to use the β_2-adrenergic agonist, terbutaline, as a pharmacological tool to investigate the involvement of the Na-K pump in fatigue. It is known that β-adrenergic agonists stimulate the sarcolemmal Na-K pump resulting in increased transport of K^+ into and Na^+ out of fibres, in resting muscle (Clausen & Flatmann 1977; McArdle & D'Alonzo 1981; see Chapter 4.3c). Furthermore, an accumulation of extracellular K^+ and/or depletion of extracellular Na^+ are thought to contribute to fatigue (Bigland-Ritchie et al. 1979; Jones et al. 1979; Juel 1986). Therefore, it was postulated that β-adrenergic stimulation of the Na-K pump would provide some protection against fatigue by either (i) reducing the stimulation-induced decline of the trans-sarcolemmal Na^+ and K^+ gradients or (ii) reducing membrane depolarization by an electrogenic contribution from the Na-K pump. Based on this hypothesis, Juel (1988) demonstrated that terbutaline induced a small increase in the resistance to intermittent tetanic fatigue in isolated mouse soleus muscle. He also found that the changes in the trans-sarcolemmal ion concentrations and membrane depolarization were attenuated by terbutaline and subsequently concluded that the increased fatigue resistance was due to increased ion transport by the Na-K pump. He did note, however, that the effect of terbutaline on fatigue resistance was small, i.e. a 10% smaller reduction in peak tetanic force. The small size of this effect is possibly because metabolic factors are more important than ionic shifts during intermittent

tetanic fatigue (Dawson et al. 1978; Hermansen 1981; Cady et al. 1989a). It was postulated that terbutaline might provide a greater increase in fatigue resistance during high-frequency fatigue, by stimulating the Na-K pump, where trans-sarcolemmal ionic shifts are thought to make a major contribution (Bigland-Ritchie et al. 1979; Jones et al. 1979; see Chapter 5).

6.2 METHODS

The muscle preparations, methodology, solutions and drugs used are described in Chapters 2, 3, 4 and 5. High-frequency fatigue was induced by continuous stimulation at 50 Hz for 20 s. This protocol minimized any frequency-dependent depression of peak force and produced severe fatigue, i.e. force was reduced to less than 50% of peak value. All experiments were performed in Solution 1 (Table 2.1) at 24°C. The fatigue parameters measured were: plateau time (PT); 95-50% fatigue time (95-50%FT); and fatigue indices at 5, 10, and 20 s of stimulation (FI_5, FI_{10}, FI_{20}). These parameters are all defined in Chapter 2.

6.3 RESULTS

(a) Effect of terbutaline and dibutyryl-cAMP on high-frequency fatigue

Terbutaline (10 μM) produced two effects on force in prolonged tetani: (i) peak tetanic force was increased (see Chapter 4) and (ii) the decline of tetanic force occurred more slowly. Figure 6.1A and C show the potentiation of tetanic force by terbutaline in normal and denervated soleus fibres respectively. The fatigue curves (Figure 6.1B and D), produced by normalizing the records in A and C, show a typical terbutaline-induced slowing of fatigue in a normal soleus preparation and one of the largest responses in a denervated soleus preparation respectively. Average effects of terbutaline on the rate of fatigue (FI_{10}) are presented in Table 6.1.

In normal soleus fibres, terbutaline increased the FI_{10} by 10.0 ± 2.8% ($n = 7$, $P < 0.02$, paired t-test). This represented a 19.8 ± 3.9% reduction of the tetanic force loss in control tetani (range 9.5 - 34.9%). The FI_{20} and 95-50%FT also indicated an increased fatigue resistance. However, the FI_5 and PT showed no significant change. In denervated soleus fibres, the FI_{10} was increased with terbutaline by 4.7 ± 0.5% ($n = 25$, $P < 0.001$, paired t-test); a 9.4 ± 1.0% reduction of the tetanic force loss (range 0 - 20.7%). All other fatigue parameters in denervated soleus fibres indicated a significant increase in fatigue resistance with terbutaline. These effects were fully reversible on wash-out. The increase in fatigue resistance was significantly greater in normal than in denervated soleus fibres ($P < 0.05$, ANOVA). This difference would, in fact, have been anticipated if terbutaline had produced this effect by stimulating the

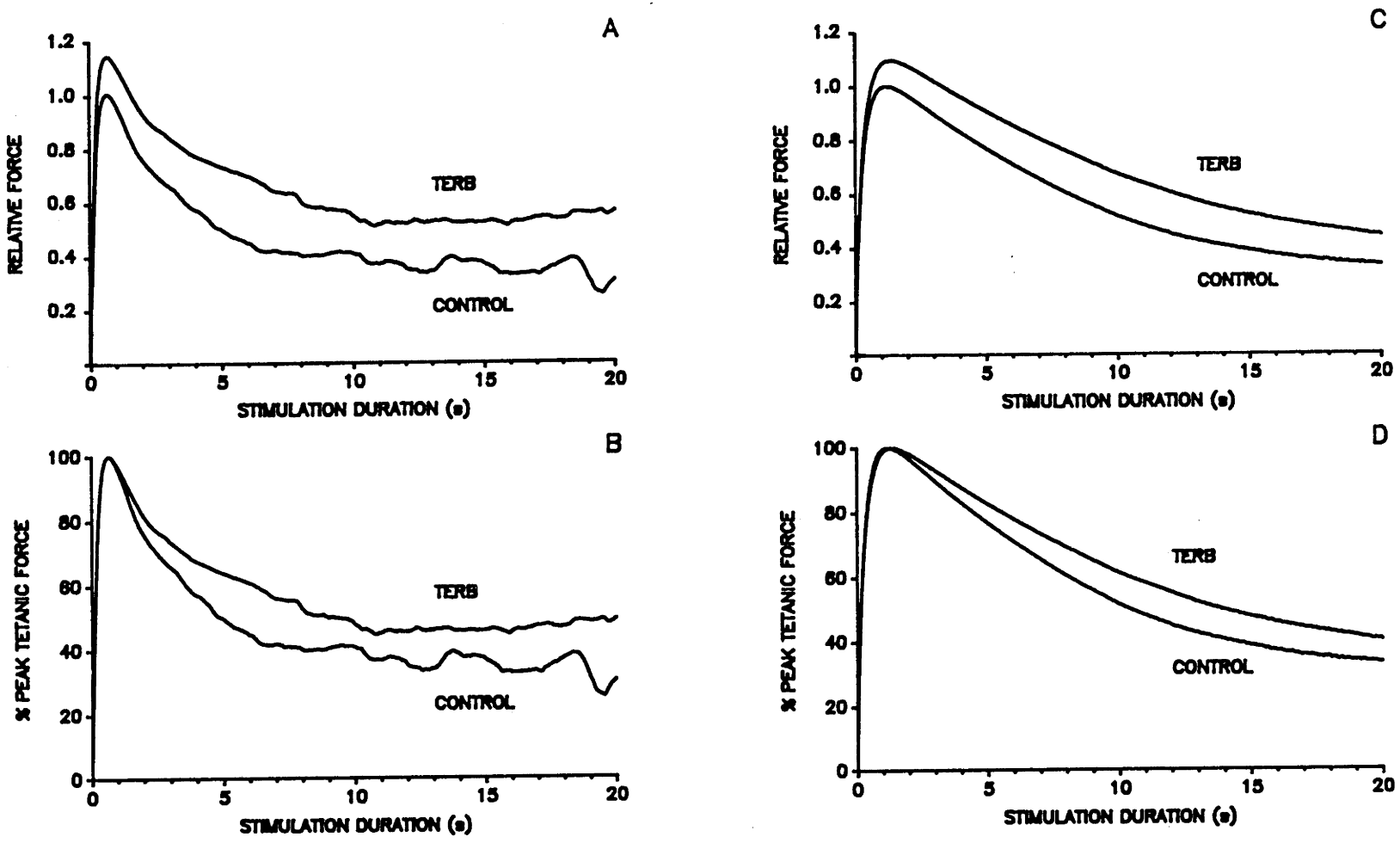


FIG 6.1: Effect of 10 μM terbutaline on prolonged tetani in a normal soleus preparation (A & B) and a denervated soleus preparation (C & D). Tetani were evoked at 50 Hz. Solution 1, 24°C. A and C are superimposed records which show the relative differences in tetanic force. B and D are fatigue curves produced from A and C respectively. CONTROL is the record immediately prior to the addition of terbutaline. TERB is the record showing the maximum effect of terbutaline on tetanic force in each preparation.

TABLE 6.1: Effect of 10 μM terbutaline and 2 mM dibutyryl-cAMP on the rate of high-frequency fatigue in normal and denervated soleus fibres

MUSCLE PREPARATION	DRUG	RATE OF FATIGUE (FI_{10})		%Reduction In Force Loss
		Control	+ Drug	
Normal Soleus	Terbutaline	51.4 ± 8.5%	61.4 ± 6.0% *	19.8 ± 3.9% (n = 7)
Denervated Soleus	Terbutaline	50.2 ± 3.0%	54.8 ± 2.8% **	9.4 ± 1.0% (n = 25)
	DBcAMP	46.7 ± 4.8%	54.1 ± 4.4% **	14.2 ± 1.7% (n = 8)

Shown are the mean values (± s.e.m.). n = number of preparations. Solution 1, at 24°C. Fatigue was induced by continuous stimulation at 50 Hz for 20 s. The effect of each drug was significant . * $P < 0.02$ and ** $P < 0.001$ (paired *t*-test).

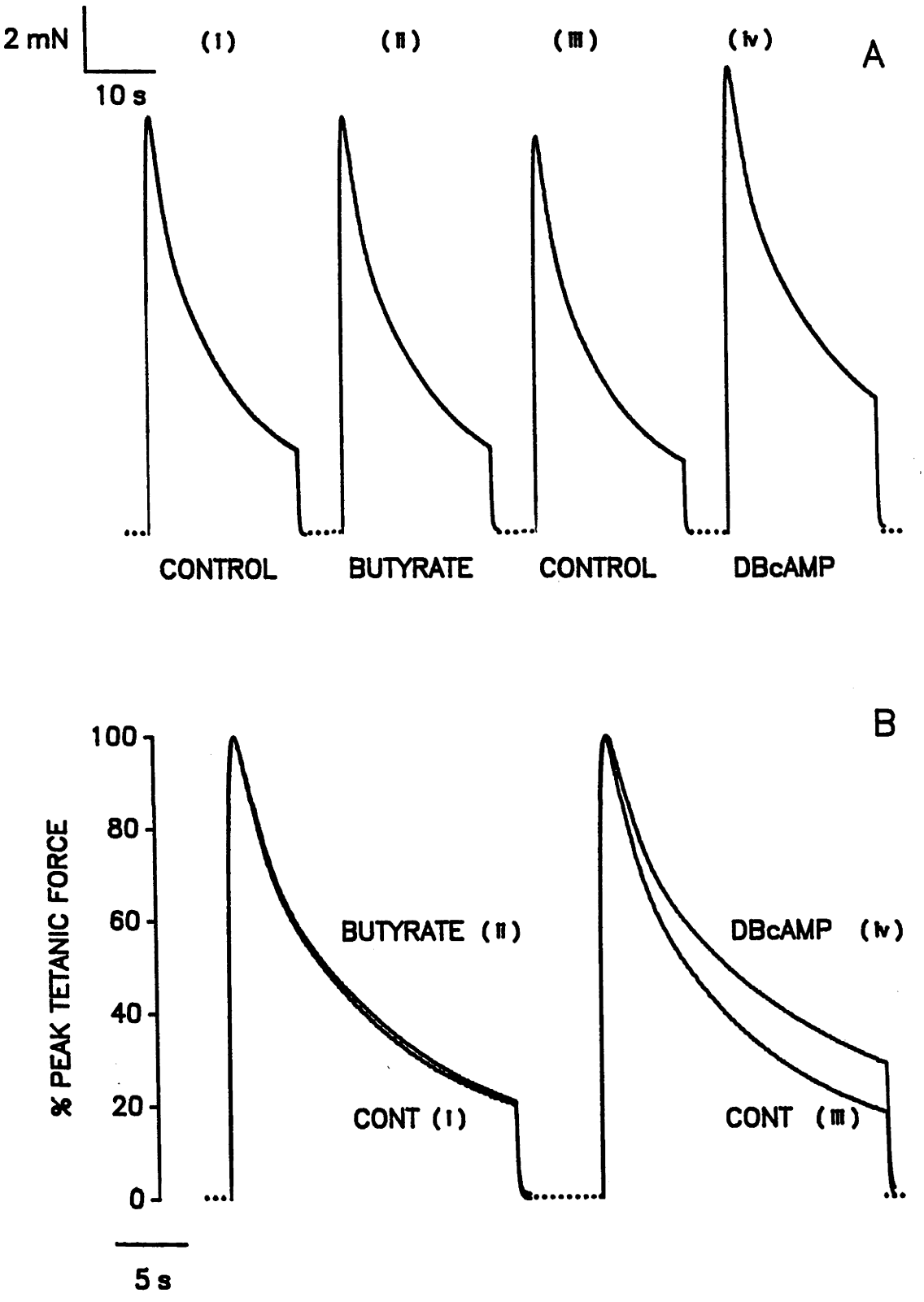


FIG 6.2: Representative effects of 2 mM butyrate and 2mM dibutyryl cyclic-AMP on prolonged tetani in a single denervated soleus preparation. Tetani were evoked at 50 Hz. Solution 1, 24°C. (A) absolute force records and (B) fatigue curves produced from the same records. CONTROL (& CONT) are described in the legend for Fig. 6.1. BUTYRATE is the record showing the effect of butyrate (ii) after 30 min. DBcAMP is the record showing the maximum effect of dibutyryl cyclic-AMP (iv) after 40 min.

Na-K pump because the number of Na-K pumps was reduced following denervation (Clausen et al. 1981, 1983). Denervated soleus preparations were used in most of the following studies because of the reproducibility of the response on successive applications of terbutaline (see Chapter 3.3c).

DBcAMP, a membrane permeable derivative of cAMP, was used to test if cAMP was the intracellular messenger responsible for the terbutaline-induced slowing of fatigue. Figure 6.2 shows a representative example of the effect of 2 mM DBcAMP on a prolonged tetanus in a denervated soleus preparation. DBcAMP increased peak tetanic force (Figure 6.2A (iv)) and decreased the rate of HFF (Figure 6.2B (iv)). On average the FI_{10} was increased by 7.4 ± 1.0% (n = 8, $P < 0.001$, paired t-test); representing a 14.2 ± 1.7% reduction of the tetanic force loss (range 6.7 - 20.8%). The maximum effect of DBcAMP took longer to be established than with terbutaline, sometimes taking 30-40 min. This observation is consistent with a slow rate of entry of DBcAMP across the sarcolemma, as observed in cardiac muscle (Drummond et al. 1974). The protective effect of DBcAMP was not due to butyrate, an hydrolysis product of DBcAMP, because butyrate neither increased force nor slowed fatigue (Figures 6.2A (ii) and 6.2B (ii) respectively).

There was no obvious correlation between the peak force potentiation and the slowing of fatigue (percentage reduction of control force loss) with either terbutaline or DBcAMP when different preparations were compared. Certainly, some preparations produced only a small increase in peak tetanic force while at the same time, revealing a dramatic slowing of fatigue. However, none of the preparations displayed a large peak force increase without a notable slowing of fatigue. Moreover, it was noted, especially with DBcAMP, that at the time when peak force potentiation was maximal, the protective effect against fatigue was sometimes still increasing. This suggests that the potentiation of peak tetanic force and slowing of fatigue were separate phenomena.

In summary, these results show that terbutaline can induce a small increase in the resistance to HFF, by a direct action on the muscle fibres (10-20% reduction of the control tetanic force loss), and this effect is likely to be mediated by cAMP.

(b) Effect of terbutaline on high-frequency fatigue: Role of the sodium-potassium pump

The main cause of HFF is thought to be a change in the ionic distribution across the sarcolemma, specifically an increase of $[K^+]_o$ and decrease of $[Na^+]_o$ (Bezanilla et al. 1972; Bigland-Ritchie et al. 1979; Jones et al. 1979; see Chapter 5). It has been hypothesized that limitation of Na-K pump function to transport K^+ back into and Na^+ back out of the fibres is responsible for the change in the ionic distribution (Clausen &

TABLE 6.2: Time-dependent effects of 1 mM ouabain on peak tetanic force and the rate of high-frequency fatigue in denervated soleus fibres

INCUBATION TIME	PEAK TETANIC FORCE	RATE OF FATIGUE (FI_{10})		
	Ratio to control	Control	+ Ouabain	Diff
10 min	0.696 ± 0.031 (n = 7)	39.2 ± 2.1%	36.4 ± 2.0%	-2.9 ± 1.5% * (n = 9)
20 min	0.640 ± 0.019 (n = 7)	39.8 ± 2.2%	39.0 ± 2.2%	-0.8 ± 1.3% ** (n = 8)
30 min	0.564 ± 0.036 (n = 7)	40.2 ± 2.8%	38.0 ± 1.9%	-2.2 ± 3.0% ** (n = 6)

Shown are the mean values (± s.e.m.). n = number of preparations. Solution 1, at 24°C. Fatigue was induced by continuous stimulation at 50 Hz for 20 s. * $0.1 > P < 0.05$ and ** $P > 0.1$ (paired *t*-test). Effect of ouabain on peak tetanic force is paired data only, from short (2 s) and long duration (20 s) tetani.

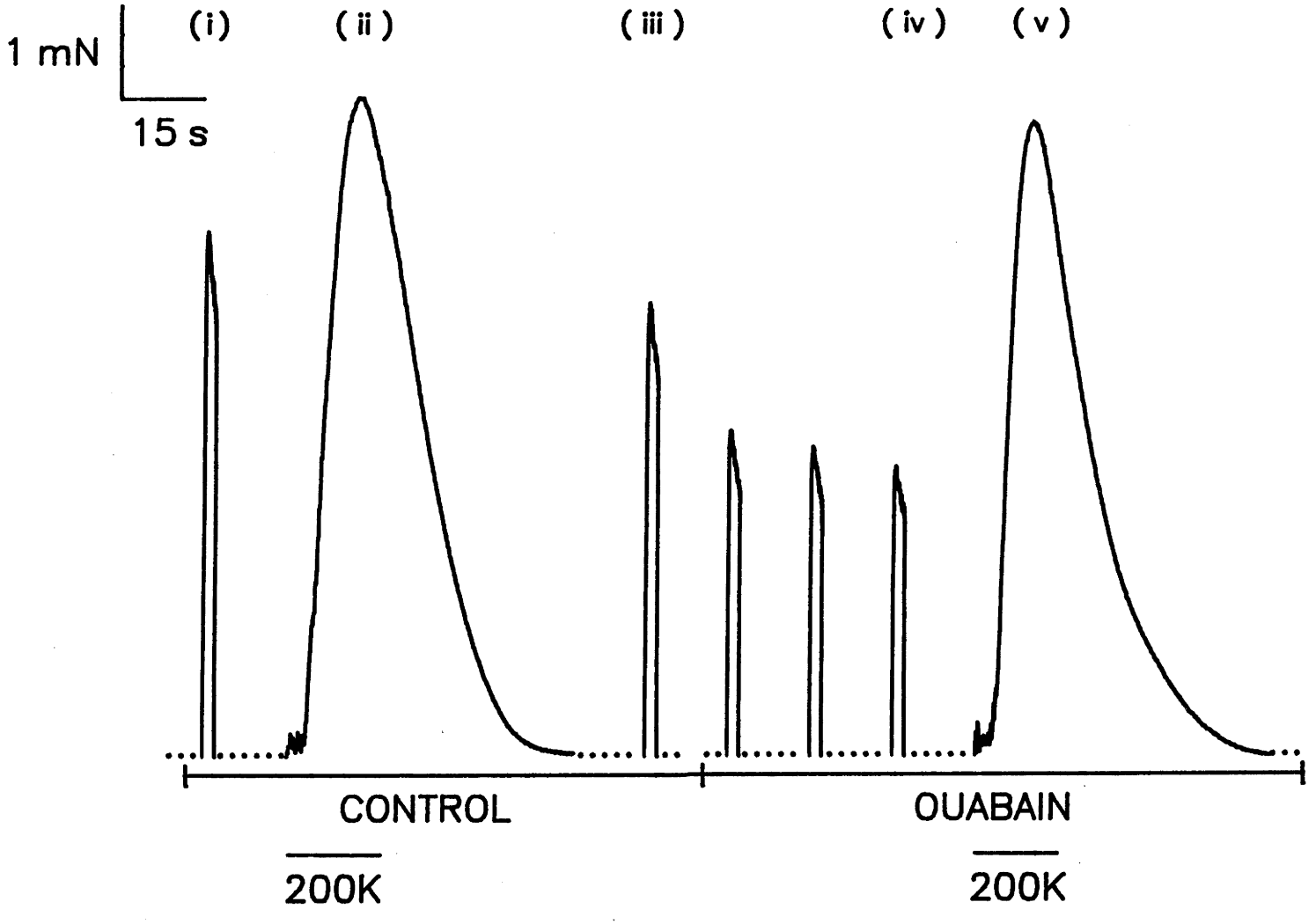


FIG 6.3: 200K-contractures and tetani in the absence and presence of 1 mM ouabain in a denervated soleus preparation. Tetani were evoked every 10 min at 50 Hz for 2 s. Solution 1, 24 °C. Ouabain depressed peak tetanic force (iv) to 59% of the control tetanus (iii) in 30 min. The ratio of the peak force in 200K to peak tetanic force was 1.33 in the absence of ouabain (ii/i) and 1.45 in the presence of ouabain (v/iii).

Everts 1988, 1989; Sejersted 1988). β-adrenoceptor activation stimulates the sarcolemmal Na-K pump resulting in (i) an increased K^+ influx and Na^+ efflux (Clausen & Flatmann 1977; McArdle & D'Alonzo 1981; Pfliegler et al. 1983; Molnár et al. 1986) and (ii) an electrogenic hyperpolarization due to a Na-K pump current (Clausen & Flatman 1977; Hicks & McComas 1989). Therefore, it was hypothesized that terbutaline would slow HFF by either of these two mechanisms. The observation that activation of β-adrenoceptors attenuated the increase in plasma $[K^+]$ during exercise supports this hypothesis (Williams et al. 1985). Furthermore, the effect of reducing the stimulation frequency from 50 to 30 Hz resulted in a reduction of $14.1 \pm 1.5\%$ ($n = 16$) of the tetanic force loss (from FI_{10}) in denervated soleus fibres, which is quantitatively similar to the effect of terbutaline.

(i) Effect of ouabain on high-frequency fatigue:

The involvement of the Na-K pump in HFF was tested with ouabain; a specific inhibitor of the Na-K pump (Albers et al. 1968; Clausen 1986; Clausen & Everts 1989; Sjodin 1989). Ouabain (1 mM) induced time-dependent effects on force in prolonged tetani. Figure 6.3 shows the depression of force in short duration tetani during exposure to ouabain in a denervated soleus preparation. Peak tetanic force was markedly reduced in the first 5-10 min after application of ouabain and then declined more slowly. Average ouabain-induced effects on peak tetanic force are presented in Table 6.2. Peak tetanic force was reduced by 30-45% in the 10-30 min following application of ouabain. This effect reversed slowly when ouabain was washed out, taking 1-2 hours. Ouabain caused only a small but non-significant increase in the rate of fatigue during exposure for up to 30 min (calculated from the fatigue curves) (Table 6.2). One notable effect of ouabain, seen in five out of nine preparations, was the appearance of oscillations during the decline of tetanic force (Figure 6.4). This observation, along with those features on oscillations noted in Chapter 5.3c, indicate that oscillations might be linked to greater changes in the trans-sarcolemmal ionic distribution although the precise mechanism is unknown. The main effect of ouabain thus appears to be the loss of force (or failure to activate) during the early stages of stimulation.

High $[K^+]$ solutions were used to investigate the mechanism of the ouabain-induced depression of peak tetanic force. Figure 6.3 shows that the amplitude of 200K-contractures was unaffected during exposure to ouabain at a time when peak tetanic force had fallen to about 60% of that in the control tetanus. Similar effects were also seen in three other denervated soleus preparations. Therefore, it is concluded that during the ouabain-induced depression of peak tetanic force (i) there is impaired SR Ca^{2+} release and (ii) the voltage sensors and voltage-dependent activation processes of E-C coupling can still operate maximally. The force depression might be caused by a

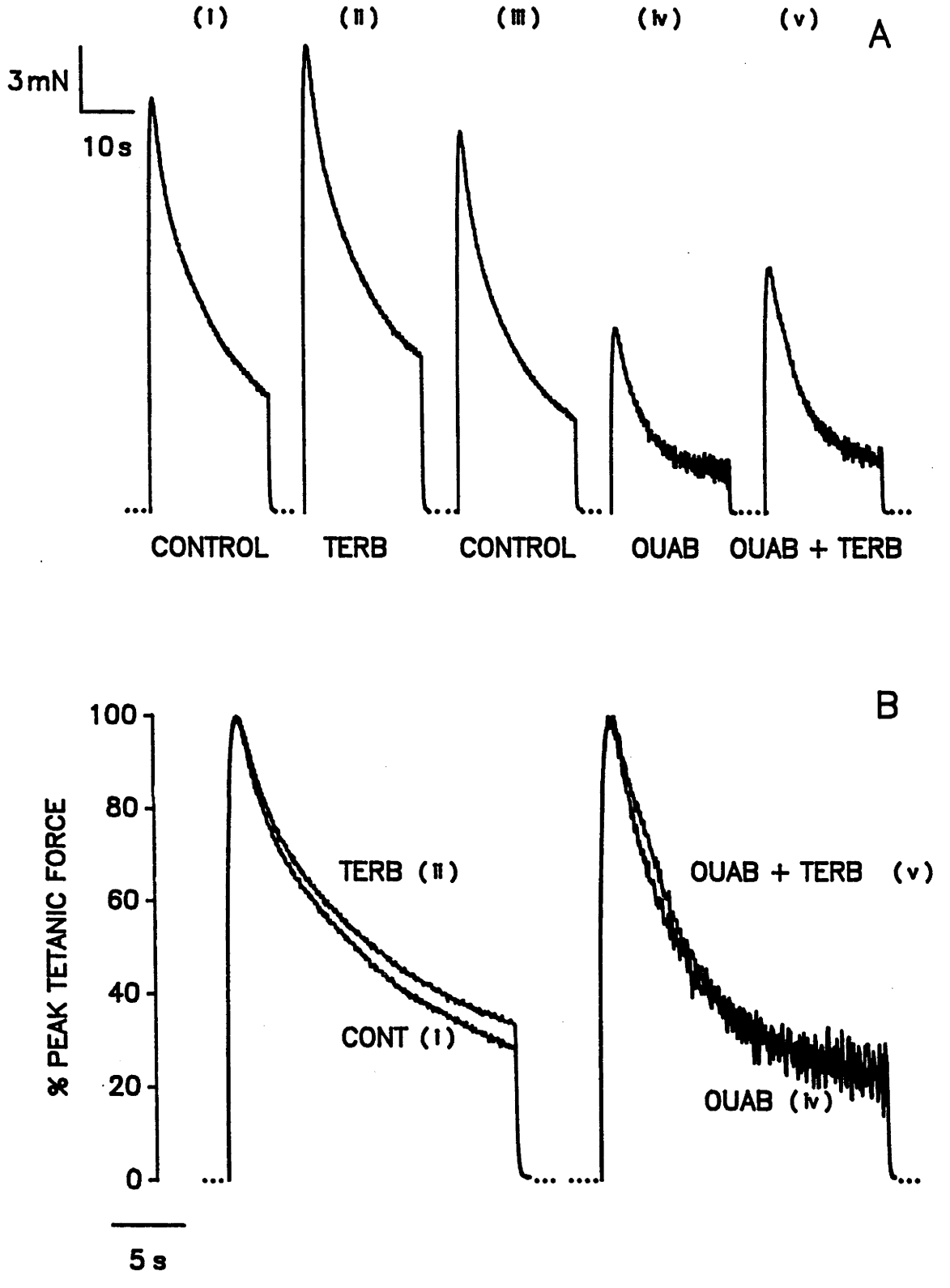


FIG 6.4: Effect of 10 μM terbutaline on prolonged tetani in the absence and presence of 1 mM ouabain in a denervated soleus preparation. Solution 1, 24°C. Tetani were evoked at 50 Hz for 20 s. (A) absolute force records and (B) the same records presented as fatigue curves. CONTROL and TERB are described in the legend for Fig. 6.1. The second control (iii) was obtained 30 min after wash-out. OUAB is the record showing the effect of 1 mM ouabain (iv) at 30 min incubation. OUAB + TERB is the record showing the maximum effect of terbutaline in the presence of ouabain (v).

reduction of the amplitude of tetanic action potentials, possibly because of a ouabain-induced decrease in the resting membrane potential, which will result in the slow inactivation of Na^+ channels (Ruff et al. 1987, 1988). The resting membrane potential was measured during exposure to ouabain in normal soleus fibres. The resting potential of -83.9 ± 0.6 mV (66/7; fibres/muscles) in control fibres was not reduced when ouabain was added for up to 30 min, i.e. -81.7 ± 0.7 (49/4), at a time when ouabain had markedly depressed peak tetanic force (Table 6.2). However, ouabain did induce a small depolarization to -76.2 ± 0.9 mV (31/2) when added for 2.5-3 hours. This indicates that a membrane depolarization was unlikely to be responsible for the depression of peak tetanic force. It should be noted, however, that the membrane potential was not recorded in denervated soleus fibres because of the presence of fibrillations when fibres were bathed in Solution 1 (see Chapter 4.3c).

(ii) Effect of terbutaline on high-frequency fatigue in the presence of ouabain:

Figure 6.4 shows that terbutaline increased peak tetanic force and slowed fatigue in the presence of 1 mM ouabain, indicating that the protective effect of terbutaline occurred even though Na-K pump activity was inhibited. Peak force potentiation by terbutaline was significantly greater in the presence of ouabain, 41.8 ± 12.9%, than in the control state, 12.1 ± 2.1%, ($n = 8$, $P < 0.5$, ANOVA). The slowing of fatigue is best shown by the average data. In five denervated soleus preparations, terbutaline increased the FI_{10} from 37.4 ± 2.8% (ouabain) to 42.2 ± 2.4% (ouabain + terbutaline); a reduction of 7.6 ± 1.4% of the tetanic force loss. Control responses to terbutaline alone were obtained in four of the five preparations where terbutaline increased the FI_{10} from 45.1 ± 2.7 (control) to 48.9 ± 3.4% (terbutaline); a reduction of 7.0 ± 1.5% of the force loss.

In summary, these results suggest that normal function of sarcolemmal Na-K pumps is required to prevent an early loss of tetanic force. However, the β-adrenoceptor mediated increase in fatigue resistance is not due to enhanced Na-K pump activity.

(c) Effect of terbutaline on high-frequency fatigue: Role of the calcium release channel

Since the hypothesis that terbutaline increases the resistance to HFF by stimulating the Na-K pump could be rejected, it was necessary to try to establish which cellular processes were involved. Because of a similarity in some of the actions of caffeine and terbutaline (Chapter 4.4b), it was speculated that both drugs may increase fatigue resistance by a similar mechanism, perhaps by an effect on E-C coupling.

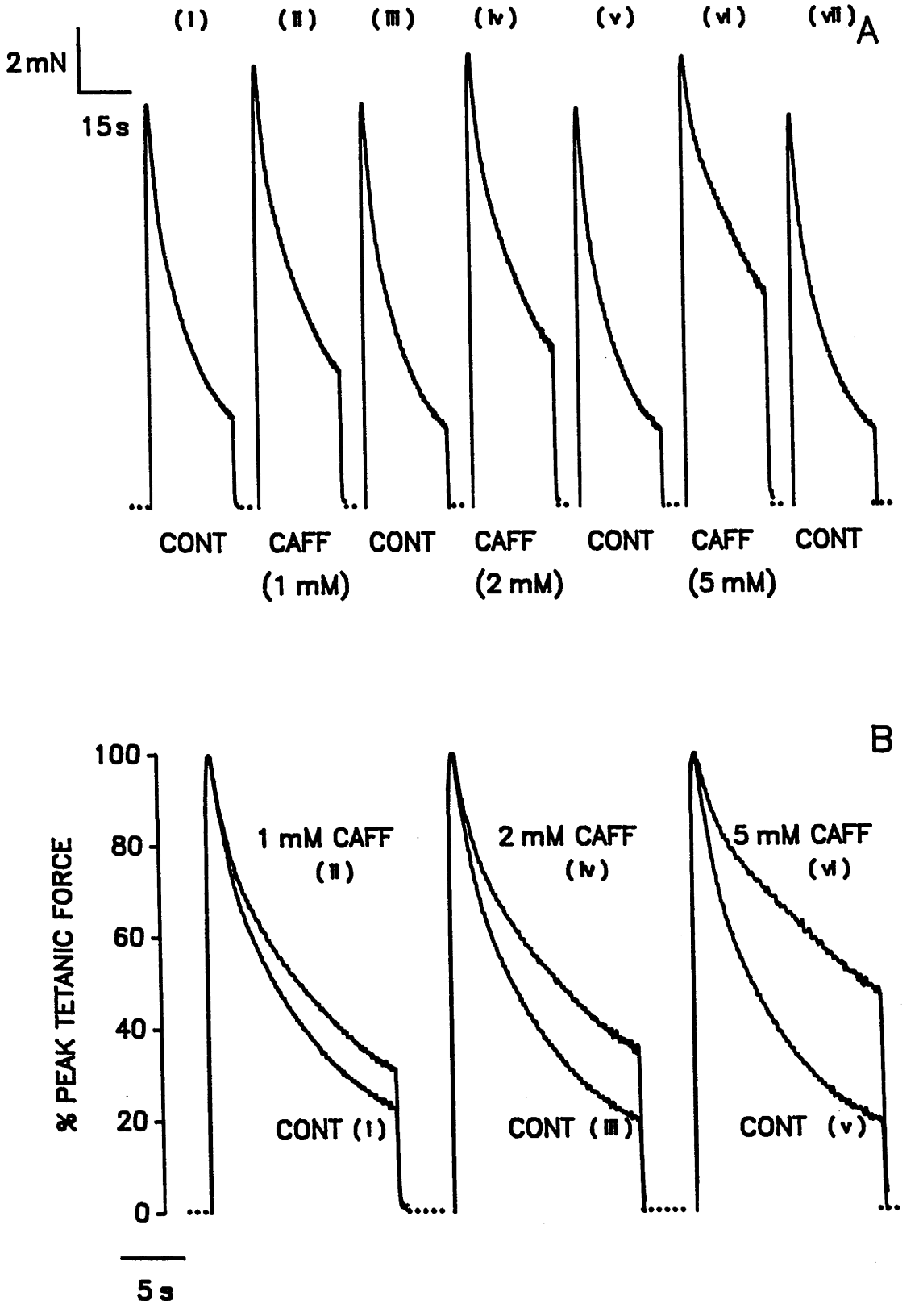


FIG 6.5: Concentration-dependent effects of caffeine on prolonged tetani in a denervated soleus preparation. Solution 1, 24°C. Tetani were evoked at 50 Hz for 20 s. (A) absolute force records and (B) fatigue curves from the same records. CONT is described in the legend for Fig. 6.1. CAFF is the record showing the maximum effect of caffeine on tetanic force at each concentration, recorded 10 min after application of caffeine. Thirty minutes was allowed between successive caffeine applications.

TABLE 6.3: Concentration-dependent effects of caffeine on the rate of high-frequency fatigue in denervated soleus fibres: comparison with the effect of terbutaline

DRUG		RATE OF FATIGUE (FI_{10})		%Reduction In Force Loss
	Concentration	Control	+ Drug	
Caffeine	(1 mM)	46.2 ± 3.8%	54.0 ± 3.2%	13.9 ± 1.3% (n = 15)
	(2 mM)	39.6 ± 1.2%	52.4 ± 1.3%	21.1 ± 3.6% (n = 3)
	(5 mM)	40.0 ± 5.2%	66.2 ± 5.0%	45.2 ± 4.7% (n = 5)
Terbutaline	(10 μM)	50.2 ± 3.0%	54.8 ± 2.8%	9.4 ± 1.0% (n = 25)

Shown are the mean values (± s.e.m.). n = number of preparations. Solution 1, at 24°C. Fatigue was induced by continuous stimulation at 50 Hz for 20 s.

(i) Effect of caffeine on high-frequency fatigue:

Caffeine increases $[Ca^{2+}]_i$ and force during submaximal membrane depolarization (Lüttgau & Oetliker 1968; Delay et al. 1986). If the SR could release more Ca^{2+} during diminished voltage activation (due to action potential failure) in HFF, then it might be expected that tetanic $[Ca^{2+}]_i$ and force would decline more slowly. Caffeine (1-5 mM) induced two effects on force in prolonged tetani. Figure 6.5A shows that caffeine increased peak tetanic force (also see Chapter 4.3g) and the decline of tetanic force occurred more slowly. Caffeine increased fatigue resistance in a concentration-dependent manner which is best illustrated in the fatigue curves (Figure 6.5B). Average values are shown in Table 6.3. Caffeine (1 mM) increased the FI_{10} by 7.8 ± 0.8% (n = 15); a reduction of 13.9 ± 1.3% of the control force loss which was quantitatively similar to the effect of terbutaline. When the caffeine concentration was increased to 2-5 mM, much larger effects were seen (Figure 6.5 iv and vi): 5 mM caffeine prevented almost half of the tetanic force loss. Higher caffeine concentrations were not tried. There was no obvious relation between the increase of peak tetanic force and the slowing of fatigue by caffeine. This is demonstrated in Figure 6.5A where increasing the caffeine concentration from 2 to 5 mM produced no additional increase in peak tetanic force but fatigue resistance increased dramatically. These results suggest that it is possible to slow HFF through modulatory influences on E-C coupling, possibly via increased Ca^{2+} release (Delay et al. 1986; Klein et al. 1990) and/or increased Ca^{2+} sensitivity of the myofilaments (Wendt & Stephenson 1983).

(ii) Effect of terbutaline on high-frequency fatigue in the presence of caffeine:

To test the hypothesis that terbutaline and caffeine slow fatigue by a similar mechanism, the effect of 10 μM terbutaline (which produces maximal effects on fatigue resistance) was tested in the presence of 1 mM caffeine (a concentration that produced a submaximal slowing of fatigue, but one in which the only important effect would be on SR Ca^{2+} release (Delay et al. 1986; Fryer & Neering 1989). It was predicted that the effect of terbutaline would be attenuated if both drugs work by a similar mechanism. Figure 6.6 shows a representative experiment in which pretreatment with 1 mM caffeine completely masked the ability of terbutaline to potentiate peak tetanic force (A) and slow fatigue (B). In these experiments the addition of terbutaline in the absence and presence of caffeine was performed in a random sequence. In seven experiments in denervated soleus fibres terbutaline increased the FI_{10} from 46.1 ± 4.2% to 51.8 ± 4.4% in the absence of caffeine. Caffeine (1 mM) increased the FI_{10} from 46.3 ± 4.2% to 55.1 ± 3.8%. Terbutaline caused no further increase in the FI_{10} (55.7 ± 3.9%) in the presence of caffeine ($P > 0.1$, paired t-test). Clearly, caffeine pretreatment prevented terbutaline from inducing a further slowing of fatigue. Thus, it is likely that caffeine

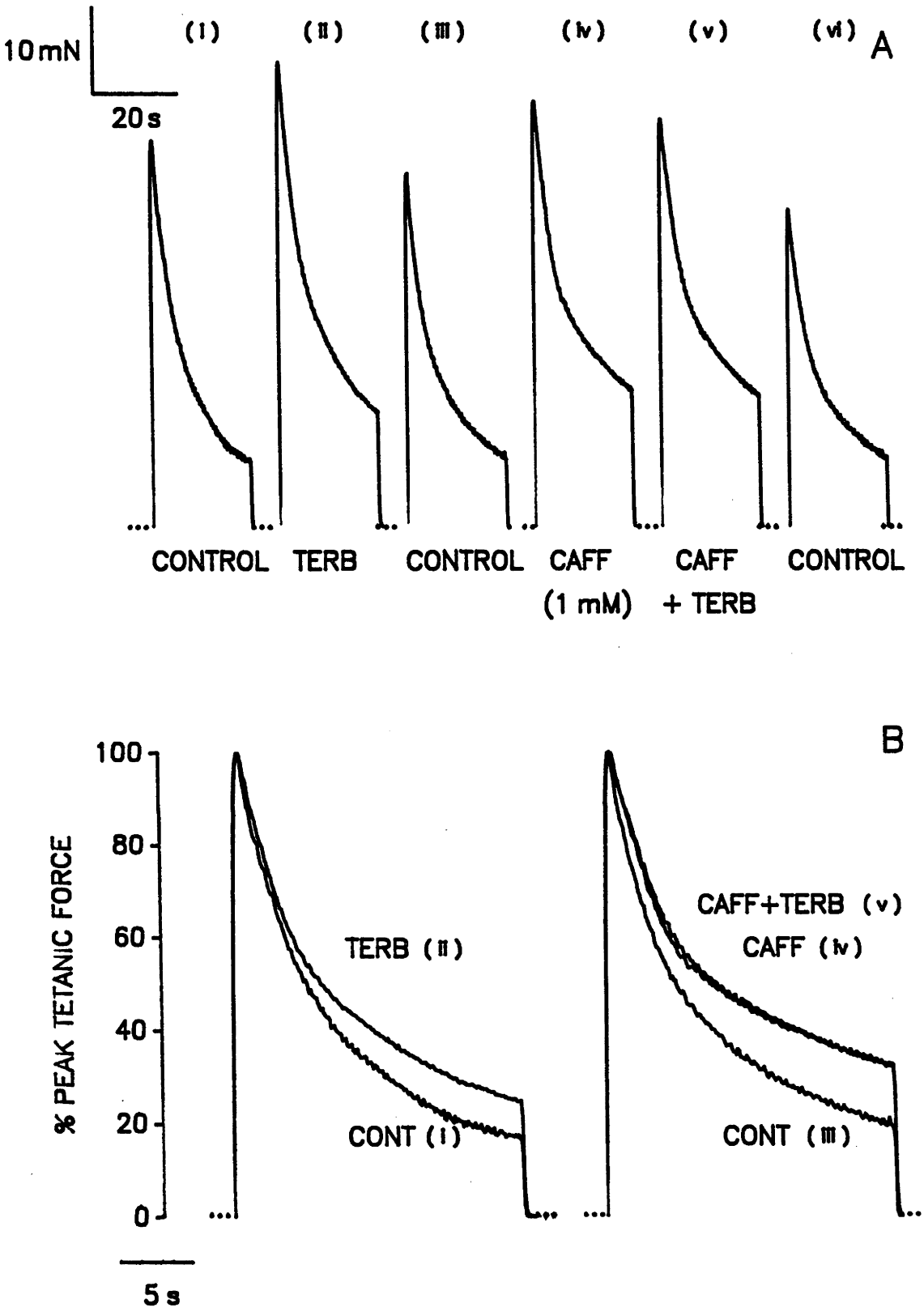


FIG 6.6: Effect of 10 μM terbutaline on prolonged tetani in the absence and presence of maximum effects of 1 mM caffeine in a denervated soleus preparation. Tetani were evoked at 50 Hz. Solution 1, 24°C. (A) absolute force records and (B) fatigue curves from the same records. CONTROL (or CONT), TERB, CAFF are described in the legends to Figs. 6.1 and 6.5. CAFF + TERB is the record 10 min after application of terbutaline in the presence of 1 mM caffeine.

saturated the intracellular processes responsible for the terbutaline-induced increase in fatigue resistance. Since cAMP-PK can phosphorylate and modulate the activity of the SR Ca^{2+} release channel (Seiler et al. 1984; Hymel et al. 1989; Timerman et al. 1990), a possibile explanation for the terbutaline-induced slowing of fatigue is that terbutaline, via cAMP, modulates the activity of the Ca^{2+} release channel to better maintain tetanic $[Ca^{2+}]_i$ during fatigue.

6.4 DISCUSSION

The results in this study confirm that β-adrenoceptor activation can increase fatigue resistance by a direct action on the muscle fibres, independent of neuromuscular transmission or blood flow, and is probably mediated by increased myoplasmic [cAMP]. In contrast to previous studies where catecholamine levels were experimentally increased during fatigue resulting in a small recovery of force (Bülbring & Burn 1939, 1940; Jami et al. 1984), the present study showed a protective effect during continuous exposure to terbutaline which indicates a slowing of fatigue processes rather than a reversal of fatigue. The terbutaline-induced increase in resistance to HFF was small; a reduction of 10-20% of the force loss in control tetani. Notably, this effect was quantitively similar to that for intermittent tetanic fatigue (Juel 1988).

These observations may be related to the excessive fatigue which occurs during exercise while using β-blockers (Fellenius 1983). In this case, central fatigue mechanisms may also have an important role (Fellenius 1983; Cooper et al. 1988), although β-blockade can still cause excessive fatigue in muscles stimulated by their motor nerve, as long as blood flow continues (Hughson et al. 1987; Cooper et al. 1988). This indicates that processes in the muscle can also be affected by β-blockade.

In principle β-adrenoceptor activation could increase resistance to fatigue by a number of cellular mechanisms including: (i) enhanced carbohydrate metabolism; (ii) activation of Na-K pumps to reduce the decline of the trans-sarcolemmal ion gradients; (iii) modulation of ionic conductances to reduce action potential fatigue; (iv) increased sensitivity of the voltage sensors to diminished action potential activation; (v) increased efficacy of other processes in E-C coupling leading to increased tetanic $[Ca^{2+}]_i$; and (vi) increased myofilament force production.

(a) Role of the sodium-potassium pump in high-frequency fatigue

The role of the Na-K pump in regulating force in prolonged tetani was examined by blocking its effect with ouabain. The most notable effect with ouabain was a reduction of peak tetanic force by about 40-50%. The mechanism for this effect may be related to

that causing HFF. Certainly, K-contracture experiments during exposure to ouabain indicate an impaired Ca^{2+} release and that the voltage sensors and voltage-activation of E-C coupling can still function maximally (compare with Chapter 5.3c). Thus, a likely explanation for the ouabain-induced depression of peak tetanic force is that it is caused by action potential failure during the early stages of stimulation. This hypothesis is consistent with the reduced action potential amplitude seen in ouabain (Smith & Thesleff 1976). Action potential depression is unlikely to be caused by a depolarization-induced inactivation of Na^+ channels in the resting state since ouabain did not reduce the resting membrane potential at a time when force was depressed. Previous studies have also shown little influence of ouabain on the resting potential in denervated fibres (Locke & Solomon 1967; Bray et al. 1976; Smith & Thesleff 1976) although a rapid depolarization is sometimes seen in normal fibres (Bray et al. 1976; Smith & Thesleff 1976; Clausen & Flatmann 1977). Action potential failure may have occurred early during tetanic stimulation if the blocked Na-K pumps were unable to restore the trans-sarcolemmal Na^+ and K^+ gradients after the first few action potentials. Increasing the stimulation frequency from 50 to 100 Hz in short term denervated fibres (2 weeks) had the same effect on prolonged tetani as ouabain, i.e. a reduction in peak tetanic force with no increase in the rate of fatigue, suggesting that both effects may be related to a failure to restore the trans-sarcolemmal Na^+ and K^+ gradients. Thus, functioning Na-K pumps appear to control force only during the early stages of prolonged tetanic stimulation. At long stimulation durations fatigue appears to be independent of Na-K pump activity.

(b) Effect of β-adrenoceptor activation on high-frequency fatigue: Role of the sodium-potassium pump

The terbutaline-induced increase in fatigue resistance did not occur via the Na-K pump since ouabain did not abolish its effect. Several recent studies support this conclusion. Firstly, stimulation *per se* increases the activity of the Na-K pump (Everts et al. 1988; Hicks & McComas 1989) and adrenaline did not add to this response (Everts et al. 1988). This suggests that terbutaline might not increase Na-K pump activity above that produced by tetanic stimulation alone. Secondly, terbutaline did not attenuate the excessive K^+ efflux from working muscle during exhaustive exercise, indicating that the catecholamine-induced stimulation of the Na-K pump might only occur in resting muscle (Rolett et al. 1990). Thirdly, the density of Na-K pumps in T-tubular membrane is less than in the surface membrane (Venosa & Horowicz 1981; Clausen 1986). This might mean that only a small effect could occur via the Na-K pumps in the T-system; a site where it is essential to increase Na^+ and K^+ transport.

(c) Effect of β-adrenoceptor activation on high-frequency fatigue: Role of the calcium release channel

A mechanism by which terbutaline could increase fatigue resistance is by modulating E-C coupling so that more Ca^{2+} is released from the SR during fatigue. Caffeine was used as a tool to investigate if such a possibility could occur. Application of high concentrations of caffeine (10-20 mM) during fatigue results in the restoration of force and $[Ca^{2+}]_i$ in amphibian twitch fibres (Eberstein & Sandow 1963; Grabowski et al. 1972; Gonzalez-Serratos et al. 1978; Allen et al. 1989; Lännergren & Westerblad 1989). However, the present experiments differed from the caffeine contracture studies because fatigue was examined in the presence of low concentrations of caffeine (1-5 mM). A new finding was that caffeine dramatically slowed fatigue during prolonged tetanic stimulation. In a similar experiment, low concentrations of caffeine prolonged the time course of maximal K-contractures in frog fibres by better maintaining the plateau phase and slowing the force decline which suggests an involvement of E-C coupling (Caputo 1976).

The mechanism by which caffeine slows HFF does not involve the action potential (Delay et al. 1986: Lüttgau & Oetliker 1968) nor asymetric charge movement (Kovács & Szucs 1983; Klein et al. 1990). It is possible that caffeine slows fatigue by producing a higher tetanic $[Ca^{2+}]_i$ during fatigue. This could be due to (i) an increased rate of SR Ca^{2+} release at each level of voltage activation (Lüttgau & Oetliker 1968; Delay et al. 1986) and/or (ii) a slower "turnoff" of Ca^{2+} release when action potential activation of the voltage sensors is impaired (Simon et al. 1989; Klein et al. 1990). Both of these effects might be explained by a modulation of the SR Ca^{2+} release channel (Rousseau et al. 1988). Another possibility, especially with 5 mM caffeine, is that force is better maintained during fatigue due to an increased Ca^{2+} sensitivity of the myofilaments (Wendt & Stephenson 1983). The measurement of tetanic $[Ca^{2+}]_i$ during HFF in the presence of caffeine might help to elucidate the mechanism for this effect.

The ability of caffeine to mask the protective effect of terbutaline on HFF could be explained if 1 mM caffeine either (i) saturated the process by which cAMP exerted its effect or (ii) desensitized this process to further effects by cAMP (Koshita & Oba 1988). The first possibility assumes that 1 mM caffeine produced near maximal effects on the process responsible for the increased fatigue resistance (e.g. via the Ca^{2+} release channel) and that the further increase in fatigue resistance with 2-5 mM caffeine is due to some other mechanism such as an increased myofilament Ca^{2+} sensitivity. Since cAMP-PK can phosphorylate and modulate the activity of the Ca^{2+} release channel (Seiler et al. 1984; Hymel et al. 1989; Timerman et al. 1990), it is possible that terbutaline produced the slowing of fatigue via this channel. Thus, terbutaline might be

able to maintain tetanic $[Ca^{2+}]_i$ during fatigue by a mechanism similar to that proposed for caffeine. As discussed in Chapter 4.4d, caffeine might mask the fatigue resistant effect of terbutaline by increasing $[cAMP]_i$ through its phosphodiesterase activity (Kramer & Wells 1980). This question may be answered by measurement of $[cAMP]_i$ under the same experimental conditions as in these contraction studies.

The possibility that both terbutaline and caffeine potentiate peak tetanic force and slow fatigue by modulating the Ca^{2+} release channel cannot be rejected on the grounds of a lack of correlation between these two effects. The observation of a small potentiation of peak tetanic force in association with a large slowing of fatigue might have been expected if the myofilaments became saturated with Ca^{2+}, so that an effect on force could only be seen when $[Ca^{2+}]_i$ was submaximal i.e. during fatigue.

Terbutaline may increase fatigue resistance by enhancing carbohydrate metabolism (Richter et al. 1982), modulating ion channel activity, or increasing the sensitivity of the voltage sensors to smaller action potentials. However, these possibilities cannot explain the finding that caffeine blocks the terbutaline response.

In conclusion, it is proposed that terbutaline slows HFF by a cAMP-dependent phosphorylation of the SR Ca^{2+} release channel so that tetanic $[Ca^{2+}]_i$ remains elevated at each level of voltage activation during HFF.

6.5 SUMMARY

(1) Terbutaline (10 μM) induced a small slowing of high-frequency fatigue in normal and denervated soleus fibres, i.e. a 10-20% reduction in the tetanic force loss.

(2) DBcAMP (2 mM) also slowed HFF in denervated soleus fibres suggesting that cAMP is the intracellular messenger involved in the fatigue-resistant effect of terbutaline.

(3) Ouabain (1 mM) depressed peak tetanic force but had little subsequent effect on the decline of force. This suggests that functioning Na-K pumps are important in controlling force only during the early stages of prolonged tetanic stimulation.

(4) Terbutaline slowed HFF in the presence of ouabain suggesting that a terbutaline-induced increase in Na-K pump activity was not responsible for the increased fatigue resistance.

(5) Caffeine (1-5 mM) induced a concentration-dependent slowing of HFF. This response might be due to a modulation of SR Ca^{2+} release resulting in increased tetanic

$[Ca^{2+}]_i$ during fatigue. An increased myofilament Ca^{2+} sensitivity at the higher caffeine concentrations may also contribute to the slowing of fatigue.

(6) The terbutaline-induced slowing of HFF was abolished in the presence of 1 mM caffeine. It is postulated that terbutaline increases the resistance to HFF by modulating SR Ca^{2+} release so that more Ca^{2+} is released at each level of voltage activation. Thus, diminishing the effect of action potential failure during HFF.

CHAPTER 7
GENERAL DISCUSSION

The sympatho-adrenal system is extremely important for the regulation of whole animal performance, especially during exercise. The naturally occurring catecholamines, adrenaline and noradrenaline, have well documented effects on many physiological systems. They are involved in the regulation of: (i) cardiac output (Christensen & Galbo 1983; Galbo 1983); (ii) blood flow to the working muscles via effects on vascular smooth muscle (Bülbring 1976; Christensen & Galbo 1983); (iii) body temperature via increased sweat production (Galbo 1983); (iv) metabolism in the liver, adipose tissue and muscle (Galbo 1983; Richter 1984); (v) neuromuscular transmission (Bowman & Nott 1969; Bülbring 1976; Carmon 1982); and (vi) hormonal release during exercise (Galbo 1983; Richter 1984). These catecholamine-induced effects are mediated by either α- or β-adrenoceptors depending on the process which is influenced and the tissue involved.

In addition, catecholamines directly influence processes occurring in skeletal muscle. This thesis is specifically concerned with the effects of catecholamines on the contractile performance of skeletal muscle following the activation of β-adrenoceptors. Three β-adrenoceptor mediated responses were observed: (i) an increased isometric force production; (ii) an increased resistance to high-frequency fatigue; and (iii) changes in the rate of relaxation. A number of processes are thought to be influenced by catecholamines in skeletal muscle as summarized in Figure 7.1. Results in this thesis suggest that the important processes for the enhancement of contractile performance are all associated with E-C coupling. A cAMP-dependent modulation of Ca^{2+} release from the SR could explain both the force potentiation and the increased fatigue resistance. In addition, an increased rate of Ca^{2+} transport by the SR Ca^{2+}-pump, subsequent to the cAMP-dependent phosphorylation of phospholamban, could explain the acceleration of relaxation in the slow-twitch fibres. The effects of increased force production and slowing of fatigue on whole animal performance are obvious. Changes in the rate of relaxation by individual fibres are also likely to be important. The increased rate of relaxation in slow-twitch fibres would reduce their contraction duration making it closer to that in fast-twitch fibres. Because whole muscles are usually composed of a mixture of fibre-types, at least in human muscle (Burke & Edgerton 1975), the changed relaxation rates of individual fibres may result in a more coordinated and efficient whole muscle contraction. The enhancement of other processes including Na-K pump activity, trans-sarcolemmal Ca^{2+} currents and carbohydrate metabolism are possibly important for maintaining ionic and metabolic homeostasis during the elevated mechanical activity. Certainly, without such a stimulation, these secondary processes may seriously affect contractile function. In summary, catecholamines influence

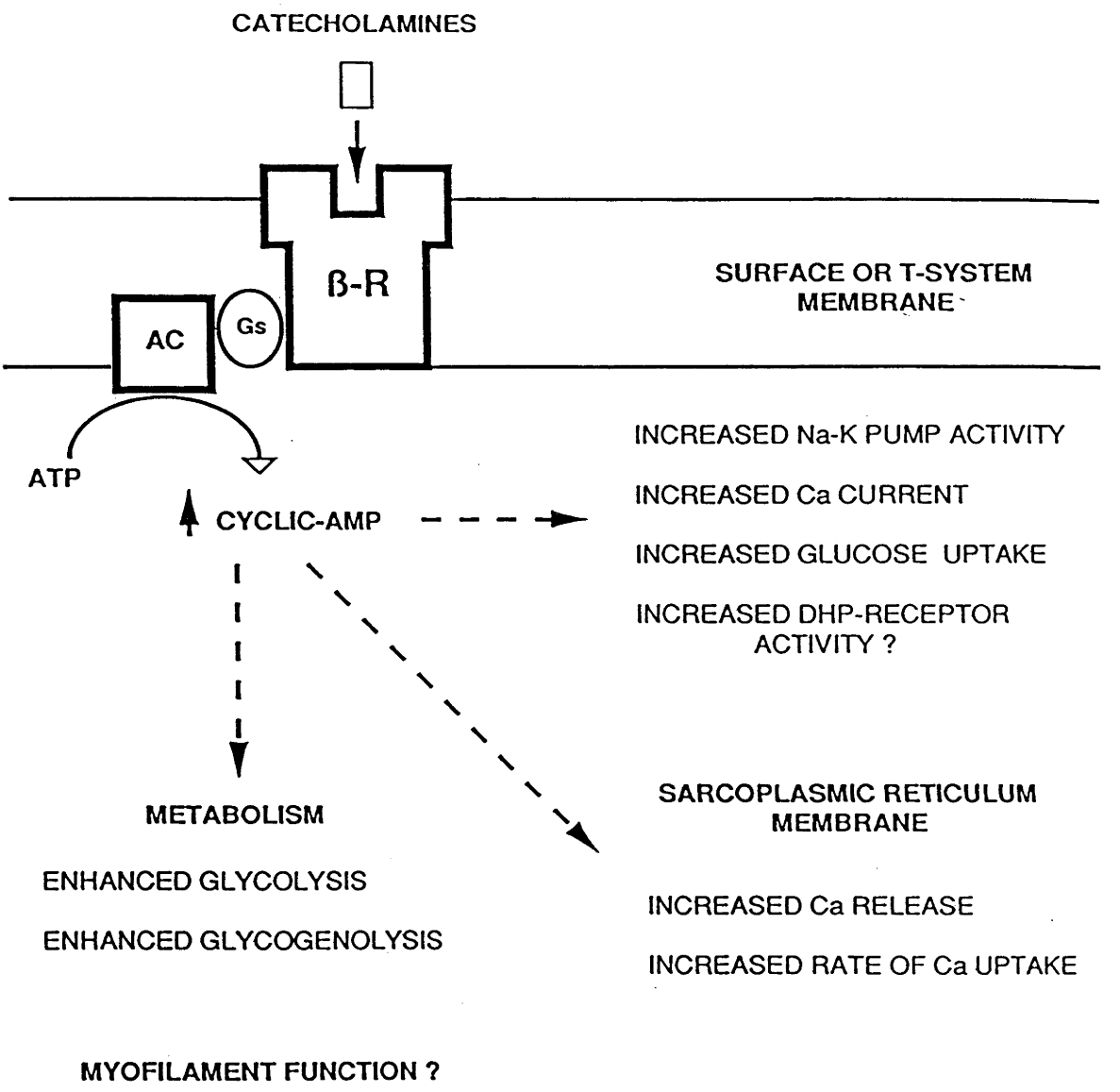


FIG. 7.1: Summary of the cellular processes known or speculated to be influenced by catecholamines in skeletal muscle.

isometric contraction as well as other processes in skeletal muscle. Enhancement of these processes might contribute to increased whole animal performance, especially in regards to the well documented fight-or-flight situation.

However, one has to be cautious when extrapolating the present experimental results on isolated preparations to human performance. Terbutaline and adrenaline were used as pharmacological tools in the present study. Hence, the concentrations used were of several orders of magnitude higher than that reported for circulating catecholamines in humans (Christensen & Galbo 1983; Galbo 1983). Certainly, an interesting area for future research would be to extend the present studies to anaesthetized animals to examine more fully the functional importance of catecholamines.

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