The Effect of Cadmium on Excitation-Contraction Coupling in Mammalian Skeletal Muscle

A thesis submitted for

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by

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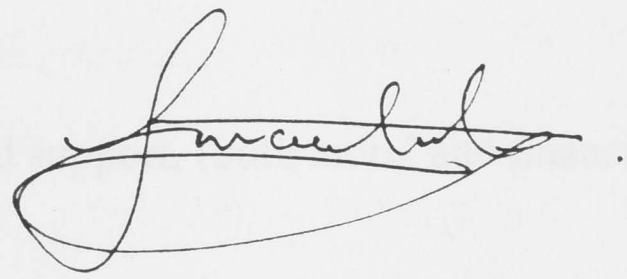
I declare that this thesis contains no published or written material belonging to other persons except where reference has been made in the text.



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Abstract

The effect of 3 mM Cd^{2+} on excitation-contraction (E-C) coupling was examined in small bundles of rat soleus fibres. Shortly following the addition of Cd^{2+} to the bath a slow, increase in tension "the Cd^{2+}-induced contracture" (CIC) was observed. If Cd^{2+} was washed out of the bath after 5 minutes or more, a rapid, transient "Cd^{2+}-withdrawal contracture" (CWC) occurred. Neither of these contractures could be attributed to fibre depolarisation since Cd^{2+} addition and washout did not affect membrane potential. The CIC appears to be the result of a Ca^{2+} influx into the muscle fibres since the contracture was abolished in the absence of external Ca^{2+} and did not depend on activation of the voltage sensor for E-C coupling. The CWC on the other hand, is believed to be the result of voltage sensor activation since the contracture was reduced by factors which depress voltage sensor activation and was reversibly abolished by inactivating the voltage sensor in 40 mM K^{+}.

In addition to these contractures, Cd^{2+} also had a major effect on twitch, tetanic and K contracture tension. 3 mM Cd^{2+} initially caused twitch potentiation which was followed by both twitch and tetanic depression. Cd^{2+} also depressed submaximal K contracture tension indicating both a positive shift and a decrease in the slope of the curve relating K contracture tension to membrane potential. The potentiation of twitches was attributed to Cd^{2+} causing an increase in action potential duration whereas the depression of twitches and tetani was attributed to both a depression of action potentials and a positive shift in the voltage dependence of contractile activation.

Cd^{2+} was also found to cause tension development in skinned fibres by directly activating the contractile apparatus. The tension increase was not the result of SR Ca^{2+} release since the SR in all preparations was disrupted using Triton X-100. It was suggested, therefore, that Cd^{2+} leaking into the myoplasm could contribute to the CIC.

It was concluded that the effects of Cd^{2+} on E-C coupling are complex. The cation most probably exerts its effects by a combination of: binding to and screening negative charges on the fibre surface membrane affecting both action potential propagation and activation of the voltage sensor; a direct binding of Cd^{2+} to the voltage sensor; and finally although not likely, by entering the myoplasm and directly activating the contractile apparatus and or causing SR Ca^{2+} release.

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List of abbreviations

UNITS

mM	millimolar
mN	millinewton
mm	millimetre
msec	millisecond
mV	millivolt
MΩ	megaohm
min	minute
sec	second
gms	grams
M	molar
Hz	hertz
pCa	$-\log_{10}[Ca^{2+}]$
pH	$-\log_{10}[H^{+}]$
μM	micromolar

Chemicals

IP_3	inositol 1,4,5-triphosphate
ATP	adenosine 5'-triphosphate
CP	phosphocreatine
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid])
TES	(N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid)
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
HDTA	1,6 Diaminohexane-N,N,N',N'-tetraacetic acid

Symbols and terms

[x]	concentration of x
#	number
Ω	Ohm
~	approximately

Mr	relative molecular weight.
V_m	membrane potential
%T	percentage tetanic tension
T-tubule	transverse tubule
Fig.	figure
n =	number equals
NB:	note briefly
ATPase	adenosine 5'-triphosphate phosphatase
E-C coupling	excitation-contraction coupling
et al	and others
CIC	cadmium-induced contracture
CWC	cadmium-withdrawal contracture
CICR	calcium-induced calcium release
RyR	ryanodine receptor
SR	sarcoplasmic reticulum
DHP	dihydropyridine
DHPR	dihydropyridine receptor
K contracture	potassium contracture
EDL	extensor digitorum longus
JCSMR	John Curtin School of Medical Research
ANU	Australian National University

Chapter 1

General Introduction

1.1 What is excitation-contraction coupling?

The term excitation-contraction (E-C) coupling describes the series of events in skeletal muscle that link the excitation of a voltage sensitive molecule situated in the surface membrane to the release of internal Ca^{2+} ions that activate contraction. The link between voltage sensor excitation and internal Ca^{2+} release in skeletal muscle is not known and continues to be the topic of an intense investigation that has spanned nearly 50 years.

1.2 The general structure and function of skeletal muscle

1.2.1 *Structure*

Skeletal muscle is composed of individual multinucleate cells (muscle fibres) ranging from 1-40 mm in length and 10-100 μM in diameter (Fig. 1.1). Each fibre is surrounded by an electrically conductive membrane called the sarcolemma which invaginates at regular intervals to form the transverse (T)- tubule system. T-tubules allow the transmission of electrical signals deep into the muscle fibre. Adjacent to the T-system lies an internal network of membranous vesicles called the sarcoplasmic reticulum (SR) which functions as an internal Ca^{2+} store and is arranged in a latticework surrounding the fibre's contractile elements (myofibrils). The SR and myofibrils are bathed in the fibres intracellular fluid, the myoplasm.

1.2.2 *The mechanism of contraction in skeletal muscle*

The signal for contraction is initiated in the CNS and is transmitted as an action potential along a motor neuron to the neuromuscular junction. Signal transduction across the junctional gap is mediated by acetylcholine (ACh) which is released from vesicles in the presynaptic terminal. The ACh diffuses across the gap and binds to ACh receptors on the postsynaptic membrane (motor end plate) causing localised depolarisation. This results in an action potential which is propogated along the surface of the muscle fibre. The action potential spreads deep into the fibre via T-tubules and causes the excitation of a voltage sensitive protein, a dihydropyridine receptor (DHPR)

located in the T-tubule membrane. Excitation of the voltage sensor somehow triggers the release of Ca^{2+} from
the SR into the myoplasm which causes myofibrillar shortening and contraction. Contractile relaxation occurs when the myoplasmic Ca^{2+} concentration is lowered by the re-uptake of Ca^{2+} into the SR via an ATP driven pump, the Ca^{2+}-ATPase. The contractile force resulting from a single action potential, a "twitch", is not adequate to generate maximum isometric tension. However, the summation of many twitches leads to a "tetanus" whereby tension reaches a plateau which, at optimal stimulation frequency, is at the maximum tension that the fibre can produce.

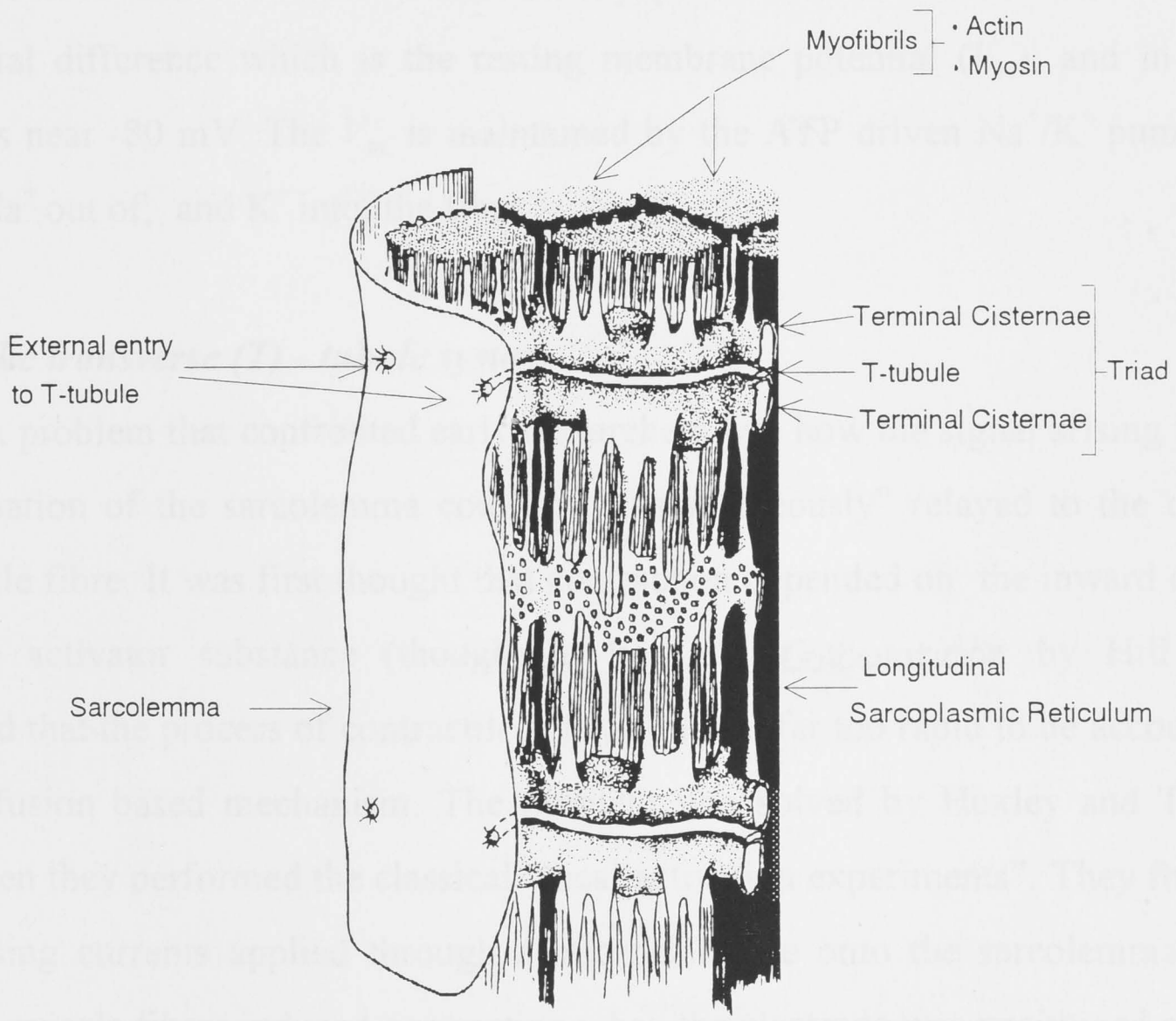


Figure 1.1 *Structure of a muscle fibre*
A diagram depicting the structural components of a frog skeletal muscle fibre. Only part of the fibre (4 myofibrils) is shown. Each myofibril is composed of actin and myosin myofilaments and is surrounded by a network of SR and T-tubule membranes. Also shown (on the left) is the fibre surface membrane (sarcolemma) which has been cut away in the remainder of the diagram so that the internal components can be seen. (Modified Peachey, 1965, and Hille, 1992).

1.3 Individual components of skeletal muscle

1.3.1 *The sarcolemma*

The sarcolemma is a lipid bilayer containing various proteins, many of which form ion channels, and functions as an ion selective barrier which maintains the ionic composition of the myoplasm. The membrane is polarised by virtue of the concentration difference between Na^+ and K^+ ions inside and outside the cell, and it's greater selectivity for K^+ than Na^+ ions. An increase in selectivity for Na^+ produces an action potential which is conducted along the fibre surface. The ionic composition of the myoplasm and fluid surrounding each muscle fibre is shown in Fig.1.2 At rest, the difference in K^+ concentration between the myoplasm and the outside of the fibre gives a potential difference which is the resting membrane potential (V_m), and in skeletal muscle is near -80 mV. The V_m is maintained by the ATP driven Na^+/K^+ pump which pumps Na^+ out of, and K^+ into, the fibre.

1.3.2 *The transverse (T) - tubule system*

A problem that confronted early researchers was how the signal arising from the depolarisation of the sarcolemma could be "instantaneously" relayed to the centre of the muscle fibre. It was first thought that contraction depended on the inward diffusion of some activator substance (thought to be Ca^{2+}). Calculations by Hill (1948), suggested that the process of contractile activation was far too rapid to be accounted for by a diffusion based mechanism. The problem was solved by Huxley and Taylor in 1958 when they performed the classical "local activation experiments". They found that depolarising currents applied through a microelectrode onto the sarcolemma of frog sartorius muscle fibres induced contraction when the electrode was positioned at "active spots" along the length of the fibre. The "active spots" corresponded to the location of the T-tubules that had been seen using electron microscopy (Porter and Palade, 1957; Robertson, 1960). Evidence that the lumen of the T-system was continuous with the extracellular environment came from studies in which muscle fibres were incubated in solutions containing large molecules such as ferretin which accumulated in the T-tubule system and could be visualised under the electron microscope (Huxley, 1964; Page,

1964). The same studies also provided a rough estimation of the T-tubules dimensions, which vary from 17-80 nm in diameter (Page, 1964; Huxley, 1964; Franzini-Armstrong & Porter, 1964; Franzini-Armstrong, Landmesser & Pilar, 1975; Luttgau & Stephenson, 1986). Using fluorescent dyes, Endo (1964) estimated that the T-system occupies approximately 1 percent of total fibre volume.

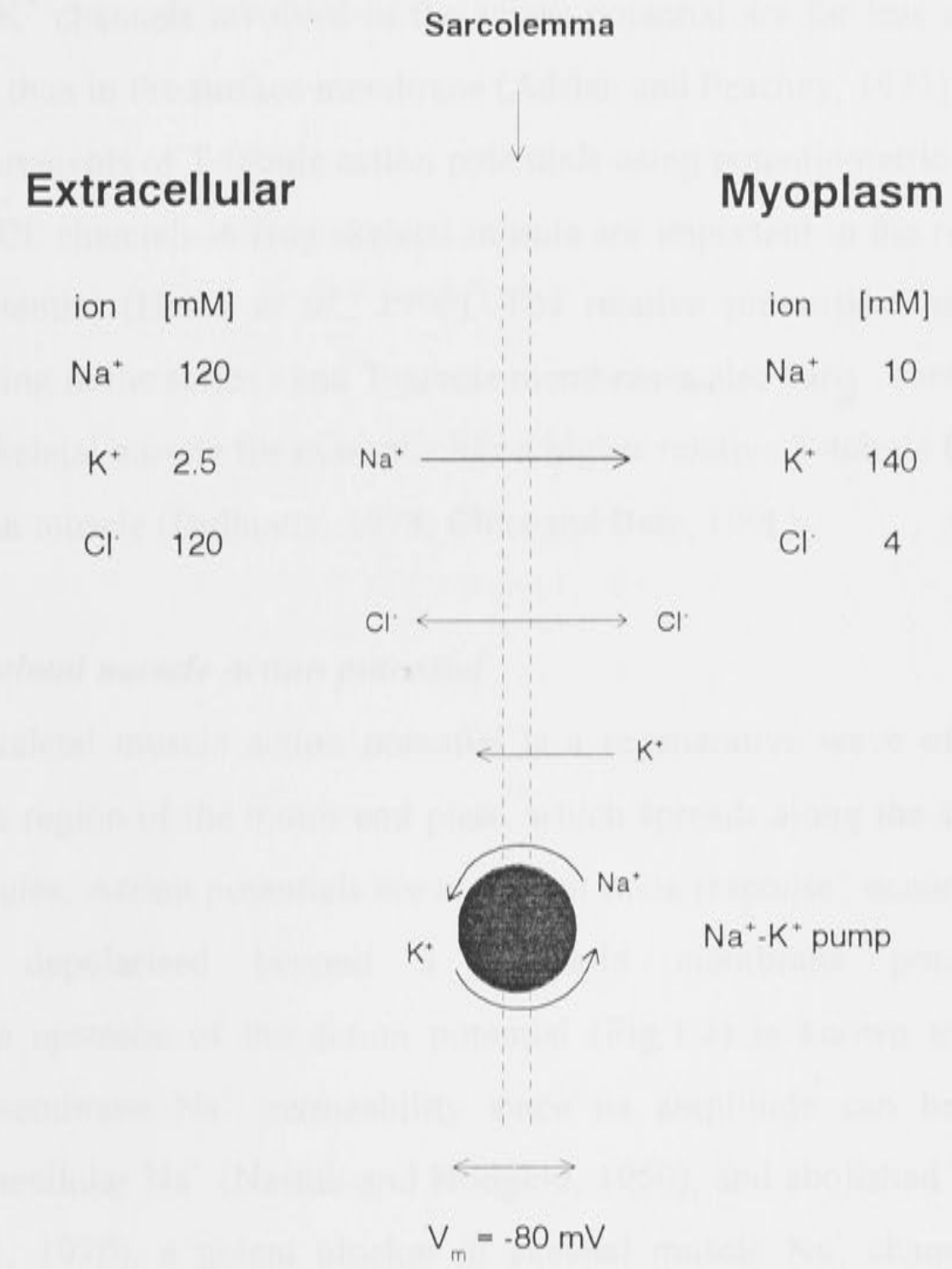


Figure 1.2 *The ionic composition of the myoplasm and extracellular fluid*
A diagram showing the typical ionic composition of the myoplasm and extracellular fluid in a frog sartorius muscle fibre. At rest, the difference in $[K^+]$ between the inside and outside of the fibre and high K^+ permeability, gives rise to the resting membrane potential (V_m) which is approximately -80 mV. This concentration difference is maintained by the ATP-driven Na^+-K^+ pump. (Values derived from Eckert and Randall, 1986).

1.3.3 *Ion channels of the sarcolemma and T-system*

Even though the T-tubule membranes are continuous with the sarcolemma, the distribution of various ion channels between these two membranes differs (Ebashi, 1976; Caille *et al.*, 1985).

1. In frog skeletal muscle, the T-system has a low resting Cl^- conductance (Eisenberg and Gage, 1969), calculated to be $1/36^{th}$ of the sarcolemmal Cl^- conductance (Adrian and Peachey, 1973).
2. Na^+ and K^+ channels involved in the action potential are far less abundant in the T-system than in the surface membrane (Adrian and Peachey, 1973).

Optical measurements of T-tubule action potentials using potentiometric dyes show that the T-system Cl^- channels in frog skeletal muscle are important in the repolarisation of the action potential (Heiny *et al.*, 1990). The relative proportions of different ion channels residing in the surface and T-tubule membranes also vary between species. Mammalian skeletal muscle for example, has a higher relative T-tubule Cl^- conductance than amphibian muscle (Dulhunty, 1978; Chua and Betz, 1991).

1.3.4 *The skeletal muscle action potential*

The skeletal muscle action potential is a regenerative wave of depolarisation initiated in the region of the motor end plate, which spreads along the sarcolemma and into the T-tubules. Action potentials are an "all or none response" occurring only when a fibre is depolarised beyond a threshold membrane potential, usually ~ -50mV. The upstroke of the action potential (Fig.1.3) is known to be due to an increase in membrane Na^+ permeability since its amplitude can be decreased by lowering extracellular Na^+ (Nastuk and Hodgkin, 1950), and abolished by tetrodotoxin (Adrian *et al.*, 1970), a potent blocker of skeletal muscle Na^+ channels (Narahashi *et al.*, 1964). The action potential reaches a peak near the Na^+ equilibrium potential (about + 20mV). Fibre repolarisation during action potential decay is due to an efflux of K^+ through delayed rectifier K^+ channels (Adrian *et al.*, 1970) and the inward movement of Cl^- ions (Hutter and Noble, 1960; Heiny *et al.*, 1990).

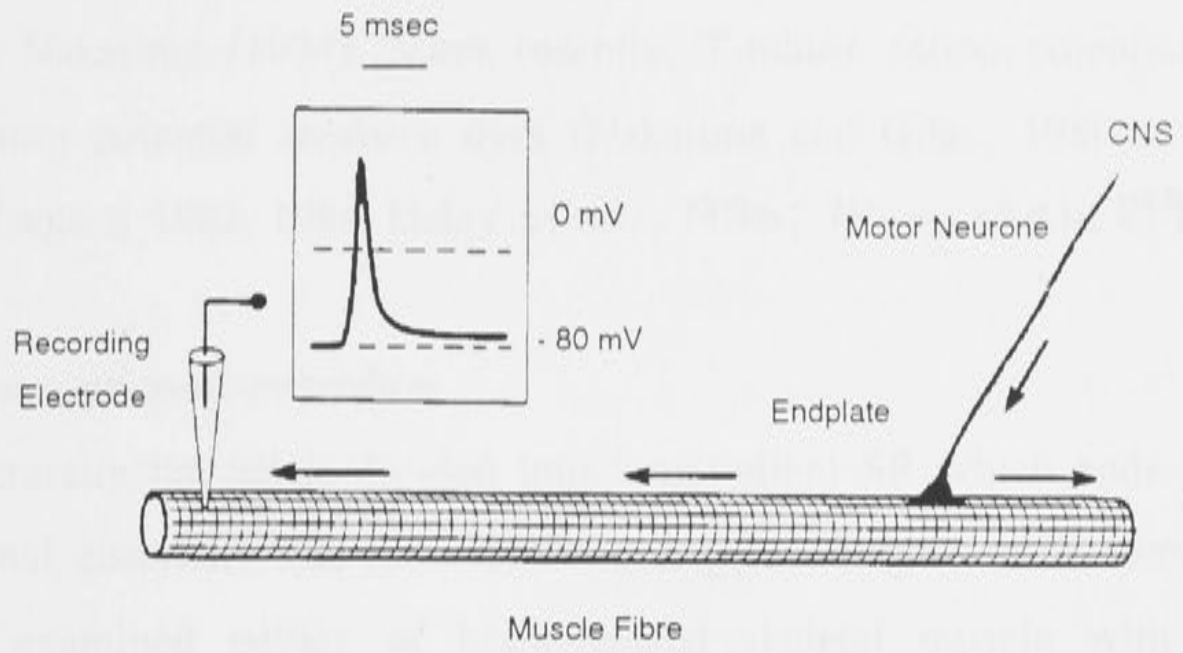


Figure 1.3 *The skeletal muscle action potential*
A schematic diagram showing the propagation of an action potential in a skeletal muscle fibre. An action potential is a self propagating wave of depolarisation which is initiated at the motor endplate in response to a signal from the CNS. The action potential travels in either direction along the surface of the fibre and spreads deep into the fibre via the T-tubule system initiating contraction. The rising phase of the action potential (inset) is due to an influx of Na^+ through voltage gated Na^+ channels. Action potential decay occurs as a result of Na^+ inactivation and an efflux of K^+ from the fibre. (Modified from Eckert and Randall, 1986).

1.3.5 *How does the action potential travel deep into the muscle fibre?*

The definitive experiments implicating the T-tubule as the site of contractile activation were performed by Gage and Eisenberg, (1967). Frog skeletal muscle fibres treated with glycerol, which selectively disrupts the T-system, failed to contract in response to electrical stimulation even though action potentials could still be recorded. Passive, electrotonic, spread of excitation throughout the T-tubule was proposed (Falk, 1968). Contrary to this proposal, Adrian *et al* (1969), demonstrated that a passive spread of excitation via the T-tubule system would only just activate the innermost parts of the fibre. T-tubules are too small to allow a measurement of their membrane potential by microelectrode recording techniques. The spread of excitation along T-tubules was therefore measured indirectly by looking at myofibrillar shortening (Constantin, 1970; Gonzalez-Serratos *et al.*, 1971). From these experiments it was proposed that the inward spread of excitation into and along the T-tubule was in the

(1). At rest, crosslinking between F-actin and myosin is prevented by the presence of another protein Tropomyosin which is tightly associated with F-actin.

(2). and causes Tropomyosin to move from its inhibitory position on F-Actin which uncovers the F-Actin -

form of a Na^+ -dependent action potential. This hypothesis was later confirmed by Bastian and Nakajima (1974). More recently, T-tubule action potentials have been measured using potential sensitive dyes (Nakajima and Gilai., 1980*a*, 1980*b*, 1981; Heiny and Vergara, 1982, 1984; Delay et al., 1986; Heiny et al., 1990).

1.3.6 *The sarcoplasmic reticulum*

Structurally the SR is divided into longitudinal SR which ends in blind sacs called terminal cisternae. The SR was characterised by Nagai and co-workers (1960), when they examined pellets of homogenised skeletal muscle with an electron microscope. They noticed small vesicles which were later found to accumulate Ca^{2+} in the presence of ATP and Mg^{2+} (Hasselbach, 1964). Active pumping allows Ca^{2+} in the lumen of the SR to reach higher concentrations than that of the myoplasm. The SR Ca^{2+} is released during contraction via the ryanodine receptor (RyR) Ca^{2+} release channel which is located primarily but not exclusively in the terminal cisternae.

1.3.7 *The skeletal muscle contractile apparatus*

Two major proteins, actin and myosin, make up the contractile element of skeletal muscle cells (Fig.1.4). Myosin, Mr 500,000 has ATPase properties which are activated by Ca^{2+} and inhibited by Mg^{2+}. Actin has no associated ATPase activity, and can be further divided into G-actin, a globular protein of Mr 42,000, and F-actin, which is a fibrous polymer of G-actin molecules. In the absence of ATP, cross linking occurs between actin and myosin leading to an increase of tension called rigor. If ATP is present, the cross bridges are broken and the muscle relaxes. At rest, the myosin ATPase is inhibited by a complex of proteins called troponins. During contraction, Ca^{2+} released from the SR binds to troponin C and causes a conformational change. The change in conformation of troponin C removes the inhibitory effect of the troponin complex on the myosin ATPase, allowing cross linking to occur and hence tension development. Relaxation occurs by the reverse process when the myoplasmic Ca^{2+} concentration has been sufficiently lowered due to attenuation of SR Ca^{2+} release and the active pumping of Ca^{2+} back into the SR.

The relative arrangements of actin and myosin give rise to banding patterns (Fig. 1.4) which are characteristic of skeletal muscle and can be seen using normal, phase contrast or interference microscopy (Aidley, 1989). The two main bands that alternate along the length of the muscle fibre are: the dark A band which encompasses the region of overlap between actin and myosin filaments as well as myosin not in the overlap, and the lighter I band, comprised of a region containing actin filaments only. Each I band is bisected by the Z line and the region between two consecutive Z lines makes up the fundamental unit of skeletal muscle, the sarcomere.

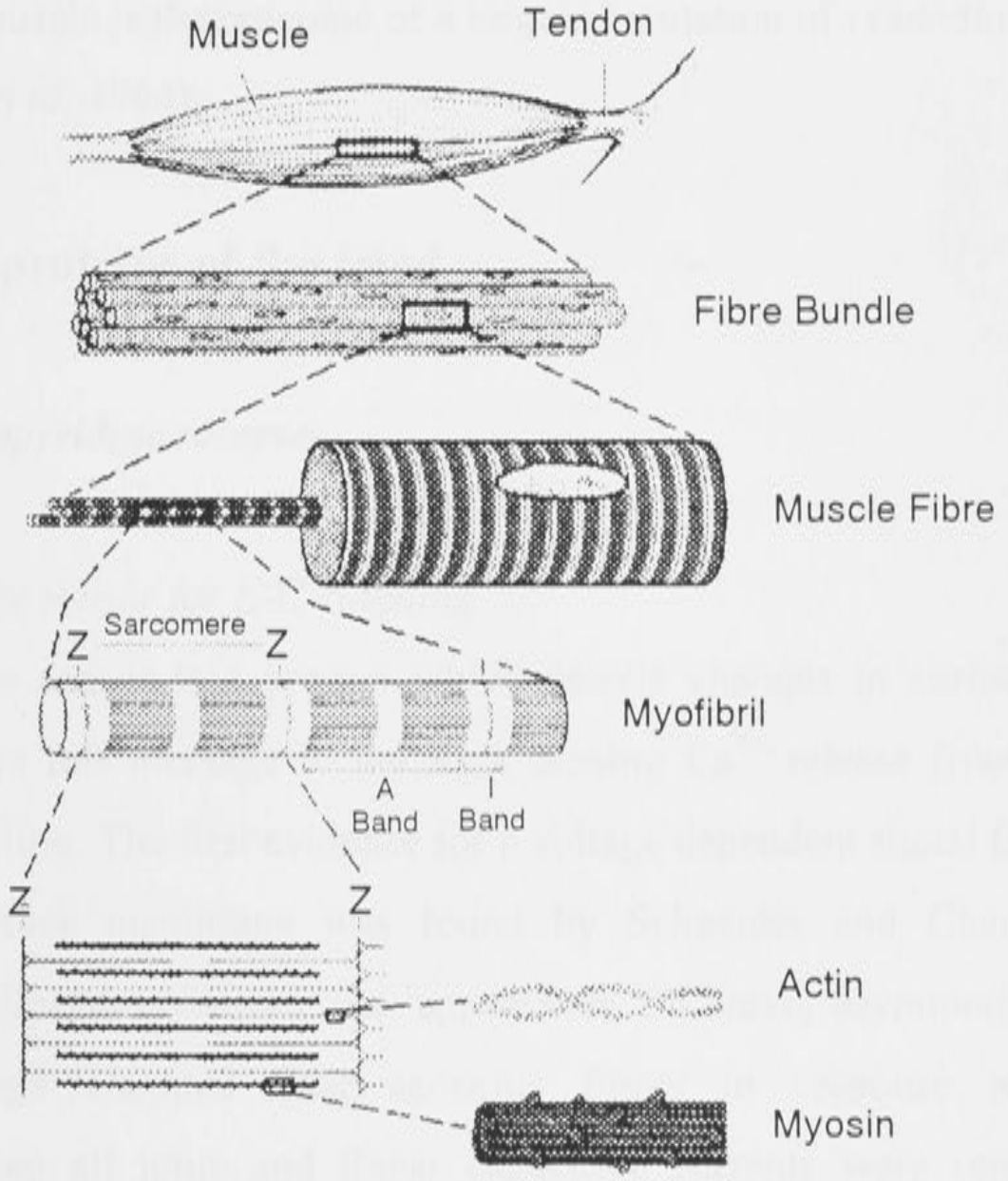


Figure 1.4 *The contractile apparatus*
A diagram illustrating the sequential breakdown of skeletal muscle into its contractile components: actin and myosin. Also shown is the characteristic banding pattern of skeletal fibres which arises from the relative arrangement of actin and myosin filaments. The dark A band includes the region of actin and myosin overlap, whilst the lighter I band contains actin filaments only. Each I band is bisected by a Z line and the region bounded by two consecutive Z lines constitutes the fundamental skeletal muscle unit, the sarcomere. (Modified from Eckert and Randall, 1986).

1.3.8 *The triad*

Electron microscopy reveals that each T-tubule is flanked on either side by SR terminal cisternae forming a 3 component unit called a triad. The terminal cisternae are separated from the T-tubule by a 12 nm gap called the triadic junction (Franzini-Armstrong, 1970). The gap is occluded by regularly spaced, electron-dense, footlike structures which are continuous with the SR but not the T-tubule membrane (Franzini-Armstrong, 1970). These footlike structures are the cytoplasmic region of the ryanodine receptor (RyR) Ca^{2+} release channel (Inui *et al.*, 1987). The feet are thought to play a role in E-C coupling since contractile ability is lost in dyspedic muscle which lacks "feet". Dyspedic muscle is the outcome of a targeted mutation of ryanodine receptors in mice (Takeshima *et al.* 1994).

1.4 Important proteins of the triad

1.4.1 *The dihydropyridine receptor*

1.4.1A *The voltage sensor for E-C coupling*

The voltage sensor is a protein which detects changes in surface membrane potential and relays this message to the RyR causing Ca^{2+} release from the SR and subsequent contraction. The first evidence for a voltage dependent signal from a voltage sensor in the surface membrane was found by Schneider and Chandler (1973). Schneider and colleagues noticed the appearance of small, asymmetric, capacitive currents in voltage clamped frog sartorius fibres in response to membrane depolarisation, when all ionic and linear capacitive currents were removed. These asymmetric charge movements were thought to reflect the movement of a voltage sensitive molecule within the surface membrane. The observation that asymmetric charge movement and voltage activated contraction occurred over a similar range of membrane potentials led to the proposal that charge movement is produced by the voltage sensor for E-C coupling. The voltage sensor is a dihydropyridine receptor (DHPR). Dihydropyridines are a class of compounds which were developed as specific blockers of L-type voltage dependent Ca^{2+} channels (Curtis and Catterall, 1983, 1986;

Flockerzi *et al.*, 1986; Kim *et al.*, 1990*a*; Spedding and Paoletti, 1992; Catterall, 1995). Binding studies have identified the T-tubule as being a rich source of DHPR's (Fosset *et al.*, 1983; Galizzi *et al.*, 1984). Blockers of L-type Ca^{2+} channels such as gallapomil (D600), diltiazem and the dihydropyridine nifedipine, paralyse skeletal muscle (Eisenberg *et al.*, 1983; Gottschalk and Luttgau, 1985; Gallant and Goettl, 1985; Lamb, 1986*a*; Avila-Sakar *et al.*, 1986; Berwe *et al.*, 1987; Dulhunty and Gage, 1988; Cognard *et al.*, 1990; Neuhaus *et al.*, 1990). This paralysis suggests an important role for L-type Ca^{2+} channels in E-C coupling. The role of the DHPR is not to provide a Ca^{2+} current since skeletal muscle contraction occurs in the absence of extracellular Ca^{2+} (Armstrong *et al.*, 1972; Luttgau and Spiecker, 1979; Cota and Stefani, 1981; Miledi *et al.*, 1984; Dulhunty and Gage, 1988; Dulhunty, 1991), and less than 5% of DHPR's form functional Ca^{2+} channels (Schwartz *et al.*, 1985)*. Observations that charge movement (Section 1.8.5.2) is also blocked by dihydropyridines and other Ca^{2+} channel antagonists led to the proposal that the DHPR might be the skeletal muscle voltage sensor (Rios *et al.*, 1986; Beam *et al.*, 1986; Lamb and Walsh, 1987; Rios and Brum, 1987; Brum *et al.*, 1988 ; Pizzaro *et al.*, 1988; Dulhunty and Gage, 1988). The definitive evidence that the voltage sensor is the DHPR came with the use of a lethal mutation of the dihydropyridine receptor, muscular dysgenesis, which results in a lack of E-C coupling in mice. Microinjection of the cDNA encoding for the α1-subunit of the skeletal muscle DHPR restored E-C coupling, charge movement, and voltage-dependent Ca^{2+} currents to dysgenic myocytes (Tanabe *et al*, 1987; 1988; 1990; Adams *et al.*, 1990; Beam *et al.*, 1986). Thus, whilst only a small proportion of DHPR's act as L-type Ca^{2+} channels, their main function is to serve as the T-tubule voltage sensor.

1.4.1B *Structure of the dihydropyridine receptor.*

The DHPR is a multimeric protein, Mr~ 390 KDa, consisting of α1, α2, β, δ and γ subunits (Takahashi *et al.*, 1987). The α1 is the main functional subunit and is associated with the following functions (Fig. 1.5):

(a) the binding sites for dihydropyridines (Kim *et al.*, 1990*b*);

(b) a functional L-type Ca^{2+} channel (Tanabe *et al.*, 1987);

(c) the voltage sensor for E-C coupling (Tanabe, *et al.*, 1990; Adams *et al.*, 1990).

*This number has been disputed (Lamb, 1992).

There is a striking homology between the α1 subunit structure and the voltage dependent Na^+ channel (Trimmer and Agnew, 1989; Tanabe *et al.*, 1987). Both proteins are made up of 4 homologous domains, each containing six putative membrane spanning α helices which, in the DHPR, supposedly form the pore of an L-type Ca^{2+} channel (Fig.1.6). Of these, helix number 4 is thought to be the voltage sensor, (Noda *et al.*, 1984, 1986; Stuhmer *et al.*, 1989 ; Adams *et al.*, 1990; Adams and Beam, 1990). The cytoplasmic loop linking helices 2 and 3 appears to be the main region that communicates with the ryanodine receptor during E-C coupling (Tanabe *et al.*, 1988, 1990; Adams *et al.*, 1990; Lu et al., 1995) and is the region that determines whether E-C coupling is skeletal or cardiac in character (Tanabe *et al.*, 1990). The remaining subunits appear to modulate the α1 subunit function (Singer *et al.*, 1991; Wei *et al.*, 1991; Varadi *et al.*, 1991).

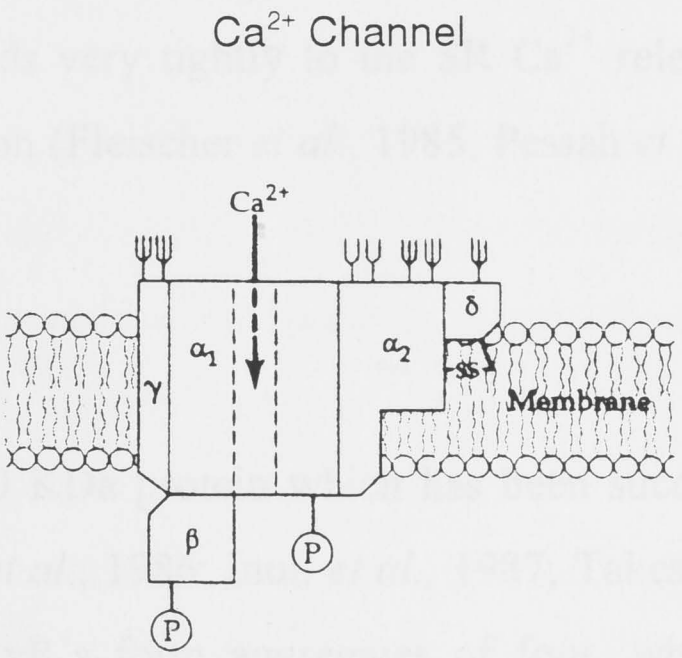


Figure 1.5 *Dihydropyridine receptor subunits*
A diagram depicting the spatial arrangement of Ca^{2+} channel (DHPR) subunits in a membrane. The α1 is the main functional subunit whereas the other subunits serve to modulate the α1 function. The contacts shown between the subunits are based on biochemical data, otherwise the arrangement is purely hypothetical. Also shown are disulphide bonds between various subunits (S-S), glycosylated regions (Ψ) and phosphorylated regions (P). (From Hille, 1992 and modified from Catterall, 1988).

In cardiac and smooth muscle, E-C coupling depends on an influx of external Ca^{2+} through L-type Ca^{2+} channels. This Ca^{2+} influx triggers a much larger release of

Ca^{2+} from the SR through ryanodine receptors, a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (Winegrad, 1979; Fabiato, 1983). In contrast to skeletal muscle, contraction in these muscles does not occur in the absence of external Ca^{2+} (Section 1.8.1). The most popular theory for the mechanism of E-C coupling in skeletal muscle is that the DHPR is mechanically coupled to the ryanodine receptor. Electron microscopy studies show particles in the T-tubule or surface membranes arranged into groups of four, called tetrads. The arrangement of the tetrads corresponds exactly to the spacing of every second RyR complex in the opposing membrane of the SR (Block *et al.*, 1988). The tetrads are thought to be clusters of DHPR's since they are absent in dysgenic mice (Franzini-Armstrong *et al.*, 1991), and are restored to dysgenic myotubes following transfection with DHPR cDNA (Takekura *et al.*, 1994).

1.4.2 *The ryanodine receptor Ca^{2+} release channel*

Ryanodine is a plant alkaloid and naturally occurring insecticide (Jenden and Fairhurst, 1969) that binds very tightly to the SR Ca^{2+} release channel and has been instrumental in its isolation (Fleischer *et al.*, 1985; Pessah *et al.*, 1985, 1986; Inui *et al.* 1987).

1.4.2A *Structure*

The RyR is a 560 KDa protein which has been successfully isolated from SR membranes (Kawamoto *et al.*, 1986; Inui, *et al.*, 1987; Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). Isolated RyR's form aggregates of four, which appear similar to the junctional foot proteins (Franzini -Armstrong, 1970, Franzini-Armstrong *et al.*, 1975; Ferguson *et al.*, 1984). Inui and colleagues (1987) confirmed that the junctional foot protein and the RyR were the same and Imagawa *et al.*, (1987) demonstrated that the RyR was the SR Ca^{2+} release channel.

The RyR appears in electron micrographs as a tetrameric structure which extends 12 nm from the surface of the terminal cisternae membrane into the junctional gap. The tetramer has a central pore of 2 nm diameter which is connected to 4 other smaller pores located at the centre of each subunit. The cytoplasmic portion is thought to be anchored by a 14x14x14 nm baseplate inserted into the terminal cisternae

membrane and it is suggested that Ca^{2+} first passes through the central pore and then into 4 radial channels formed between the cytoplasmic portions of the RyR protein before entering the junctional gap (Wagenknecht *et al.*, 1989; Radermacher *et al.*, 1992, 1994; Wagenknecht and Radermacher, 1995).

1.4.2B *Distribution of the RyR*

The RyR is found in many tissues. In mammals there are three different isoforms: RyR1 present mainly in skeletal muscle (Takeshima *et al.*, 1989), RyR2 the predominant form found in cardiac muscle (Nakai *et al.*, 1990) and RyR3 isolated from brain and non-muscle tissue (Hakamata *et al.*, 1992). Different isoforms of the RyR are found in other species and more than one isoform can occur within the same cell: in mammalian skeletal and cardiac muscle and in nonmammalian muscle such as chicken, frog and toadfish (Airey *et al.*, 1990; Olivares *et al.*, 1991; O'Brien *et al.*, 1993; Percival *et al.*, 1994; Zorzato *et al.*, 1994; Ivanenko *et al.*, 1995).

1.4.2C *Modulation of the RyR*

(See Ogawa, 1994; and Coronado *et al.*, 1994; for further details)

Various ligands modulate the skeletal muscle RyR. Low concentrations of ryanodine (< 10μm) lock the Ca^{2+} release channel into an open conformation and higher concentrations (>10 μm) block the channel (Fleischer *et al.*, 1985; Meissner, 1986*a*; Lattanzio *et al.*, 1987). The skeletal muscle RyR is activated by adenine nucleotides, micromolar Ca^{2+} and millimolar PO_4^{2-}, and is inhibited by millimolar Ca^{2+}, Mg^{2+}, and micromolar ruthenium red (Ebashi, 1976; Morii and Tonomura, 1983; Smith *et al.*, 1985, Fruen *et al.*, 1994). A number of drugs also affect Ca^{2+} currents through the RyR including: caffeine (Chu *et al.*, 1990; Lee *et al.*, 1991; Lee, 1993), various anaesthetics (procaine (Meissner, 1984), tetracaine (Shoshan-Barmatz and Zchut, 1993)), polyamines such as spermine (Zarka and Shoshan-Baramatz, 1992), scorpion toxins (Valdivia *et al.*, 1992), immunosppressants such as FK506 and rapamycin (Timerman *et al.*, 1993; Ahern *et al.*, 1994), and bastadin, a novel compound isolated from sponges (Mack *et al.*, 1994).

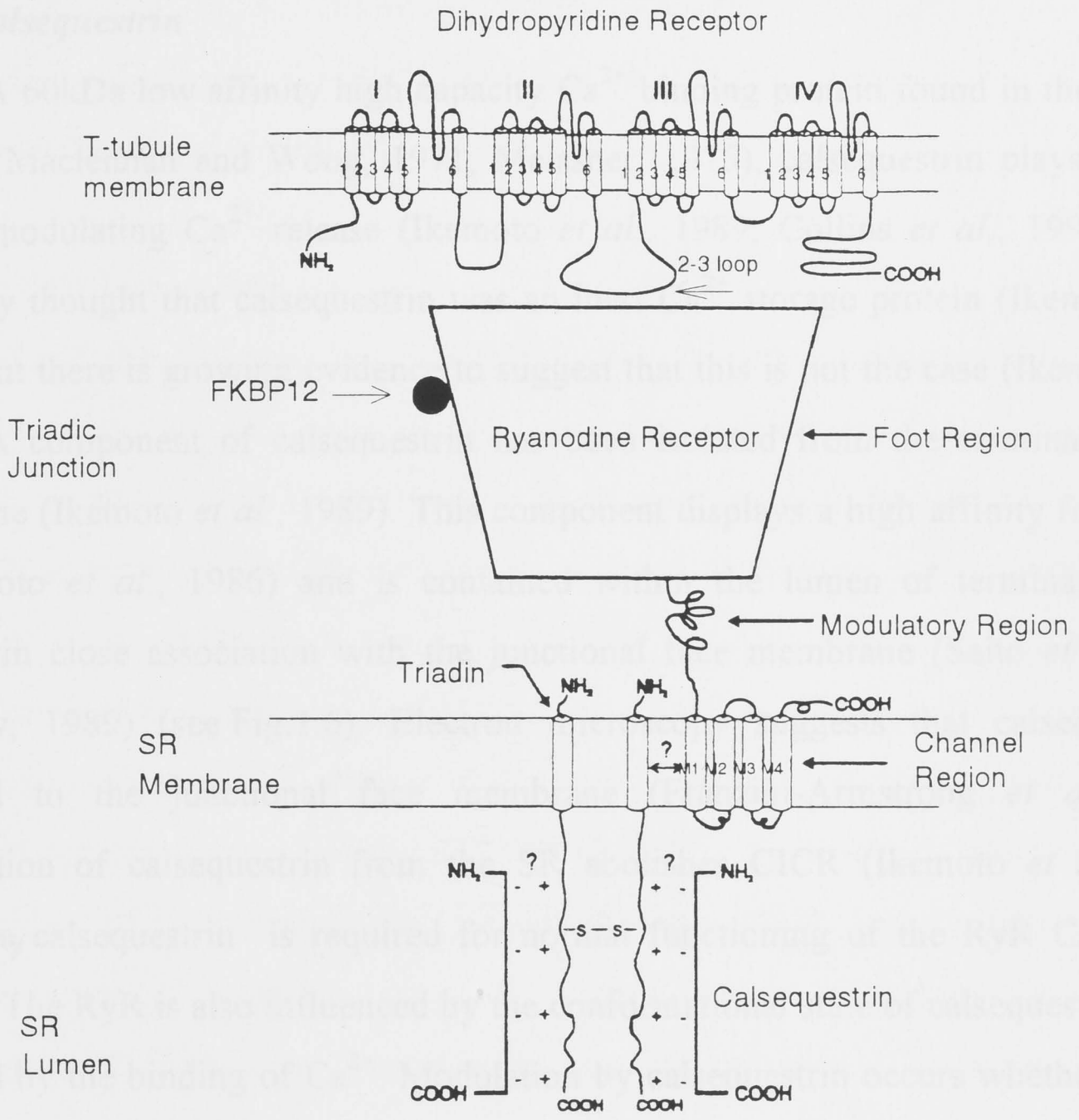


Figure 1.6 *Structure of the DHPR α1 subunit and RyR*
A diagram showing the transmembrane structure of the DHPR α1 subunit and its position in the T-tubule membrane relative to the RyR Ca^{2+} release channel of the SR. The DHPR α1 subunit is composed of 4 identical domains each containing 6 putative membrane spanning α helices of which helix number 4 is proposed to be the voltage sensor for E-C coupling. The cytoplasmic loop between domains 2 and 3 is thought to communicate with the RyR receptor during E-C coupling. The RyR Ca^{2+} release channel is associated with the membranes of the terminal cisternae. The large cytoplasmic "foot" region of the receptor occludes much of the triadic junction, whilst the Ca^{2+} conducting pore region of the receptor is embedded firmly within the junctional face membrane. Also shown are proteins which have less well defined roles in E-C coupling including the FKBP12, triadin, and calsequestrin (see below). (Modified from McPherson and Campbell, 1993).

1.5 Proteins that modulate Ca^{2+} uptake, storage and release in the SR

1.5.1 *Calsequestrin*

A 60kDa low affinity high capacity Ca^{2+} binding protein found in the lumen of the SR (Maclennan and Wong, 1971; Meissner, 1975), calsequestrin plays an active role in modulating Ca^{2+} release (Ikemoto *et al.*, 1989; Collins *et al.*, 1990). It was originally thought that calsequestrin was an inert Ca^{2+} storage protein (Ikemoto *et al.*, 1974), but there is growing evidence to suggest that this is not the case (Ikemoto *et al.*, 1991). A component of calsequestrin has been isolated from the terminal cisternae membrane (Ikemoto *et al.*, 1989). This component displays a high affinity for the RyR (Kawamoto *et al.*, 1986) and is contained within the lumen of terminal cisternae vesicles in close association with the junctional face membrane (Saito *et al.*, 1984; Dulhunty, 1989) (see Fig.1.6). Electron microscopy suggests that calsequestrin is anchored to the junctional face membrane (Franzini-Armstrong *et al.*, 1987). Dissociation of calsequestrin from the SR abolishes CICR (Ikemoto *et al.*, 1989). Therefore, calsequestrin is required for normal functioning of the RyR Ca^{2+} release channel. The RyR is also influenced by the conformational state of calsequestrin, which is altered by the binding of Ca^{2+}. Modulation by calsequestrin occurs whether the RyR is opened by T-tubule depolarisation or directly by CICR (Ikemoto *et al.*, 1974; Ohnishi, and Reithmeier 1987). However, the precise role of calsequestrin in E-C coupling has yet to be elucidated.

1.5.2 *Calmodulin*

Photoaffinity labelling studies show that calmodulin, a protein of Mr 17 kDa (Klee and Vanaman, 1982) binds to high molecular weight proteins (RyR) in native SR vesicles (Seiler *et al.*, 1984). Yang *et al.*, (1994) showed a direct interraction of calmodulin with the RyR. Multiple calmodulin binding sites are present on each RyR subunit: the tetrameric channel binds 4 calmodulin molecules in the presence of Ca^{2+} and 16 in the absence of Ca^{2+} (Menegazzi *et al.*, 1994; Guerrini *et al.*, 1995; Tripathy *et al.*, 1995). Some calmodulin binding sites have been identified on the cytoplasmic surface of the RyR using electron microscopy (Wagenknecht *et al.*, 1994). The

modulation of the RyR by calmodulin varies depending on the Ca^{2+} concentration: calmodulin activates the Ca^{2+} release channel at submicromolar free Ca^{2+} levels (Tripathy *et al.*, 1995; Buratti *et al.*, 1995), and calmodulin inhibits the channel at micromolar to millimolar free Ca^{2+} (Smith *et al.*, 1989; Tripathy *et al.*, 1995; Ikemoto *et al.*, 1995; Buratti *et al.*, 1995).

1.5.3 *The FK506 binding protein*

FK506 binding proteins (FKBP's) are *cis-trans* peptidyl-prolyl isomerases which bind with high affinity to FK506, an immunosuppressant drug (Standaert *et al.*, 1990). A number of different FKBPS have been isolated and a 12 kDa protein (FKBP12) is found in close association with the skeletal muscle RyR (Collins, 1991; Jayaraman *et al.*, 1992), in a ratio of 4 FKBP's per RyR tetramer (Timerman *et al.*, 1993). Terminal cisternae vesicles depleted of FKBP12 display a decreased threshold for caffeine activated Ca^{2+} release and a decreased rate of Ca^{2+} uptake. This was thought to correspond to a greater efflux of Ca^{2+} through the RyRl because the effect could be blocked by ruthenium red (Timerman *et al.*, 1993). These results suggest that FKBP12 serves to modulate the Ca^{2+} release channel (Timerman *et al.*, 1993; Coronado et al., 1994). RyR's which have been chemically stripped of, or expressed without FKBP12 show that FKBP12 has profound effects in coordinating RyR single channel opening to the maximum conductance (Brill antes *et al.*, 1994; Ahern *et al.*, 1994; Chen *et al.*, 1994). skinned fibe experiments by LAMB and stephenson (1996) suggest that the FK506-binding protein may play a vital role in skeletal muscle E-C coupling.

1.5.4 *The Ca^{2+}-Mg^{2+}-ATPase*

Muscle relaxation following contraction is mediated by the reuptake of Ca^{2+} into the SR by an ATP driven pump, the Ca^{2+}-ATPase. The presence of the Ca^{2+}-ATPase was suggested by the finding that isolated SR could accumulate Ca^{2+} in the presence of ATP, Mg^{2+}, and Ca^{2+} (Hasselbach, 1964; Hasselbach and Oetliker, 1983; Martonosi and Beeler, 1983) in a manner dependent on ATP hydrolysis (Hasselbach and Makinose, 1963). The pump is a Ca^{2+}-Mg^{2+} activated ATPase of Mr~ 110kDa embedded into the SR membrane (Franzini-Armstrong et al., 1986; Aidley, 1989). The activity of the pump is controlled by the concentration of external Ca^{2+}. Hasselbach and Makinose (1963),

calculated that two Ca^{2+} ions are taken up for every molecule of ATP split at (10^{-7} M) Ca^{2+} concentrations, whereas Ca^{2+} uptake is almost abolished at lower (10^{-8} M) Ca^{2+} concentrations. The action of the pump may be aided by the binding of SR luminal Ca^{2+} to calsequestrin, suggesting that the internal Ca^{2+} concentration of the SR may modulate Ca^{2+}-ATPase activity (Maclennan and Wong, 1971). Ca^{2+}-ATPases occurring in the SR and endoplasmic reticulum (ER) are collectively encoded for by the SERCA (SR-ER-Ca^{2+}-ATPase) gene family. SERCA genes are widespread and occur in many other tissues such as smooth muscle, kidney and brain (Inesi and Kirtley, 1992).

1.6 Other junctional proteins with less defined roles in E-C coupling

A direct interaction between the T-tubule voltage sensor and the RyR during E-C coupling has not been established even though the two proteins are arranged in a fashion that would support a mechanical link (Block *et al.*, 1988). Other triadic proteins have been isolated and a role for these proteins in signal transduction across the junctional gap cannot be ruled out. Triadin, a 95 KDa glycoprotein, may be involved in coupling of the RyR to the DHPR (Brandt *et al.*, 1992). A 36 kDa protein, glyceraldehyde 3-phosphate dehydrogenase, has been shown to promote junction formation between isolated T-tubules and terminal cisternae (Corbett *et al.*, 1985) and aldolase, a 40kDa protein, binds to RyR and is released by inositol polyphosphates (Thieleczek *et al.*, 1989).

1.7 Excitation-contraction coupling

Contractile activation occurs as a result of T-tubule depolarisation which can be experimentally evoked in several ways. By exploiting the fact that V_m is determined by the extracellular [K^+], Kuffler (1946), Hodgkin, and Horowicz (1960a) demonstrated that skeletal muscle contraction could be achieved by depolarising muscle fibres in elevated K^+ solutions. The amplitude of these potassium (K) contractures was graded, and depended on the [K^+] of the depolarising solution. K contracture amplitude depends only on the response of the E-C coupling mechanism to depolarisation. Hence, K contractures differ from twitches and tetani which depend firstly on the generation of an

action potential and then on the response of E-C coupling to the action potential depolarisation. V_m can also be controlled electronically using microelectrodes (Marmont, 1949; Cole, 1949; Hodgkin *et al.*, 1952). This technique, "voltage clamping", has contributed much to the understanding of voltage-activated contraction.

1.7.1 *The skeletal muscle voltage sensor and asymmetric charge movement*

In the early seventies, voltage clamp studies revealed that voltage sensitive molecules in the T-tubule membrane generated small asymmetric capacative currents in response to membrane depolarisation. These capacitive currents are absent during membrane hyperpolarisation (Schneider and Chandler, 1973). This phenomenon is thought to reflect the conformational changes of the voltage-sensitive protein for E-C coupling in the T- tubule membrane in response to changes in the membrane electrical field. Chandler and coworkers (1976) found that charge movement was sigmoidally related to voltage in an identical manner to the activation of contraction. Maintained depolarisation abolished charge movement, which recovered upon subsequent membrane repolarisation. This "immobilisation" of charge occurred over the same voltage range as "contractile inactivation" (Hodgkin and Horowicz, 1960; Luttgau, 1963). The activation and inactivation of charge movement can be represented in simplest terms by a 3 state model proposed by Chandler *et al.*, (1976), (Fig. 1.7):

1.7.2 *Components of asymmetric charge movement*

Charge movement can be divided into two major components, charge 1 and charge 2 (Adrian and Almers, 1976; see Huang, 1988; and Rios and Pizarro, 1991 for reviews). Charge 1 has been localised to the T-tubule, and is sigmoidally related to membrane potential (Chandler *et al.*, 1976*a*&*b*; Dulhunty and Gage, 1983, 1985; Lamb, 1986*a*; Melzer *et al.*, 1986). In fibres held at -100 mV, charge 1 has the same voltage dependence as the activation/inactivation of tension (Schneider and Chandler, 1973; Chandler *et al.*, 1976; Horowicz and Schneider, 1981*a*&*b*; Adrian *et al.*, 1976; Huang, 1982; Hui, 1983; Adrian and Huang, 1984). Simultaneous measurement of Ca^{2+} transients reveal that charge 1 precedes and is proportional to Ca^{2+} release (Kovacs, 1979; Melzer *et al.*, 1986). A second component of charge movement, charge 2, was

independently identified by Adrian and Almers (1976 *a&b*) and Schneider and Chandler (1976). Charge 2 can be isolated by blocking other charge species with lidocaine (Huang, 1982), or by immobilisation of other charge species with sustained depolarisation (Adrian *et al.*, 1976). It was suggested by Brum and Rios (1987) that charge 1 and 2 interconvert when the membrane is conditioned at different potentials. This idea was also considered by Adrian *et al.*, (1976) but was rejected on the grounds that a decrease in charge 2 wasn't seen upon the repriming of charge 1 following inactivation by sustained depolarisation. Instead, Haung (1993), suggests that charge 1 and charge 2 are separate systems.

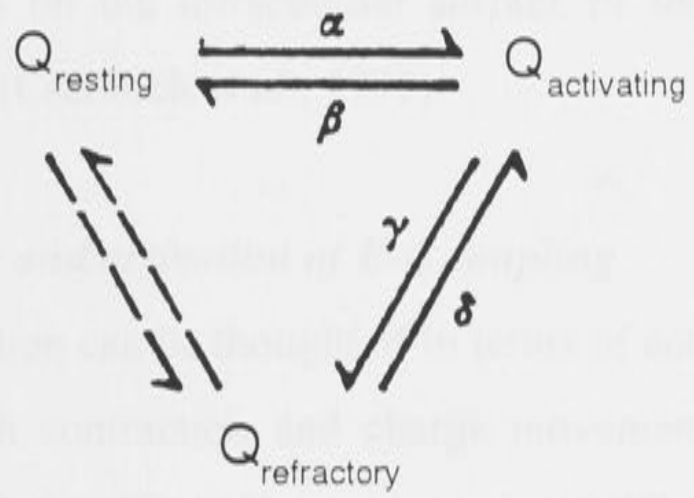


Figure 1.7 *A 3 state model of charge movement*
On depolarisation from the resting membrane potential, charge (Q), distributes rapidly between the resting ($Q_{resting}$) and active ($Q_{activating}$) configurations. The value of the rate constant for this transition, ($\alpha+\beta$) is ~ 0.1 msec^{-1} at 0 °C. Prolonged depolarisation results in the slower conversion of charge from the active state to an inactive state ($Q_{refractory}$) which has a rate constant ($\gamma+\delta$) in the order of 0.1 sec^{-1} at 0 °C. Charge converts from the refractory state back to the resting state only when the membrane has been repolarised. A direct link between the refractory and resting states has been marked with a dashed line since such a step was not required in order to explain the experimental data. (Modified from Chandler *et al.*, 1976*b*).

Charge 1 is further subdivided into a rapid component Q_{β}, and a slower "hump-like" component Q_{γ} (Adrian and Peres, 1979). Q_{γ} appears at the threshold for contraction (about -60 mV), and inactivates when the fibre is maintained at -40 mV, the potential at which contractile inactivation occurs (Horowicz and Schneider, 1981*b*;

Huang, 1981b; Adrian and Huang, 1984 *a&b*). Q_γ is blocked by tetracaine, which also blocks contraction (Huang, 1981a; Hui 1982; Hui, 1983*a&b*). Q_β on the other hand, is less voltage dependent, inactivates with prolonged depolarisation to -20mV and is insensitive to tetracaine (Hui, 1982). Q_γ is not always seen as a distinct component of charge movement, is virtually absent in the rabbit (Lamb, 1986*a*), and has not been demonstrated in the rat (Hollingworth and Marshall, 1981; Dulhunty and Gage, 1983; Melzer *et al.*, 1986; Hollingworth *et al.*, 1990). Later studies found Q_γ to be influenced by factors acting on Ca^{2+} release from the SR (Csernoch *et al.*, 1991; Garcia *et al.*, 1991; Szucs *et al.*, 1991a; Pizzaro *et al.*, 1991). Q_β is thought by some authors to be responsible for releasing Ca^{2+} from the terminal cisternae whereas Q_γ is the result of this released Ca^{2+} binding to fixed charges on the intracellular surface of the T-tubule, producing an additional depolarisation (Csernoch *et al.*, 1991).

1.7.3 *The voltage sensor and activation of E-C coupling*

Contractile activation can be thought of in terms of activation\inactivation of the voltage sensor since both contraction and charge movement share the same voltage dependence and are similarly affected by various agents (Caputo *et al.*, 1979; Luttgau and Stephenson, 1986; Luttgau *et al.*, 1987; Dulhunty and Gage, 1988; Dulhunty, 1991). However, this is not strictly correct since voltage sensor activation is the first of many steps that occur between excitation and tension development. Contractile activation can be conveniently described by a tension Vs voltage curve, in which normalised peak tension (tension at varying membrane potentials ÷ maximal tension) is plotted against membrane potential. A typical tension Vs voltage curve constructed using K contracture data is shown in Fig.1.8 The same curve could also be produced from voltage clamp experiments. The relationship between contractile tension and membrane potential is best fitted by a Boltzmann equation of the following form (Dulhunty and Gage, 1983):

$$T_a = T_{max} / [1+\mathrm{EXP}((V_a - V_m) / k_a)]$$

Where;

T_a is the normalised K contracture amplitude at membrane potential V_m.

V_a is the potential at which $T_a = 0.5\ T_{max}$ and k_a is the slope factor.

Values of T_a, V_a and k_a are often used to compare the effects of various agents on the voltage sensor.

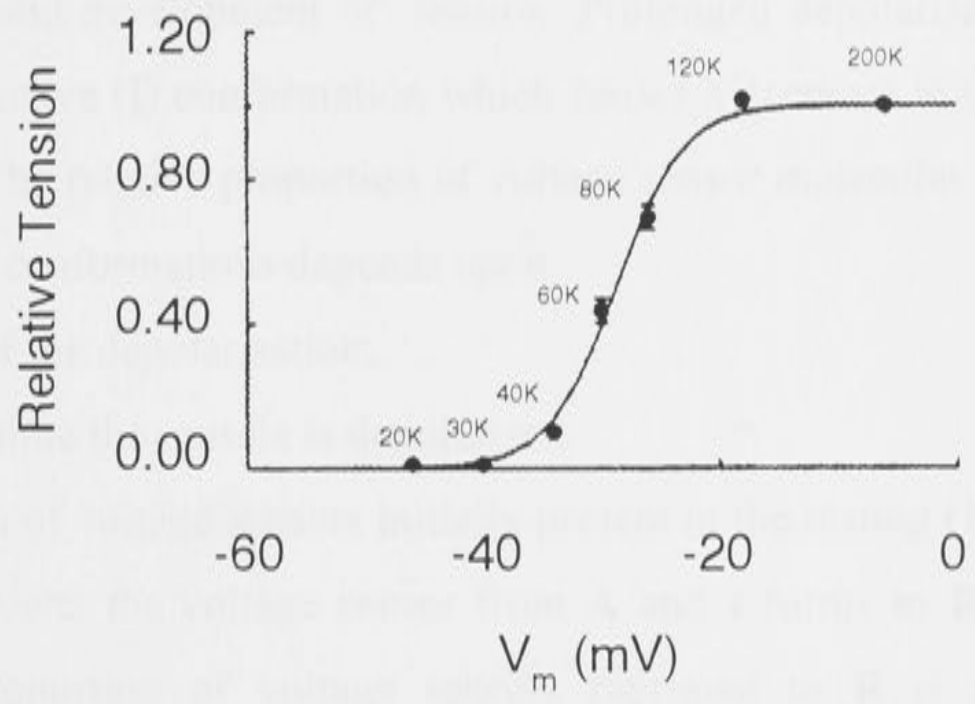


Figure 1.8 *The voltage dependence of contractile activation*
A curve showing the relationship between membrane potential, extracellular solution [K^+], and contractile tension recorded in a bundle of rat soleus fibres. Contractile tension produced by exposing preparations to elevated [K^+] solutions (and normalised to the maximal contractile response) is shown plotted against the V_m measured in each of the high [K^+] solutions. The [K^+] used are shown beside each data point. The curve is constructed using a Boltzmann function described in the text (Section 1.8.3). From the curve, tension develops when fibres are depolarised to approximately -40 mV (corresponding to a [K^+] of ~30 mM). Tension increases rapidly with larger depolarisation s and reaches a plateau when the fibres have been depolarised to ~ -20 mV ([K^+] ~120 mM). (Modified from Dulhunty, 1992).

1.7.4 *Voltage dependent inactivation*.

Prolonged depolarisation results in a spontaneous decay of contractile tension (Hodgkin and Horowicz, 1960a) which can be most simply explained if the voltage sensor is assumed to exist in three different states (Caputo and Bolanos, 1979), which

are analagous to those shown in the 3 state model for charge movement (Section 1.1.1), (Adrian and Almers, 1976[a]; Chandler *et al*, 1976[b]; Rakowski, 1981):

$$R \leftrightarrow A \leftrightarrow I$$

At rest, the voltage sensor molecules are present in a non-active or resting state (R). Subsequent T-tubule depolarisation (beyond the contractile threshold) shifts the voltage sensors into an active (A) conformation which coincides with the release of Ca^{2+} from the SR and development of tension. Prolonged depolarisation converts the molecules to an inactive (I) conformation which causes a decrease in Ca^{2+} release and a decay of tension. The relative proportion of voltage sensor molecules entering both the active and inactive conformations depends upon:

(1) the strength of the depolarisation;

(2) the length of time the muscle is depolarised;

(3) the proportion of voltage sensors initially present in the resting (**R**) stage.

Repolarisation converts the voltage sensor from **A** and **I** forms to **R**, a process called repriming. The proportion of voltage sensors reprimed to **R** is dependent on the magnitude of repolarisation and the amount of time allowed for repriming. Inactivation is measured experimentally using a two pulse protocol in which a "conditioning" submaximal depolarisation is immediately followed by a maximally activating depolarisation. The size of the second "test" contracture is smaller than normal because some of the voltage sensors are inactivated during the initial "conditioning" depolarisation (Hodgkin and Horowicz, 1960). This two pulse protocol is used to obtain inactivation curves where the tension produced by the test depolarisation (normalised against the maximal tension response) is plotted against the membrane potential measured in the conditioning solution. An example of such a curve is shown in Fig.1.9. The relationship can be described by a Boltzmann equation of the form (Dulhunty & Gage, 1983):

$$T_i = T_{max} / [1 + \mathrm{EXP}((V_c - V_i)/ k_i)]$$

Where,

T_i = test contracture amplitude at conditioning membrane potential V_c

T_{max} = maximum tension output of the preparation

V_c = conditioning membrane potential

V_i = membrane potential at which $T_i = 0.5\ T_{max}$

k_i = slope factor

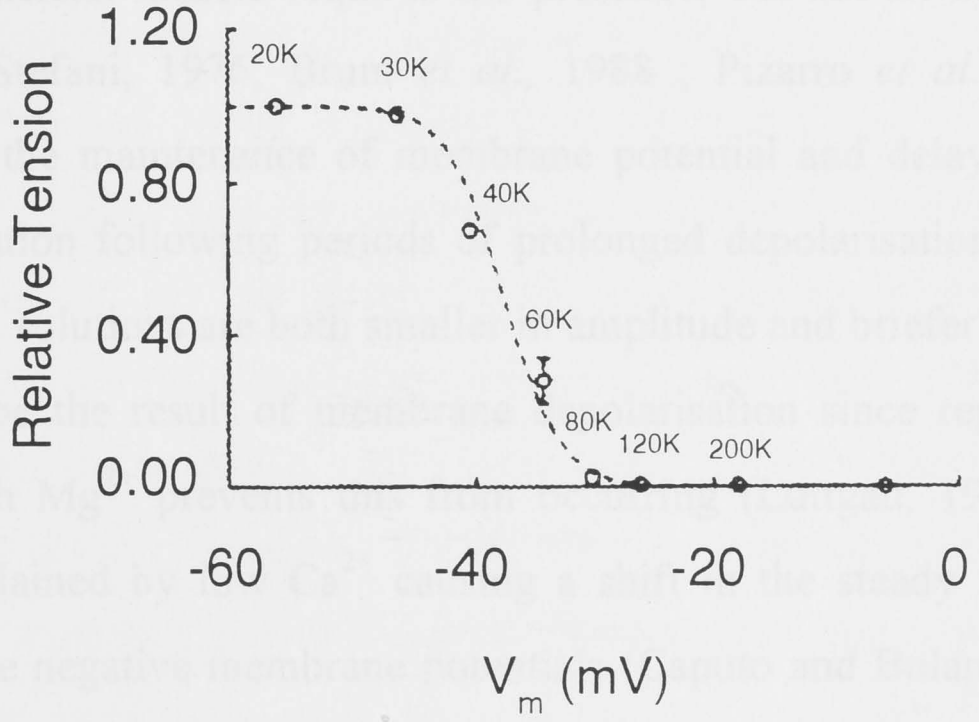


Figure 1.9 *The voltage dependence of contractile inactivation*
A curve describing the voltage dependence of contractile inactivation in bundles of rat soleus fibres. Data points were obtained using the two pulse protocol described in Section 1.7.4. The curve was constructed using a Boltzmann function (described in the text). (Modified from Dulhunty, 1992).

1.7.5 *Caffeine-induced contractures: a mechanism of contraction that bypasses the voltage sensor*

Caffeine causes contraction in skeletal muscle (Axelsson and Thesleff, 1958; Luttgau and Oetliker, 1968; Endo et al., 1970) via activation of the RyR (Erlich and Watras, 1988; also reviews by Fill and Coronado, 1988; Coronado *et al.*, 1994; Ogawa, 1994). Thus, caffeine is useful in determining whether agents affect E-C coupling before or at SR Ca^{2+} release. However, caffeine contractures are small and difficult to

elicit in mammalian muscle (Cairns and Dulhunty, 1994) and are not as useful in this preparation as in amphibia where 10 mM caffeine can elicit maximum tension (Luttgau and Oetliker, 1968).

1.8 Factors affecting E-C coupling.

1.8.1 *The role of external Ca^{2+}*

E-C coupling in skeletal muscle requires the presence, but not an influx, of external Ca^{2+} (Beaty and Stefani, 1976; Brum *et al.*, 1988 ; Pizarro *et al.*, 1989). Ca^{2+} is important both in the maintenance of membrane potential and delaying the onset of contractile inactivation following periods of prolonged depolarisation. K contractures evoked in low Ca^{2+} solutions are both smaller in amplitude and briefer in duration. This is not thought to be the result of membrane depolarisation since replacement of the omitted Ca^{2+} with Mg^{2+} prevents this from occurring (Luttgau, 1963). Instead, the results can be explained by low Ca^{2+} causing a shift in the steady state inactivation curve towards more negative membrane potentials (Caputo and Bolanos, 1987). Using EGTA, Armstrong *et al.*, (1972) was the first to demonstrate that an influx of external Ca^{2+} is not required in amphibian fibres during a twitch. This was also shown later by Luttgau and Spiecker (1979) and subsequently by several other investigators.

Similar reports on the effects of low external Ca^{2+} on E-C coupling have been obtained using mammalian skeletal muscle in which lowering external Ca^{2+} causes initial twitch potentiation followed by depression. The decay of K contracture tension also occurs more rapidly and recovery from inactivation is slowed (Graf and Schatzmann, 1984; Dulhunty and Gage, 1988). These results are explained (Dulhunty and Gage, 1988) by assuming that the transition of the voltage sensor between the resting, active and inactive conformations, as shown in the previous model (Section 1.7.4), requires the release/binding of Ca^{2+}. Thus, conversion of the voltage sensor from the resting to the active conformation involves the release of Ca^{2+}. This would be accelerated in a low Ca^{2+} solution and hence explains the initial potentiation of twitch tension. Further dissociation of Ca^{2+} converts the sensor from the active to the inactive state, thus the twitch tension is depressed and K contractures decay rapidly in low Ca^{2+}.

Likewise, repriming (ie conversion of the voltage sensor from the inactive state back to the resting state) requires the rebinding of Ca^{2+} which is retarded in low external Ca^{2+}.

1.8.2 *Mg^{2+} and other multivalent cations*

Many divalent cations modulate E-C coupling but none are fully effective in replacing Ca^{2+} (Schnier *et al.*, 1993). Luttgau (1963) showed that Mg^{2+} has similar properties to Ca^{2+} in maintenance of membrane potential and later argued that most of the twitch depression seen with low Ca^{2+} and high concentrations of EGTA was due to membrane depolarisation and could be prevented by replacing the omitted Ca^{2+} with Mg^{2+} (Luttgau & Spiecker, 1979). Similarly, Dulhunty and Gage, (1988), showed that solutions containing EGTA concentrations as high as 20 mM had little effect on contraction in mammalian skeletal muscle as long as Mg^{2+} (10 mM) was present.

Most divalent cations can partially replace Ca^{2+} and their addition can reverse the effects of removing external Ca^{2+}. Ni^{2+} for example, reverses the effects of low Ca^{2+} on K contracture tension (Caputo, 1981). This is interesting because Ni^{2+} is impermeant and so must be acting at some external site, presumably the voltage sensor. Similar observations have been made using using Ni^{2+}, Co^{2+} and La^{3+} (Lorkovic & Rudel, 1983) and with Ba^{2+} and La^{3+} (Bolanos *et al.*, 1986). An interesting report by Dulhunty and Gage (1989) showed two effects of the divalent cations Mg^{2+}, Co^{2+}, and Cd^{2+} on K contractures in mammalian skeletal muscle fibres:

(a) the cations caused a positive shift in the curve relating K contracture tension to membrane potential;

(b) K contractures were broader than controls indicating a slower onset of inactivation.

Similar effects on K contractures have also been reported for Mn^{2+} (Oota *et al.*, 1972) and La^{3+} (Parry *et al.*, 1974). The reported effects of cations on E-C coupling can be explained by an effect on voltage sensor activation/inactivation using the previous model (Section 1.7.4) which requires the release/binding of Ca^{2+} to move the voltage sensor between various states. It is possible that other cations bind to the same sites on the voltage sensor normally occupied by Ca^{2+}.

Many authors report an associated order of effectiveness for different cations in replacing Ca^{2+}. Interestingly, this order potency is often the same as that with which the cations block Ca^{2+} channels (Dulhunty and Gage, 1989).

1.8.3 *Anions*

The effect of anion species on E-C coupling has been well documented. Hodgkin & Horowicz (1960*b*) found that both nitrate and thiocyanate caused a potentiation of twitch and submaximal K contracture tension which could be attributed to a negative shift in the voltage dependence of force activation. Better studied are the actions of perchlorate, which also potentiates twitches and submaximal K contractures in amphibian muscle, (Foulks *et al.*, 1973 *a&b*; Gomolla *et al.*, 1983; Luttgau *et al.*, 1983) and mammalian muscle (Dulhunty, 1992; Dulhunty *et al.*, 1992*b*). Perchlorate is thought to cause a negative shift in the voltage dependence of force activation via the voltage sensor since perchlorate also affects charge movement in the same way (Luttgau *et al.*, 1983). A negative shift in the steady state inactivation curve has been reported in mammalian muscle (Dulhunty *et al.*, 1992*b*), but not in amphibian muscle (Foulks *et al.*, 1973*a*; Gomolla *et al.*, 1983). However, recent studies suggest that perchlorate also modulates E-C coupling by directly activating the RyR Ca^{2+} release channel (Ma *et al.*, 1993; Fruen *et al.*, 1994; Ikemoto *et al.*, 1995).

1.8.4 *Charge screening and binding*

The surface membranes of cells contain an abundance of fixed negative charges, some originating from amino acid side chains of membrane proteins, the bulk arising from sialic acid residues (Miller, 1983) and negatively charged phospholipids (Moczydlowski *et al.*, 1985). The surface charge generates an electric field which adds to the potential difference between the inside and outside of the cell (Frankenhauser and Hodgkin, 1957) (Fig.1.10). There is evidence that some surface negative charges are clustered around the entrance to voltage gated ion channels (Hille *et al.*, 1975) and are important in the gating of these channels. The local potentials produced by these charges are "invisible" in the macroscopic V_m and so do not affect the measured resting membrane potential. However, surface potentials act as a driving force on ions near the

membrane surface, and exert an effect on voltage sensors residing within the membrane (Hille, 1992).

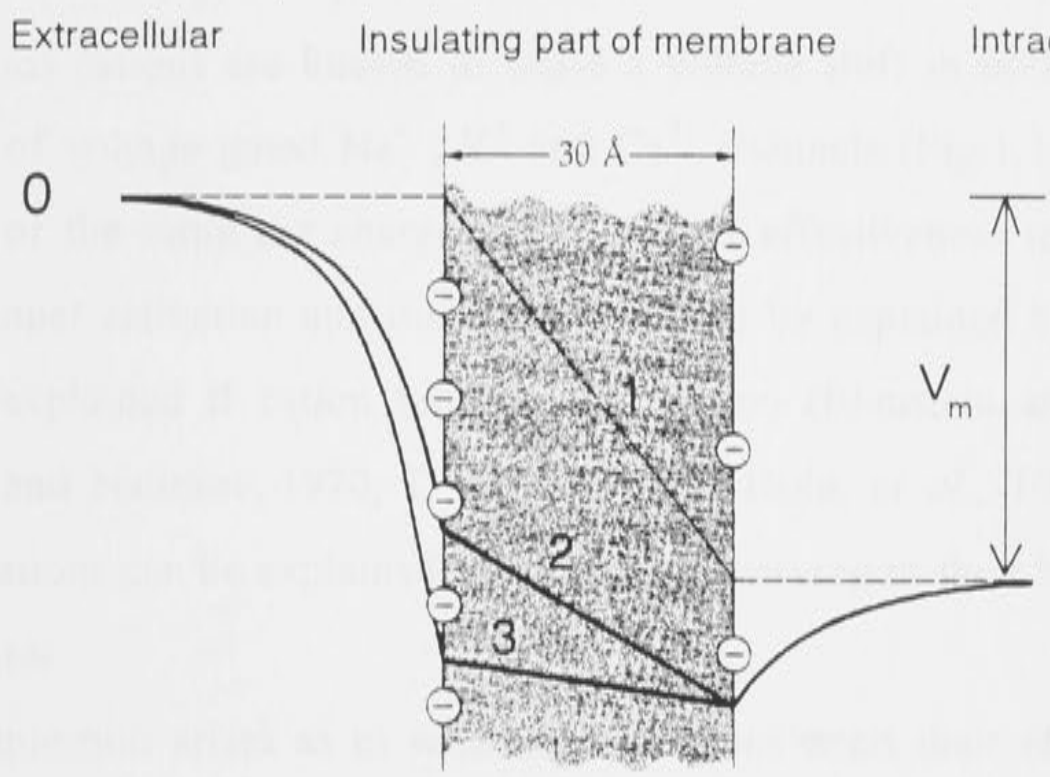


Figure 1.10 *The effect of surface charge on membrane field*
A diagram illustrating how surface charge effects the electrical field across the membrane. The dotted line (1) represents the potential profile obtained in the absence of fixed negative surface charges and lines 2 and 3 represent the effect of increasing surface charge. From the diagram, increasing the amount of surface charge effectively decreases the electrical field across the membrane that would be detected by voltage sensitive molecules but has no effect on the recorded membrane potential (V_m). Hence, addition of divalent cations which screen the negative charges, effectively hyperpolarises the membrane potential that would be seen by the voltage sensitive molecules. A larger depolarisation is thus required to achieve the same reduction in field as that achieved when negative charges are not screened. This has important consequences on the opening and closing of voltage gated channels and on activation/inactivation of the voltage sensor for E-C coupling. (Modified from Hille *et al.*, 1975 and Hille, 1992).

Cations bind to surface charges thus altering the membrane electric field (Frankenhauser and Hodgkin 1957). Gilbert and Ehrenstein (1969) proposed that similar effects could be achieved by charge screening rather than binding. A shift in surface potential by charge screening can be predicted by the Gouy-Chapman-Stern theory which describes the effect of electrolytic solutions on a uniformly charged planar

surface (Gilbert and Ehrenstein, 1969; Mclaughlin *et al.*, 1971; Begenisich and Stevens, 1975; Hille *et al.*, 1975). A problem with this model is that biological membranes are rarely planar surfaces and the charge distribution is unlikely to be uniform (see review by Mclauchlin, 1989).

Various cations are known to cause a voltage shift in both the activation and inactivation of voltage gated Na^+ , K^+ and Ca^{2+} channels (Fig.1.11). The observation that cations of the same net charge differ in their effectiveness in producing voltage shifts in channel activation and inactivation cannot be explained by charge screening, but can be explained if cation binding also occurs (Blaustein and Goldman, 1968; Mozhayeva and Naumov, 1970; D'Arrigo, 1973; Hille, *et al.*, 1975). Differences in potency of cations can be explained by the cations varying in their binding affinities for membrane sites.

The question arises as to whether the cations exert their effects by binding to charges on the membrane itself or by binding to charges on the channel proteins. To answer this question, experiments have been performed in which voltage gated Na^+ channels (Cukierman, *et al.*, 1988;) and K^+ channels (Bell and Miller., 1984; Moczydlowski, *et al.*, 1985) were reconstituted into artificial lipid bilayers composed of either neutral or negatively charged phospholipids. All authors demonstrated channel gating was different when the bilayer was composed of negatively charged phospholipids and that this difference decreased with increasing ionic strength of the bathing solutions. Mutational analysis and chemical modification have revealed that charges on channel proteins are also important for channel gating (Anderson, *et al.*,1988; Noda, *et al.*, 1989; MacKinnon and Miller, 1989; MacKinnon and Yellen, 1990). In Section 1.8.1, a model for the skeletal muscle voltage sensor which involved the binding of Ca^{2+} to a modulatory site important in permitting the transition of the sensor between various states was discussed. It is probable that the effect of external divalent cations on E-C coupling is a combination of the screening and binding to membrane surface charges as well as direct binding to the voltage sensor.

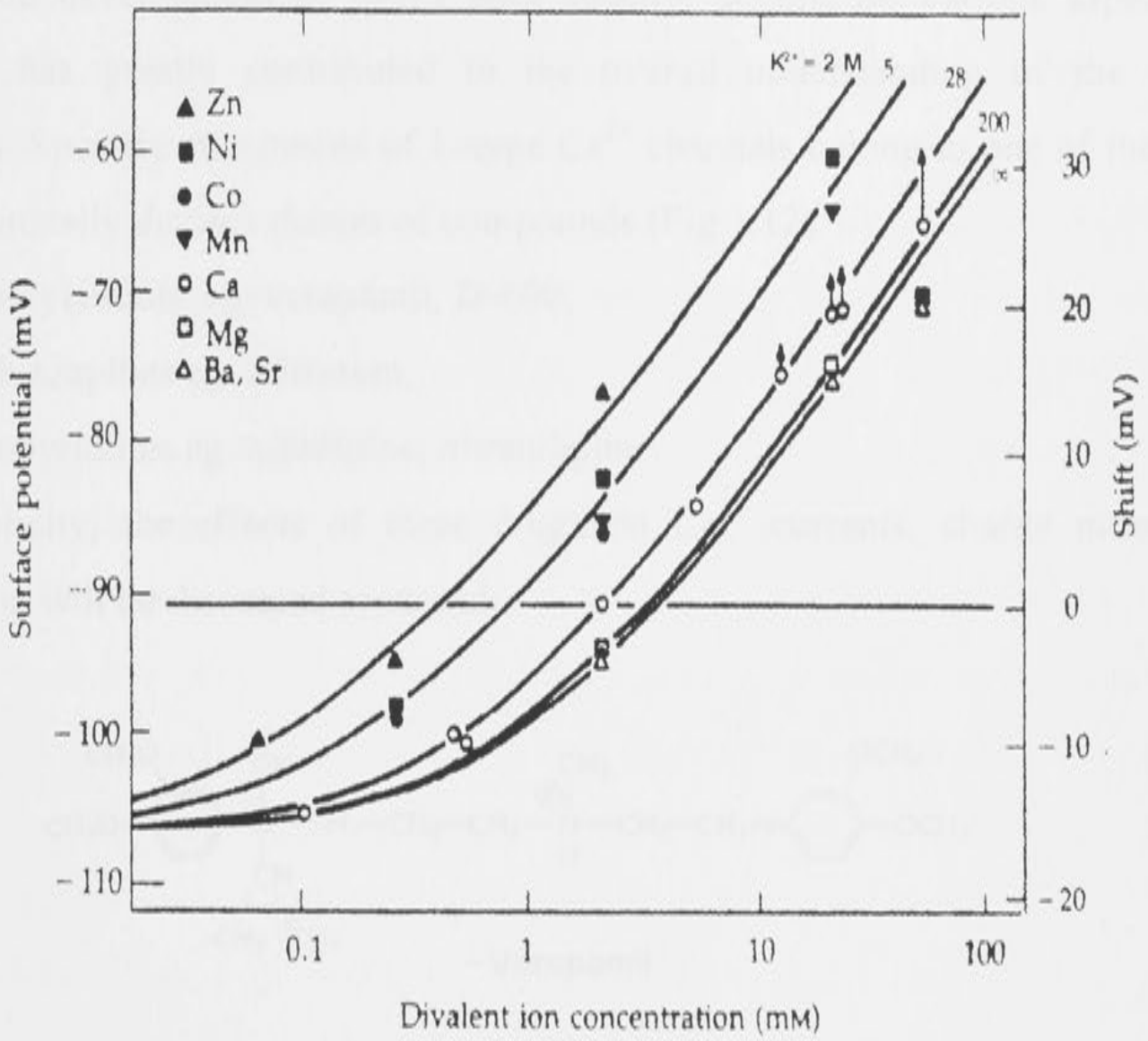


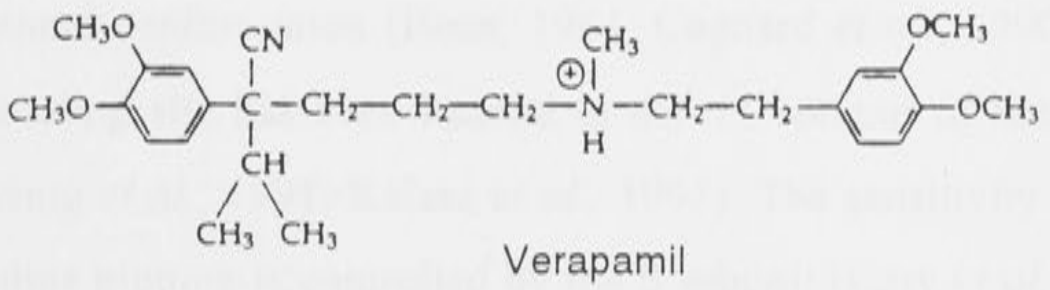
Figure 1.11 *Consequences of charge screening and binding*
Shifts of Na^+ channel activation in frog nodes of ranvier produced by different cations. The right hand scale shows the shift in Na^+ channel activation (mV). The bottom scale shows divalent cation concentration. The curves and left hand scale are surface potentials calculated using the Gouy-Chapman-Stern model of surface potentials which makes assumptions about the surface density, pK_a's, and divalent ion dissociation constants (K^{2+}) for fixed charges within a Debye length of the voltage sensor for activation gating. The curve $K^{2+} = \infty$ corresponds to no binding of divalent ions. In this case, the change in surface potential and the shift in Na^+ channel gating, as the divalent cation concentration increases are due to a combination of charge screening and binding. The horizontal line marks the control value for surface potential obtained with 2 mM Ca^{2+} only. Note also the different efficacy of various cations in causing the shift in Na^+ channel activation which can be explained by the different dissociation constants (K^{2+}) associated with each of the cations. (Modified from Hille *et al.*, 1975 and Hille, 1992).

1.8.5 *Drugs that modulate E-C coupling*

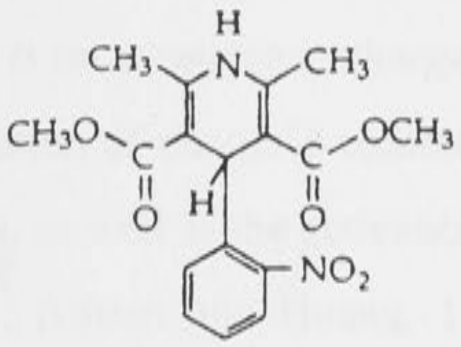
The development of drugs with specific actions on various aspects of E-C coupling has greatly contributed to the overall understanding of the underlying processes. Specific antagonists of L-type Ca^{2+} channels belong to one of the following three chemically distinct classes of compounds (Fig.1.12):

1. phenyalkylamines eg. verapamil, D-600;
2. benzothiazapines eg. diltiazem;
3. dihydropyridines eg. nifedipine, nitrendipine.

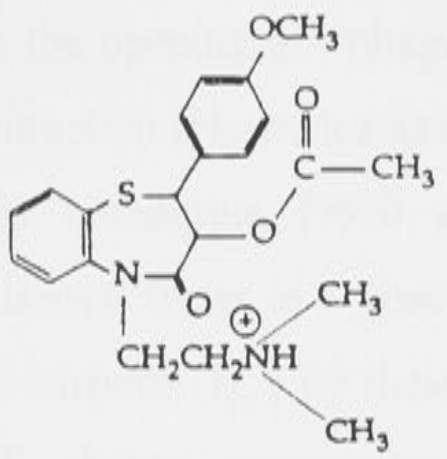
For simplicity, the effects of these drugs on Ca^{2+} currents, charge movement and contraction will be discussed separately.



Verapamil



Nifedipine



Diltiazem

Figure 1.12 *Examples of some organic L-type Ca^{2+} channel antagonists*
Molecular structures of representatives from 3 chemically distinct classes of organic L-type Ca^{2+} channel antagonists: Nifedipine (a dihydropyridine), Verapamil (a phenylalkylamine) and Diltiazem (a benzothiazepine). (Modified from Hille,1992).

1.8.5A *Effect on Ca^{2+} currents*

Ca^{2+} channel blocking drugs have been instrumental in determining the role of inward Ca^{2+} currents during E-C coupling. Slow, voltage-dependent, inward Ca^{2+} currents (a signature of L-type Ca^{2+} channels) are effectively blocked by nifedipine at concentrations of 1-10 μM in both amphibian and mammalian skeletal muscle (McCleskey, 1985; Ildefonse *et al.*, 1985; Avila-Sakar *et al.*, 1986; Rios *et al.*, 1986; Agnew, 1987; Lamb, 1986b; Luttgau *et al.*, 1987; Lamb and Walsh, 1987; Cognard *et al.*, 1990; Neuhaus *et al.*, 1990), and 100 μM D600 (Sanchez & Stefani, 1978; Almers *et al.*, 1981; Gonzalez-Serratos *et al.*, 1982; McCleskey, 1985; Avila-Sakar *et al.*, 1986). Often, as in the case of nifedipine, the block is voltage dependent, being more effective when the fibre is depolarised. This has been attributed to the drug binding to an inactive channel conformation (Bean, 1984; Cognard *et al.*, 1990). The specific Ca^{2+} antagonist binding site has been located in the α1 subunit of the DHPR (Kim *et al.*, 1990; Striessnig *et al.*, 1991; Kalasz *et al.*, 1993). The sensitivity of the α1 subunit to dihydropyridine binding is controlled by the β subunit (Lory *et al.*, 1992, Woscholoski and Marme, 1992).

1.8.5B *Effects on asymmetric charge movement*

Activation of charge 1 coincides with the opening of voltage dependent L-type Ca^{2+} channels, as well as the activation of contraction (Horowicz and Schneider, 1981*b*; Huang, 1981; Adrian and Huang, 1984*a*&*b*). Nifedipine, D600, and the anaesthetic tetracaine, suppress charge 1 in normally polarised fibres in a dose dependent manner which is similar to the block of inward Ca^{2+} currents (I_{ca}) by these drugs. Nifedipine and D600 also inhibit the recovery of charge movement following charge immobilisation by prolonged depolarisation (Hui *et al.*, 1984; Feldmeyer *et al.*, 1990). Similar actions of nifedipine on charge 1 are seen in frog and mammals (Lamb, 1986b). In contrast to charge 1, nifedipine increases charge 2 in frog skeletal muscle (Rios *et al.*, 1986) but, along with D600, has no effect in mammalian muscle (Lamb, 1987).

1.8.5C *Effects on contraction*

Reports on the effect of Ca^{2+} antagonists on E-C coupling are varied and often conflicting. It is generally thought that Ca^{2+} antagonists exert their action by altering the voltage dependence of activation/inactivation after binding to specific receptors on the voltage sensor. Studies on asymmetric charge movement support this idea. D600 causes a reversible paralysis of contraction in frog skeletal muscle following contractile inactivation by sustained depolarisation (Eisenberg *et al.*, 1983; Gottschalk & Luttgau, 1985, Caputo and Bolanos, 1987). Similar effects on contraction have been reported for the anaesthetic procaine (Heistracher and Hunt, 1969; Luttgau and Oetliker, 1968) and tetracaine (Caputo and Bolanos, 1987). Reports on the action of nifedipine vary. Nifedipine and verapamil (10^{-4} M) potentiate twitches in amphibian and mammalian muscle (Griffiths & Taylor 1982; Dulhunty and Gage, 1988) which is thought to be the result of increased action potential duration (Griffiths and Taylor, 1982). Nifedipine (10^{-6}-10^{-5} M) also blocks K contractures in frog skeletal muscle (Avila-Sakar, 1986), rat soleus (Dulhunty and Gage 1988), and contractions in voltage clamped frog fibres (Ildefonse *et al.*, 1985) and rat myoballs (Cognard *et al.*, 1990). Other reports show no effect of nifedipine (10^{-6} -10^{-5} M) on contraction (Lamb, 1986*b*; Luttgau *et al.*, 1987; Rakowski *et al.*, 1987). The paralysing action of nifedipine and D600 are more pronounced in depolarised fibres (Bean, 1984, Rios and Brum, 1987) suggesting that the drugs bind to the resting state of the voltage sensor with low affinity and to the active and inactive states with high affinity (Neuhaus *et al.*, 1990). The effect of nifedipine is modulated by Ca^{2+}: twitches are potentiated by nifedipine in the presence of 2.5 mM Ca^{2+} and depressed by nifedipine in low Ca^{2+} solutions (Dulhunty and Gage, 1988; Neuhaus *et al.*, 1990). The fact that nifedipine only depresses contraction under certain conditions may account for the discrepencies between investigators. Skeletal muscle paralysis could occur if Ca^{2+} antagonists prevent the binding of Ca^{2+} to the voltage sensor (Dulhunty & Gage, 1988; Neuhaus *et al.*, 1990). The shift of the voltage sensor from an active to an inactivated state is thought to require the release of Ca^{2+} (ie the opposite of repriming). Nifedipine may accelerate inactivation and shift the voltage dependence of inactivation towards more negative potentials by displacing Ca^{2+} from the voltage sensor (Neuhaus *et al.*, 1990).

1.9 Possible mechanisms of E-C coupling

As previously discussed, the mechanism by which the voltage sensor in the T-tubule membrane is coupled to the release of Ca^{2+} from the SR in skeletal muscle is not understood. Numerous mechanisms of T-tubule -SR coupling have been proposed and are briefly discussed below .

1.9.1 *Direct electrical continuity mechanism*

Mathais *et al.*, (1981) proposed a mechanism whereby ionic currents are propagated electrically across the triadic junction, initiating Ca^{2+} release by depolarisation of the SR. This mechanism was not popular for the following reasons:

(a) there was no evidence of a continuous membrane linking the T-tubule to the SR membranes (Gilly, 1981)

(b) Cl^- distributions both at rest and during SR Ca^{2+} release fail to support a potential change across the SR membrane during contraction (Somlyo *et al.*, 1981).

(c) the area of the membrane in the SR would increase membrane conductance and capacitance in a manner that is not observed (Somlyo *et al.*, 1977; Martonosi, 1984; Oetliker, 1982; Ashley *et al.*, 1991).

The discovery that the SR Ca^{2+} release channel and Cl^- channels are voltage dependent (Smith *et al.*, 1985, 1986; Stein and Palade, 1988, Kourie *et al.*, 1996) has led to a renewed interest in the possibility that SR membrane potential changes during contractile activation.

1.9.2 *Ca^{2+} induced Ca^{2+} release (CICR)*

The triggering of Ca^{2+} release from the SR by an influx of external Ca^{2+} ions through voltage dependent Ca^{2+} channels in the T-Tubule (CICR) is the basis of E-C coupling in cardiac but not skeletal muscle (Luttgau & Stephenson, 1986). Ashley *et al.* (1991) report evidence to support such a mechanism in skeletal muscle including:

(a) CICR occurs in both skinned skeletal and cardiac muscle (Endo *et al.*, 1970, Endo,1977; Fabiato, 1983, 1985).

(b) skeletal SR Ca^{2+} release channels incorporated into artificial lipid bilayers show an increase in channel open probability when the Ca^{2+} concentration is increased on the cytoplasmic side of the bilayer (Lai *et al.*, 1988; Smith *et al.*, 1988; Ma *et al.*, 1988; Fill *et al.*, 1990).

(c) during long depolarisations (1-2 sec) a secondary slow component of contraction appears with a time course similar to the slow inward Ca^{2+} current (Ildefonse *et al.*, 1985). However, this wouldn't contribute to twitches which reach maximal tension before the activation of the slow inward Ca^{2+} current (Ashley *et al.*, 1991), but could contribute to tetanic and K contracture tension.

There is evidence against CICR as the primary activation process in E-C coupling. Firstly, the fact that E-C coupling in skeletal muscle occurs in "Ca^{2+} free" solutions (see Section 1.8.1), argues against such a mechanism. Secondly, Baylor and Hollingworth (1988) injected the Ca^{2+} indicator and chelator, Fura-2 into the myoplasm of frog skeletal muscle and found that SR Ca^{2+} release in response to T-tubule depolarisation, wasn't depressed. Theoretically, if CICR was a part of E-C coupling, then Fura-2 should mop up the Ca^{2+} entering via the T-tubule and prevent opening of the SR Ca^{2+} release channel.

Recent studies have renewed interests in CICR as a possible mechanism for E-C coupling in skeletal muscle. Studies in which caged Ca^{2+} was released into one side of isolated Rabbit skeletal RyR incorporated into lipid bilayers revealed that RyR activation by this released Ca^{2+} could allow CICR to be involved in normal E-C coupling (Gyorke *et al.*, 1994). However, this is probably not likely since Gyorke's experiments were conducted at sub myoplasmic $[Mg^{2+}]$ and yet there is evidence to show that at normal myoplasmic $[Mg^{2+}]$ CICR is inhibited in skeletal muscle *

1.9.3 *E-C coupling mediated by a second messenger system*

The second messenger, inositol 1,4,5 -trisphospate (IP_3) has been suggested to be the initiator of E-C coupling (Sevematsu *et al.*, 1984; Vergara *et al.*, 1985). IP_3 causes Ca^{2+} release from the SR of skinned fibres (Volpe *et al.*, 1985, 1986; Donaldson *et al.*, 1987; Rojas and Jaimovich, 1990) and activates the amphibian SR Ca^{2+} release channel when incorporated into lipid bilayers (Suarez-Isla *et al.*, 1988, 1991; Liu, *et al.*, 1989). It is now generally accepted that IP_3 plays a modulatory role in skeletal muscle E-C coupling (Dulhunty, 1992), since:

* (Endo, 1985; Lamb and Stephenson, 1991).

(a) the time course of contractures due to IP_3-induced Ca^{2+} release is too slow to be physiologically important (Somlyo *et al.*, 1988)

(b) the degradation of IP_3 by enzymes would be too slow to account for the speed at which Ca^{2+} release from the SR is terminated during a twitch (Walker, 1987)

(c) IP_3 sensitive Ca^{2+} channels in skeletal muscle SR are different from the RyR (Dulhunty, 1992).

1.9.4 *Mechanical coupling*

A mechanical coupling of the voltage sensor to the Ca^{2+} release channel of the SR is the most widely accepted mechanism for E-C coupling in skeletal muscle. Chandler *et al.*, (1976) proposed that upon T-tubule depolarisation, the voltage sensor undergoes a conformational change which gates Ca^{2+} release through a direct interraction with the Ca^{2+} release mechanism. A direct link between the DHPR and RyR is supported by the following:

(a) the RyR is modulated by the 2-3 loop of DHPR α1 subunit (Tanabe *et al.*, 1988; Adams *et al.*, 1990; Lu et al, 1995).

(b) electron microscopy reveals that DHPR's are arranged in tetrads (Block *et al.*, 1988) which align with every alternate RyR (Block *et al.*, 1988, Bers and Stiffel, 1993; Margreth *et al.*, 1993). It is proposed that T-tubule depolarisation initiates the opening of RyR's immediately opposite tetrads via mechanical coupling and that the Ca^{2+} released opens the remaining RyR via CICR (Takekura *et al.*, 1994);

(c) in cardiac muscle where E-C coupling occurs by CICR, DHPR's do not form tetrads (Sun *et al.*, 1995).

1.9.5 *Mechanical coupling via intermediate proteins*

The voltage sensor may be coupled to the RyR via intermediate proteins (see review by Rios and Pizarro, 1991). Low molecular weight proteins which bind to both DHPR and RyR have been isolated from the triadic junction (Brandt *et al.*, 1990, Kim *et al.*, 1990*b*). The three dimensional structure of the RyR, elucidated by Wagenknecht *et al.*, (1989), shows the presence of a large cytoplasmic region which could act as the "plunger" that gates Ca^{2+} release in Chandler's 1976 model. Etter

(1990) proposed that arginine residues in the S4 segment of the DHPR could move such plungers, but experimental evidence to demonstrate such a mechanism is scarce. A major flaw in this type of mechanism applying to activation of all RyR's is the presence of extrajunctional RyR's (Dulhunty *et al.*, 1992*a*) and that every second junctional RyR is not aligned with a tetrad (Block *et al.*, 1988). It is unlikely extrajunctional RyR's are controlled by a direct coupling with the DHPR because of the distance between the two proteins. It is possible that there are two separate gating mechanisms for the junctional and extrajunctional Ca^{2+} release channels.

1.9.6 *E-C coupling mediated by the removal of Mg^{2+} inhibition on the RyR*

High (10 mM) Mg^{2+} inhibits depolarisation-induced Ca^{2+} release from the SR in amphibian and mammalian skinned fibres. Lowering external magnesium from 1mM (normal myoplasmic concentration) to 15 μM, causes a massive efflux of Ca^{2+} from the SR. These results are explained by a direct modulatory action of magnesium on the RyR. It is proposed that during E-C coupling, T-tubule voltage sensors cause SR Ca^{2+} release by reducing the affinity of the RyR for magnesium (Lamb and Stephenson, 1991, 1992).

1.10 The effects of Cd^{2+} on E-C coupling in mammalian skeletal muscle

Dulhunty and Gage (1989) investigated the effects of various cations on E-C coupling in rat soleus fibres. In particular, fibres bathed in solutions containing 3 mM Cd^{2+} exhibited some interesting effects which included:

1. a rapid potentiation of twitches followed by depression of both twitches and tetanic contractions;
2. the development of a slow tension increase shortly following Cd^{2+} addition called the "Cd^{2+}-induced contracture";
3. appearance of a rapid transient contracture upon Cd^{2+} washout called the "Cd^{2+}-withdrawal contracture".

In this thesis, the effects of Cd^{2+} on E-C coupling, twitches and tetani are further characterised and the mechanisms of the Cd^{2+} -induced and -withdrawal contractures are investigated.

Chapter 2

General Methods

2.1 General information

2.1.1 *Animals*

Male Wistar rats (*Rattus norvegicus*) weighing between 200-400 gms were used in all experiments. Animals were housed at the animal facility (JCSMR) where they were cared for according to animal ethics guidelines. At the facility all animals were kept in a constant temperature, stress free environment with unlimited access to commercial rat chow and clean water. Bedding material was changed regularly and the number of animals per cage was minimised to prevent overcrowding. All animals used in experiments appeared to be healthy.

2.1.2 *Ethical considerations*

All experiments using animals were performed in accordance with the guidelines setout in the original project proposal passed by the ANU animal ethics committee.

2.1.3 *Solution preparation*

Solutions were made using analytical grade reagents and water that had been passed through a Millipore water purification system incorporating reverse osmosis and ion exchange. Solutions used for whole fibre experiments were generally prepared from stocks on the day of experimentation. Reagent stocks were stored in glass bottles and refrigerated at 4°C. Skinned fibre solutions were prepared in advance and stored in plastic vials at -80 °C until use. Plastic containers were used to avoid possible contamination by the leaching of Ca^{2+} from glass. The pH of all solutions was measured using a T.P.S digital pH meter (model # 1852 A).

2.1.4 *Statistics*

All data are given as the average value ± 1 SEM. Where applicable, a significance of difference between two values was tested using an independent students T-test. Differences were considered to be significant if $P \leq 0.05$. An independent T-test analysis was for the following reasons:

1. control data was often obtained from different preparations than test data. This was necessary since preparations rarely recovered fully from prolonged (30 min or more) exposure to 3 mM Cd^{2+} and it was possible that some residual Cd^{2+} might still be bound to the preparation after Cd^{2+} washout;
2. the number of control experiments often exceeded the number of test experiments.

Wherever possible, both control and test experiments were performed on the same day with preparations dissected from the same animal.

2.2 Whole fibre studies

(Chapters 3, 4 and 5).

2.2.1 *Solution composition*

The solutions used in the whole fibre experiments were the same as those used in a previous study by Dulhunty and Gage (1989) and were of two different types:

1. Krebs solution containing Cl^- as the principal anion;
2. a low Cl^- solution containing SO_4^{2-} as the principal anion.

The majority of the whole fibre experiments were performed using the Krebs solution. The low Cl^- solution was used specifically for K contracture experiments because it permits a rapid change in membrane potential when the extracellular $[K^+]$ is increased (Hodgkin and Horowicz, 1960; Dulhunty and Gage, 1985). The composition of the Cl^- and SO_4^{2-} solutions are given in Tables 2.1 and 2.2, respectively. In the Cl^- Krebs solution it was assumed that all ions were fully dissociated so that the added [ion] was equivalent to the free [ion]. SO_4^{2-} salts do not dissociate as readily in solution and so it is important to note that the free concentrations of cations in these solutions are expected to be somewhat lower than the added concentration. The approximate free concentrations of Ca^{2+}, Mg^{2+} and Cd^{2+} in each of the SO_4^{2-} solutions listed in Table 2.2 are given in Appendix 1.

code	type	NaCl	$MgCl_2$	KCl	$CaCl_2$	$CdCl_2$	$CoCl_2$	$LaCl_3$	$ZnCl_2$
1A	**control**	150	2	2	2.5	0	0	0	0
B	**5Mg**	150	5	2	2.5	0	0	0	0
C	**3Cd**	150	2	2	2.5	3	0	0	0
D	**3Zn**	150	2	2	2.5	0	0	0	3
E	**3Co**	150	2	2	2.5	0	3	0	0
F	**3La**	150	2	2	2.5	0	0	3	0
G	**2Co**	150	2	2	2.5	0	2	0	0
H	**2Co/3Cd**	150	2	2	2.5	3	2	0	0
I	**0Mg**	150	0	2	2.5	0	0	0	0
J	**0Mg/3Cd**	150	0	2	2.5	3	0	0	0
K	**10Mg**	150	10	2	2.5	0	0	0	0
L	**10Mg/3Cd**	150	10	2	2.5	3	0	0	0
M	**30Mg**	150	30	2	2.5	0	0	0	0
N	**30Mg/3Cd**	150	30	2	2.5	3	0	0	0
O	**0Ca**	150	4.5	2	0	0	0	0	0
P	**0Ca/3Cd**	150	1.5	2	0	3	0	0	0
Q	**10Ca**	150	2	2	10	0	0	0	0
R	**10Ca/3Cd**	150	2	2	10	3	0	0	0
S	**30Ca**	150	2	2	30	0	0	0	0
T	**30Ca/3Cd**	150	2	2	30	3	0	0	0
U	**0.5Cd**	150	2	2	2.5	0.5	0	0	0
V	**1Cd**	150	2	2	2.5	1	0	0	0
W	**4Cd**	150	2	2	2.5	4	0	0	0
X	**6Cd**	150	2	2	2.5	6	0	0	0
Y	**10Cd**	150	2	2	2.5	10	0	0	0

NB: *All concentrations shown are total concentrations (in mM)
*Each solution also contains: 11 mM glucose and 2 mM TES, pH 7.4.

Table 2.1 *Composition of commonly used Cl^--containing solutions*

code	type	NaCl	KCL	Na_2SO_4	$MgSO_4$	K_2SO_4	$CaSO_4$	sucrose	$CdCl_2$
2A	**control**	16	0	32.25	1	1.75	7.6	170	0
B	**3Cd**	16	0	32.25	1	1.75	7.6	170	3
C	**30K**	0	16	85	1	7	7.6	0	0
D	**30K/3Cd**	0	16	85	1	7	7.6	0	3
E	**40K**	0	16	80	1	12	7.6	0	0
F	**40K/3Cd**	0	16	80	1	12	7.6	0	3
G	**60K**	0	16	70	1	22	7.6	0	0
H	**60K/3Cd**	0	16	70	1	22	7.6	0	3
I	**80K**	0	16	60	1	32	7.6	0	0
J	**80K/3Cd**	0	16	60	1	32	7.6	0	3
K	**120K**	0	16	40	1	52	7.6	0	0
L	**120K/3Cd**	0	16	40	1	52	7.6	0	3
M	**200K**	0	16	0	1	92	7.6	0	0
N	**200K/3Cd**	0	16	0	1	92	7.6	0	3

NB: *All concentrations shown are total concentrations (in mM)
*Each solution also contains: 11 mM glucose and 2 mM TES, pH 7.4

Table 2.2 *Composition of commonly used SO_4^{2-} -containing solutions*

NB: All elevated K^+ solutions had a high ionic strength equivalent to that of the 200K solution to avoid errors that may have been incurred by varying ionic strength with each high K^+ solution. This was unavoidable if sufficiently high K^+ to give maximum activation was to be used.

2.2.2 *Macrodissection*

Animals were killed quickly by CO_2 overdose. Skin and fur overlying the muscle of interest was removed. Muscles were quickly excised and pinned out into a shallow petri dish lined with Sylgard 184 (Dow Corning). Excised muscles were bathed in either control Krebs or SO_4^{2-} solution depending on the experiment that followed. Generally, one muscle was used whilst the other was refrigerated. Muscles stored thus, remained viable for several hours.

Two types of muscle, both originating from the rat hindlimb were used. These were the soleus and extensor digitorum longus (EDL) which are composed primarily of slow and fast twitch fibres respectively (Fryer *et al.*, 1988). The bulk of the experiments were performed on soleus fibres since the preparations were less subject to rundown and this was the muscle used in the original study by Dulhunty and Gage (1989). EDL fibres were used occasionally for comparative purposes.

2.2.2A *Soleus*

Freshly killed animals were pinned onto a corkboard with their ventral side down. Skin and fur overlying the gastrocnemious/soleus muscles was carefully removed using a pair of curved dissecting scissors (Teufel #1), exposing the gastrocnemious muscle and calcineal (achilles) tendon. The calcineal tendon was severed and the free end grasped using a pair of rat tooth forceps (Teufel #7). The free end of the calcineal tendon was gently pulled away from the limb and the connective tissue joining the muscle to the limb was cut both medially and laterally towards the knee. The tendon of origin which connects the superior end of the soleus to the posterior end of the fibula was severed and the soleus was freed from the gastrocnemius muscle by grasping the tendon of origin and cutting the connective tissue between the two muscles.

2.2.2B *EDL*

The distal tendon of the tibialis anterior was severed after first cutting through another smaller tendon which runs transversely across its surface. The external connective tissue overlying the tibialis anterior was then cut away to allow removal of

the muscle. The exposed EDL was removed by severing the proximal tendon which attaches the EDL muscle to the femur. The free end of the proximal tendon was then grasped between forceps and the EDL was gradually freed from the limb by gently pulling the tendon towards the foot whilst cutting the connective tissue at the same time. Finally, the muscle was freed from the foot by severing the distal tendons.

2.2.3 *Microdissection*

Two types of whole fibre preparations were used depending on the nature of the experiments to be performed.

2.2.3A *Preparations used for contracture studies*

Small bundles (10-15 fibres) were used for all contracture experiments. The objective was to obtain a preparation that was small enough to allow all fibres adequate exposure to the surrounding solution whilst minimising the percentage of fibres damaged during the dissection process. Ideally single fibres would have been used for these experiments but this is difficult due to the tight association between fibres in both soleus and EDL muscles. Soleus or EDL muscles dissected according to the method outlined in Section 2.5 were pinned out into a petri dish lined with Sylgard 184 (Dow Corning) and, depending on the experiment, bathed in control Krebs or SO_4^{2-} solution. Muscles were freed of any superficial connective tissue using forceps (Dumont #5) and 8 cm curved blade dissecting scissors (Teufel). A small portion of the tendon of origin with muscle fibres attached was freed from the main preparation by cutting the connective tissue between the fibres along the longitudinal axis of the muscle towards the distal tendon. Care was taken to grasp the preparation by connective tissue so that fibre damage was kept to a minimum. Superficial fibres were then trimmed away from the freed preparation until a bundle containing only 10-20 intact fibres remained.

2.2.3B *Preparations used for V_m and action potential measurements*

The dissection procedure is essentially the same as for the contracture preparations except that the end product was a flat sheet of fibres 1-2 fibres thick ×

30 -50 fibres wide. The thinner preparations allow a more even exposure of fibres to the surrounding solution, thus preventing problems arising from diffusion delays. In addition, thin preparations permit the transmission of light from a lamp positioned below the recording chamber, which allows single fibres to be seen for impalement with microelectrodes.

2.2.4 *Twitches, tetani and potassium contractures*

2.2.4A *Mounting of preparations*

A drawing of the setup used for the recording of twitches, tetani and potassium (K) contractures is shown in Fig. 2.1. Small bundles of fibres dissected by the method outlined in Section 2.2.3A, were mounted in a 2 ml rapid flow (1.2 mls/sec) perspex bath: a small stainless steel hook fashioned from a dissecting pin was inserted through the tendon at one end of the preparation and was attached to a semiconductor force transducer (Akers, model AE875, SensoNor a.s.Horten, Norway) positioned directly above the bath. The other end of the preparation was clamped between a pair of forceps which were mounted perpendicular to the bath. Both the forceps and transducer were attached to micromanipulators (Narashige, Japan), which were used to lower the preparation into the bath and to adjust the preparation resting tension. The temperature of the bath was maintained at 22.5 ± 1.0 °C by a circulating water jacket connected to a thermostatically controlled heater. The temperature of the solutions entering the bath was controlled by passing them through a chamber containing heated water which was connected in series to the water jacket surrounding the bath. Room and bath temperature could be monitored at all times by thermistor probes. Solution flow into the bath was controlled by an electronic switching solenoid (built at the ANU) which allowed a rapid flow rate of 1.2 mls/sec (giving a bath volume changeover time* of 1.5 sec). Excess solution was removed at the transducer end of the bath by an electric vacuum pump (Neuberger, miniport). Two massive platinum electrodes were positioned on either side of the preparation, along the length of the bath, for production of synchronous twitches and tetanic contractions.

* ~98% replacement, originally measured using dye dilution techniques (personal communication with Dr Dulhunty).

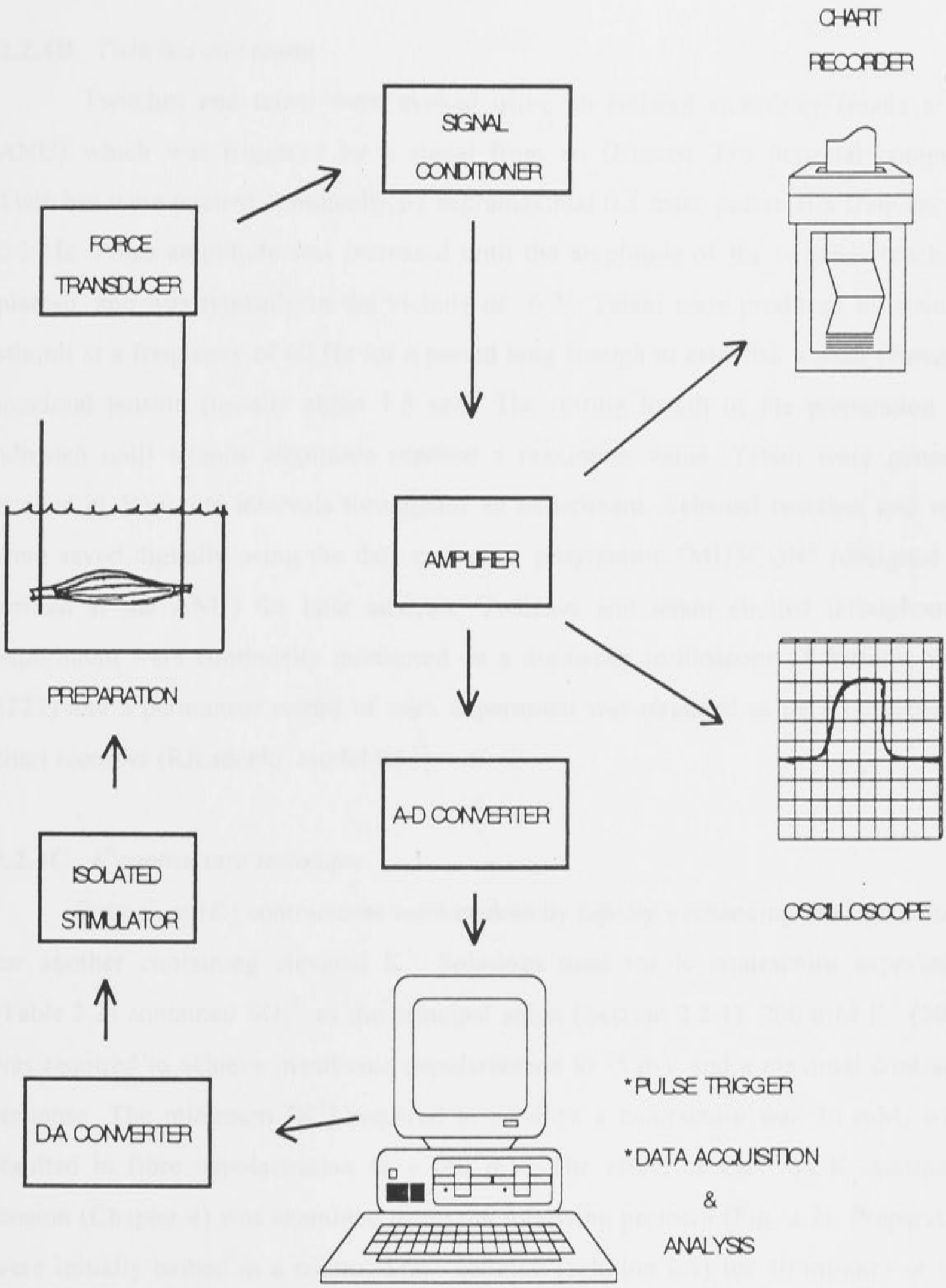


Figure 2.1 *Essential components of the setup used for whole fibre contracture studies*

2.2.4B *Twitches and tetani*

Twitches and tetani were evoked using an isolated stimulator (made at the ANU) which was triggered by a signal from an Osborne 386 personal computer. Twitches were elicited continually by supramaximal 0.5 msec pulses at a frequency of 0.1 Hz. Pulse amplitude was increased until the amplitude of the twitches reached a plateau, and was typically in the vicinity of 6 V. Tetani were produced by trains of stimuli at a frequency of 60 Hz for a period long enough to establish a clear plateau of maximal tension (usually about 1.5 sec). The resting length of the preparation was adjusted until tetanus amplitude reached a maximum value. Tetani were generally evoked at 5 minute intervals throughout an experiment. Selected twitches and tetani were saved digitally using the data collection programme "MUSCON" (designed and written at the ANU) for later analysis. Twitches and tetani elicited throughout an experiment were continually monitored on a digitising oscilloscope (Tektronix model 5223) and a permanent record of each experiment was obtained using a multichannel chart recorder (Rikadenki, model R61).

2.2.4C *K contracture technique*

Potassium (K) contractures were evoked by rapidly exchanging the bath solution for another containing elevated K^+. Solutions used for K contracture experiments (Table 2.2) contained SO_4^{2-} as the principal anion (Section 2.2.1). 200 mM K^+ (200K) was required to achieve membrane depolarisation to -5 mV and a maximal contractile response. The minimum [K^+] required to produce a contracture was 30 mM, which resulted in fibre depolarisation to ~ -40 mV. The effect of Cd^{2+} on K contracture tension (Chapter 4) was examined using the following protocol (Fig. 2.2). Preparations were initially bathed in a control SO_4^{2-} solution (solution 2A) for 10 minutes or until successive tetani were of approximately the same amplitude (± 5 %). A maximally activating 200K contracture (*200K1*) was then evoked by rapidly replacing the control bathing solution with another containing 200 mM K^+ (solution M). Once the tension had returned to baseline, the high potassium solution was replaced with the control solution and the preparation was left until the tetanic tension recovered. The process of twitch and tetanic tension recovery after the K contracture follows "repriming" of the

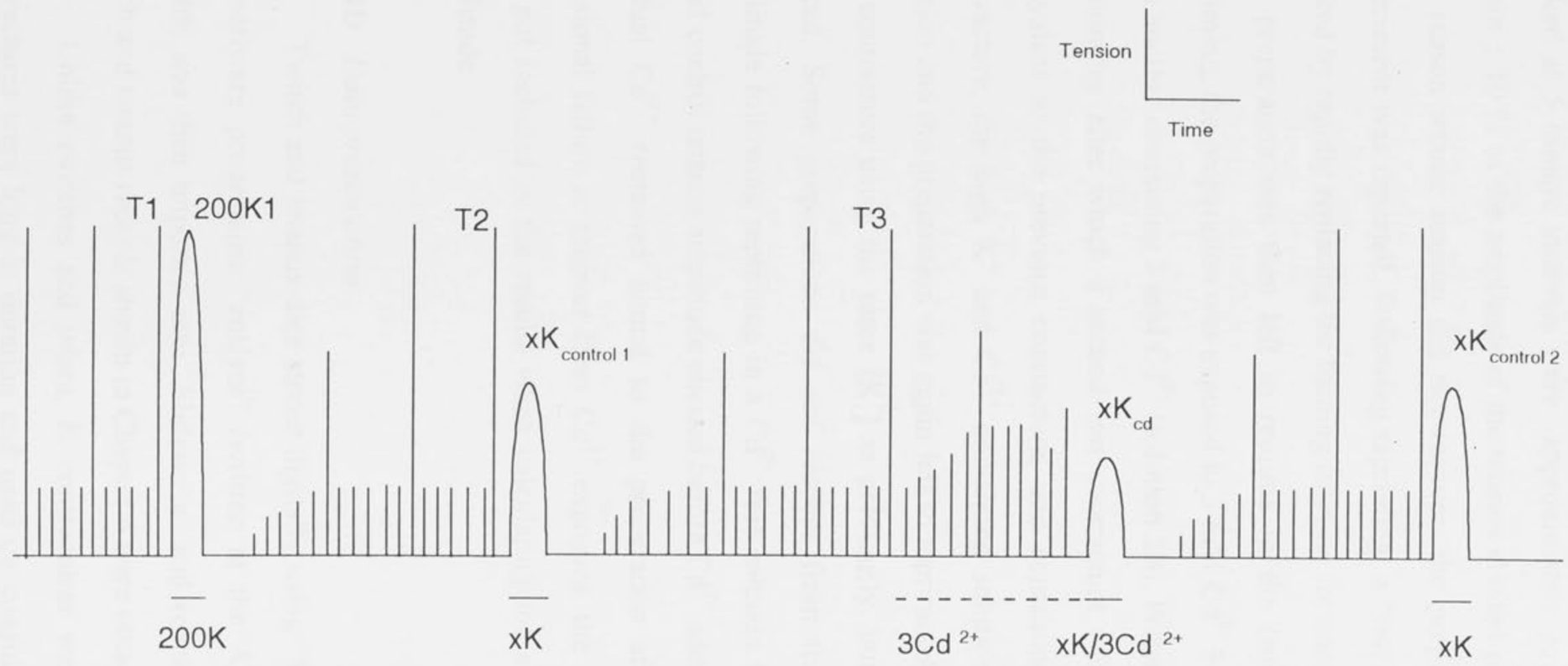


Figure 2.2

A simulated experiment illustrating the protocol used to examine the effect of 3 mM Cd^{2+} on K contracture tension in bundles of rat soleus fibres. The vertical scale is tension and the horizontal scale is time. The large vertical lines show tetani and the smaller lines are twitches. The large rounded deflections show K contractures. The simulated preparation is bathed in the control SO_4^{2-} solution (solution 1A, Table 2.2) between exposure to high K^+ and Cd^{2+} solutions which are indicated below the figure as solid or dotted lines respectively.

voltage sensor. Repriming was considered to be complete when successive tetani evoked at 5 minute intervals were approximately equal in amplitude (± 5 %), and within ± 10 % of the amplitude of the tetanus elicited prior to the K contracture. If for any reason tetanic tension did not recover, the preparation was discarded and the experiment was repeated. Following repriming, a "test" K contracture ($xK_{control\ 1}$) was evoked by rapidly replacing the bathing solution for another containing 30-120 mM K^+. The preparation was then left to reprime in the control solution as before. After repriming, the preparation was exposed to 3 mM Cd^{2+} by replacing the control solution with another containing 3 mM Cd^{2+} (solution 2B). Preparations were bathed in Cd^{2+} for 10 minutes after which a second test contracture (xK_{Cd}) was evoked using a [K^+] equivalent to the previous contracture, and containing 3 mM Cd^{2+}. Following the contracture, the high K^+ and Cd^{2+} containing solution was replaced by the control solution and the preparation was again left to reprime. After repriming, another control test contracture using the same [K^+] as previously, but without Cd^{2+} ($xK_{control\ 2}$) was evoked. Some preparations did not recover from the Cd^{2+} treatment and tetanus amplitude following repriming in a Cd^{2+} free solution was often only 40-50 % of the initial control tetanus amplitude elicited before Cd^{2+} addition. It was possible that some residual Cd^{2+} remained bound to the preparation after washout. Because of this occasional failure to recover from Cd^{2+} exposure, the second control test contracture was not included in the results when calculating the average control test contracture amplitude.

2.2.4D *Data presentation*

Twitch and tetanus data stored digitally using "MUSCON" was analysed using the software programme "analyse" (written at the ANU). The data from selected records was then imported into "Slidewrite" and redrawn as a graph. All individual twitch and tetanus records shown in Chapter 4 were obtained in this way.

Unlike twitches and tetani, K contractures were not stored digitally as the contractures were long in duration and used up considerable disk space. However, a chart record of each experiment was obtained, and all K contracture records shown throughout this report are reproductions of the chart records.

2.2.4E *Construction of force activation curves*

K contracture data obtained using the protocol shown in Fig. 2.2 was analysed in the following way:

1. the initial control maximal 200K contracture (200K1) amplitude was normalised to the control tetanus (T1) (by dividing 200K1 tension by T1), to obtain a tension ratio "R" (this ratio was typically between 0.9 and 1.2);
2. the amplitude of the control test contracture $xK_{control}1$ was normalised to the control tetanus elicited immediately before the contracture (T2) to obtain the ratio "R2", (this value depends on the $[K^+]$ in the contracture solution);
3. the amplitude of the test contracture evoked in 3 mM Cd^{2+}, (xK_{Cd}) was normalised to the control tetanus elicited just prior to the addition of Cd^{2+} to the bath, "T3" to yield the ratio "R3" (NB: the K contracture evoked in Cd^{2+} was not normalised to the immediate preceding tetanus because Cd^{2+} also affects tetanus amplitude);
4. finally, normalised control and Cd^{2+} test K contracture amplitudes were normalised to the maximal 200K response by dividing R2 or R3 by R1 respectively.

This process was repeated for each $[K^+]$ in several preparations. The average normalised control and Cd^{2+} K contracture tension obtained at each test $[K^+]$ was then plotted against the membrane potential measured in each K^+ solution, using the software graphics program "slidewrite plus" (version 3). Curves were fitted to each set of data using a Boltzmann equation of the form (Dulhunty and Gage, 1983).

$$T_a = T_{max} / [1+EXP((V_a-V_m) / k_a)]$$

Where;

T_a is the normalised K contracture amplitude at membrane potential V_m,

V_a is the potential at which $T_a = 0.5\ T_{max}$

k_a is the slope factor.

A representative contractile activation curve was shown in Chapter 1 (Fig. 1.8). Values of T_a, V_a, and k_a for each curve were obtained from the best fit of the equation to the data using a least squares analysis.

2.2.5 *Measurement of the Cd^{2+} -induced and -withdrawal contractures*

2.2.5A *Contracture protocol*

The Cd^{2+} -induced and -withdrawal contractures produced within the same preparations are examined separately in Chapters 5 and 3, respectively. For simplicity, the following protocols were used to produce and measure these contractures. The type of preparations used for Cd^{2+} -induced and -withdrawal contractures were identical to those used in K contracture experiments (Section 2.2.3A). Preparations were initially bathed in Krebs solution (solution 1A, Table 2.2). Once preparations were stable (ie the amplitude of consecutive tetani differed by no more than ± 5 %), the $[Mg^{2+}]$ of the bathing solution was increased to from 2 mM to 5 mM by replacing the control solution with another containing 5 mM Mg^{2+} (solution 1B). This was done so that when 3 mM Cd^{2+} was later added to the bath by replacing the elevated Mg^{2+} , the observed effects of Cd^{2+} could not be attributed to an increase in divalent cation concentration. After 10 minutes in the elevated Mg^{2+} solution, Cd^{2+} was introduced by replacing the 5 mM Mg^{2+} solution with another containing 3 mM Cd^{2+}/2 mM Mg^{2+} (solution 1C). Shortly (4-5 minutes) after Cd^{2+} addition, a slow increase in tension, "the Cd^{2+} -induced contracture" developed. If the Cd^{2+} -containing solution was washed out after 5 minutes or more (by replacing the Cd^{2+} solution with the 5 mM Mg^{2+} solution) a transient "Cd-withdrawal contracture" was produced.

2.2.5B *Measurement of contracture amplitude and duration*

The CIC was measured as the difference in tension between the baseline immediately preceding Cd^{2+} addition to the level after a designated time in Cd^{2+}. Since the CIC did not decay, the duration of this contracture was not measured. The CWC was measured as the difference in tension between the baseline immediately preceding Cd^{2+} withdrawal and the peak tension of the contracture. The duration of the CWC on the other hand was measured as the difference in time between the instant of Cd^{2+} withdrawal and the time at which the decay of withdrawal contracture tension reached a plateau.

2.2.5C *Data presentation*

Both the Cd^{2+}-induced and -withdrawal contracture amplitudes were normalised to the control tetanus elicited immediately prior to Cd^{2+} addition. Since the duration of both contractures was long, often lasting for many minutes, the data was not saved digitally. A chart recording of each contracture was obtained and the records shown throughout this report are reproductions of the chart records. So that a direct visual comparison could be made between records obtained from different preparations, the following criteria were used in selecting which records would be used in the figures:

1. records were chosen from preparations with control tetani of approximately equal (± 10 %) amplitude;
2. records were chosen from preparations with contracture amplitudes that most closely resembled the average response obtained for the experimental group.

Occasionally, in a series of experiments, there were no records with control tetani of equal amplitude. When this occurred, records were either enlarged or reduced using a photocopier to normalise the tetanus height. Since this changed both time and amplitude scales, appropriately adjusted scalebars were provided alongside each of the altered records. Where necessary, faint records were reinforced in ink to allow the contractures to be seen more clearly, the record was not modified by this process.

For clarity, the CWC, effects of Cd^{2+} on twitches, tetani and K contractures, and the CIC are described separately throughout this report since many experimental manipulations had differential effects on these parameters and different mechanisms are described for each. However, for most experimental manipulations, data for CWC, twitch, tetanus and CIC were obtained from the same preparation.

2.2.6 ***Recording of V_m and action potentials***

A drawing of the apparatus used for V_m and action potential recording is shown in Fig. 2.3. Small flat sheets of fibres, dissected as outlined in Section 2.2.3B, were pinned out into a 3 ml perspex bath lined with Sylgard 184 (Dow Corning). The preparations were stretched to approximately 1.6 × the normal resting length to reduce contraction and displacement of electrodes during action potential recording (stretching the preparations reduced the interaction between the actin and myosin filaments). A

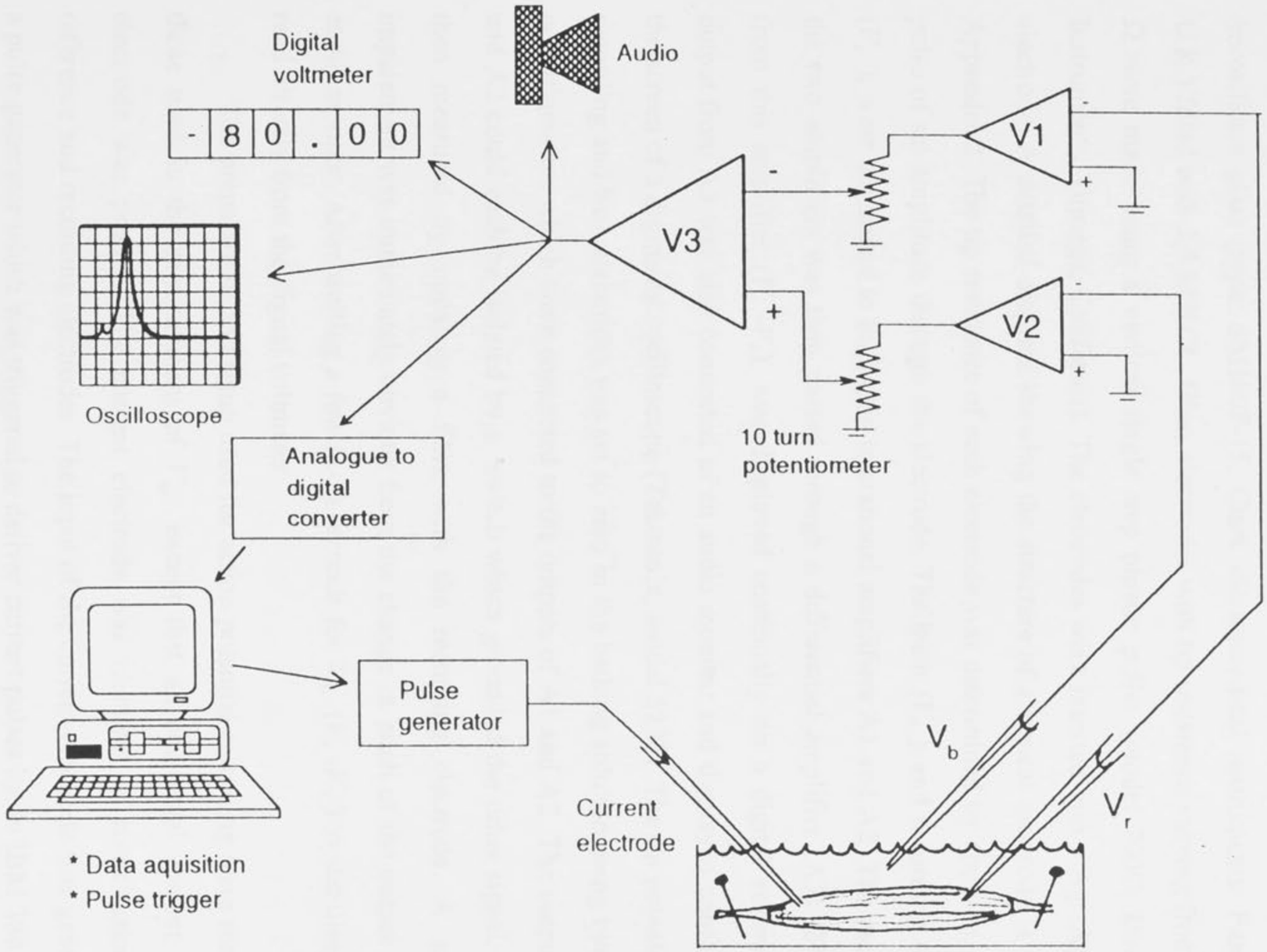


Figure 2.3 *Essential components of the setup used to record membrane and action potentials*

piece of cut silicone tubing was often placed below the preparation to produce extra tension and a stable support for fibre impalement. All electrodes were made from borosilicate glass (type: SM100F-15, Clark electromedical instruments, Pangbourne, U.K.) filled with 2.5 M KCL. Glass electrodes with tip resistance varying from 2-10 M Ω were made using a vertical single step pipette puller (model 700C, David Kopf Instruments, Tujunga, California). The electrodes were inserted into a $Ag^+/AgCl$ wire electrode. A detailed diagram showing the structure of a typical electrode is shown in Appendix 2. The tip resistance of each electrode was determined by applying a current pulse of set amplitude through the electrode. The bath (V_b), and recording electrodes (V_r), were connected to high gain operational amplifiers A1 and A2. The output from the two amplifiers was then passed through a differential amplifier (A3). The output from this amplifier (V_r -V_b), was displayed continually on a digital voltmeter. The output from A3 was also connected to an audio monitor and displayed continually on the screen of a digitising oscilloscope (Tektronix, model 5223). The tip potential of the recording and bath electrodes was set to zero in the bathing solution using two 10 turn potentiometers which were connected to the outputs of A1 and A2. The outputs of A1 and A2 could each be isolated by a switch which grounded the other signal. V_m was then measured by impaling a fibre with the recording electrode. A successful impalement was immediately obvious from the change in pitch of the output from the audio monitor. After waiting a few (2-3) seconds for V_m (V_r -V_b) to stabilise, V_m was read directly from the digital voltmeter.

The preparations and setup used for action potential recording were the same as those used in the measurement of V_m except that an additional current injecting electrode was present. The current electrode was identical in construction to the reference and recording electrodes. The input of the current electrode was generated by a pulse generator which was triggered to deliver current pulses by an IBM 386 personal computer. Small (5-10 mV) hyperpolarising pulses at a frequency of 1 HZ were used initially. A successful impalement with the current electrode was indicated by the appearance of hyperpolarising steps in the V_m. Fibres were abandoned if V_m decreased to potentials more positive than -75 mV after impalement with the current electrode. The distance between the current and recording electrodes was ~ 0.5 - 1 mm. Action

potentials were elicited by single depolarising pulses of 0.5 msec duration. The pulse amplitude was gradually increased until an action potential was generated. Action potential data was collected and stored digitally using "MUSCON". Data was analysed and selected records were displayed in the same way as twitch and tetanus data (Section 2.2.4D).

2.3 Skinned fibre studies

(Chapter 6)

All techniques and solutions used in the skinned fibre experiments were derived from a previous study by Stephenson and Theileczek, (1986).

2.3.1 *Skinning technique*

Soleus muscles (removed by the method outlined in Section 2.2.2A) were blotted dry on a piece of filter paper (Whatman #1) and then pinned out into a Petri dish lined with Sylgard and containing cold paraffin oil. The paraffin provides an inert environment which facilitates resealing of T-tubules following mechanical removal of the sarcolemma (Lamb and Stephenson, 1990*a*). All microdissection was performed under a dissecting microscope (10-40 × magnification). A small bundle of fibres was gently teased away from the main preparation (cutting was kept to a minimum). The free end of the bundle was grasped evenly between two pairs of forceps and was carefully teased into two smaller bundles of approximately equal size. This process was repeated until a single fibre remained. The sarcolemma was then mechanically removed from the fibre in the following way:

1. the free end of the fibre was grasped between a pair of forceps and held outstretched from the main preparation;
2. the free end was then pinned against the Sylgard using a pair of forceps;
3. the edge of the free end pined against the Sylgard was grasped between a second pair of forceps and pulled gently away from the fibre back towards the main preparation.

If successful, a small cuff became visible (Fig. 2.4A) which slowly enlarged with progressive sarcolemmal removal as it travelled up the fibre,. Typically, the skinning process should not result in more than a 10 -20 % reduction in fibre volume. Sometimes fibres were split and skinned at the same time (Fig. 2.4B). Split preparations are not ideal for use in experiments where contraction is activated by T-tubule depolarisation, caffeine or low Mg^{2+} since splitting fibres is thought to damage the resealed T-tubule and SR structure (Lamb and Stephenson, 1990a). Fortunately, in this study, intact SR or T-tubules were not required since elevated Ca^{2+} or Cd^{2+} solutions were used to activate the contractile apparatus directly and hence split preparations could be used. Furthermore, in accordance to the methods in Stephenson and Thieleczek (1986), skinned fibres were routinely split into small myofibrillar bundles of 30-40 μM diameter to prevent ATP depletion at the centre of the fibre during contractile activation. This was necessary since creatine kinase (which regenerates ATP) could not be added to the Cd^{2+} containing solutions because it forms a precipitate with Cd^{2+}.

2.3.2 *Mounting of preparations*

A photograph showing the major components of the apparatus used to record contractures in skinned fibres in shown in Fig. 2.5. Skinned rat soleus fibres were mounted as follows: the Petri dish containing the skinned fibre was placed on top of a perspex solution rack which contained numerous (10 or more) 2 ml perspex baths filled with various solutions. The solution rack was fixed to the top of a laboratory jack which allowed the solutions to be elevated or lowered. The preparation was elevated so that the end of a force transducer mounted directly above the solution rack was immersed in the paraffin oil bathing the preparation. The free end of the skinned fibre was tied by a single knot into the middle of a 1 cm piece of braided silk suture (#7-0, Cynamid, Australia), under the dissecting microscope set on 10 × magnification. The fibre was then tied, using a double knot, to a stainless steel pin which was fixed to the force transducer (with shellac). The steel pin was used to increase the sensitivity of the transducer. The end of the fibre still attached to the preparation was then clamped between a pair of stationary forceps mounted above the solution rack. Both the forceps

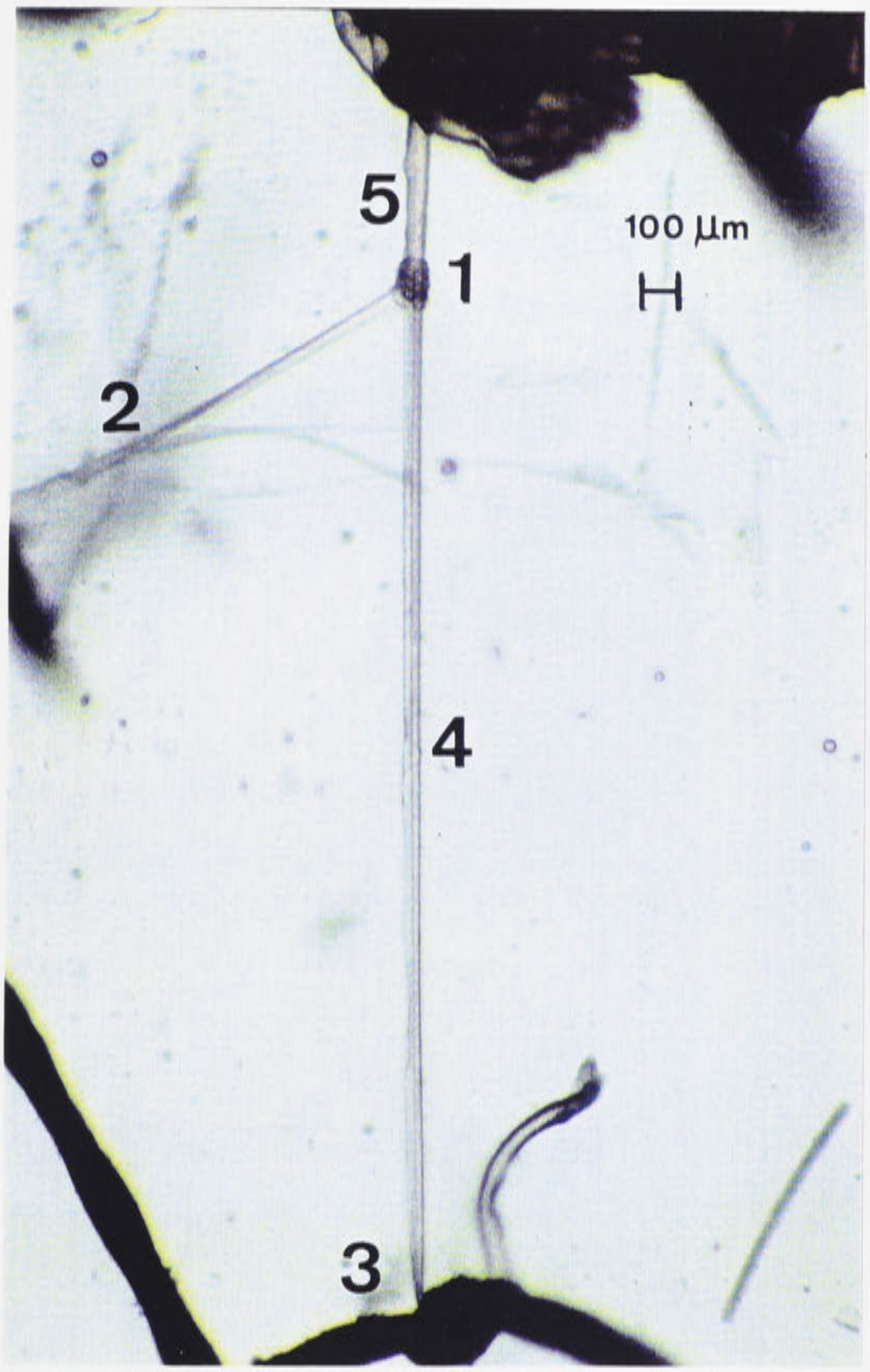


Figure 2.4A *A typical skinned fibre*

A photograph of a single skinned rat soleus fibre immersed in paraffin oil, seen under a dissecting microscope (45 × magnification). The distance indicated by the scale bar (top right corner) is approximately 100µM. The following features are numbered:

1. sarcolemmal "cuff"
2. sarcolemma (peeled away)
3. loop of silk suture securing free end of skinned fibre
4. skinned region of the fibre
5. unskinned region of the fibre

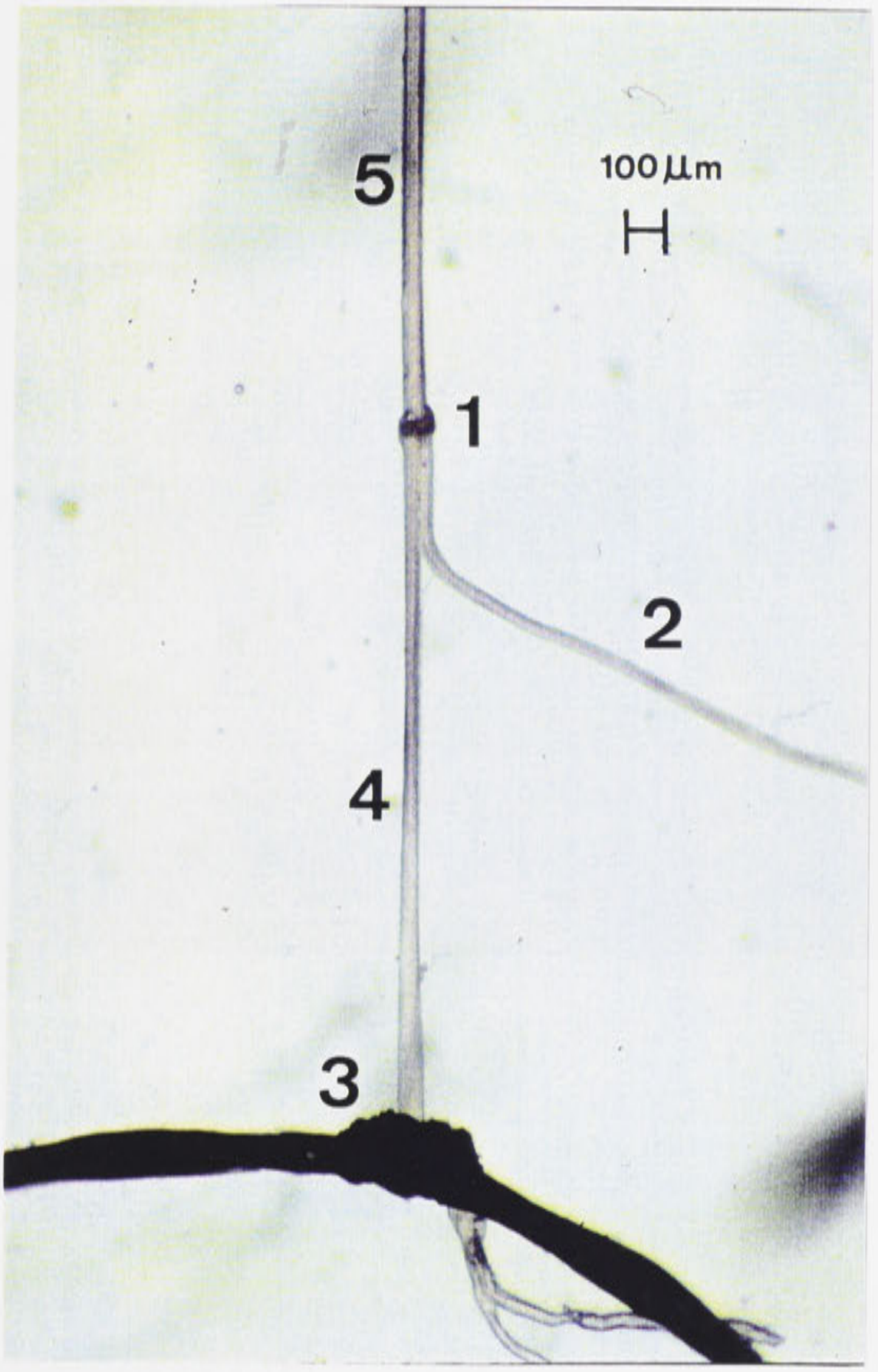


Figure 2.4B *An example of a skinned and split fibre*

A photograph showing a single skinned and split rat soleus fibre immersed in paraffin oil, seen under a dissecting microscope (45 × magnification). The distance indicated by the scale bar is approximately 100 µM. The following features are numbered:

1. sarcolemmal "cuff"
2. half of the skinned and split portion of the fibre
3. end of one half of the skinned and split fibre secured by a loop of silk suture
4. other half of skinned and split portion of the fibre
5. unskinned region of the fibre

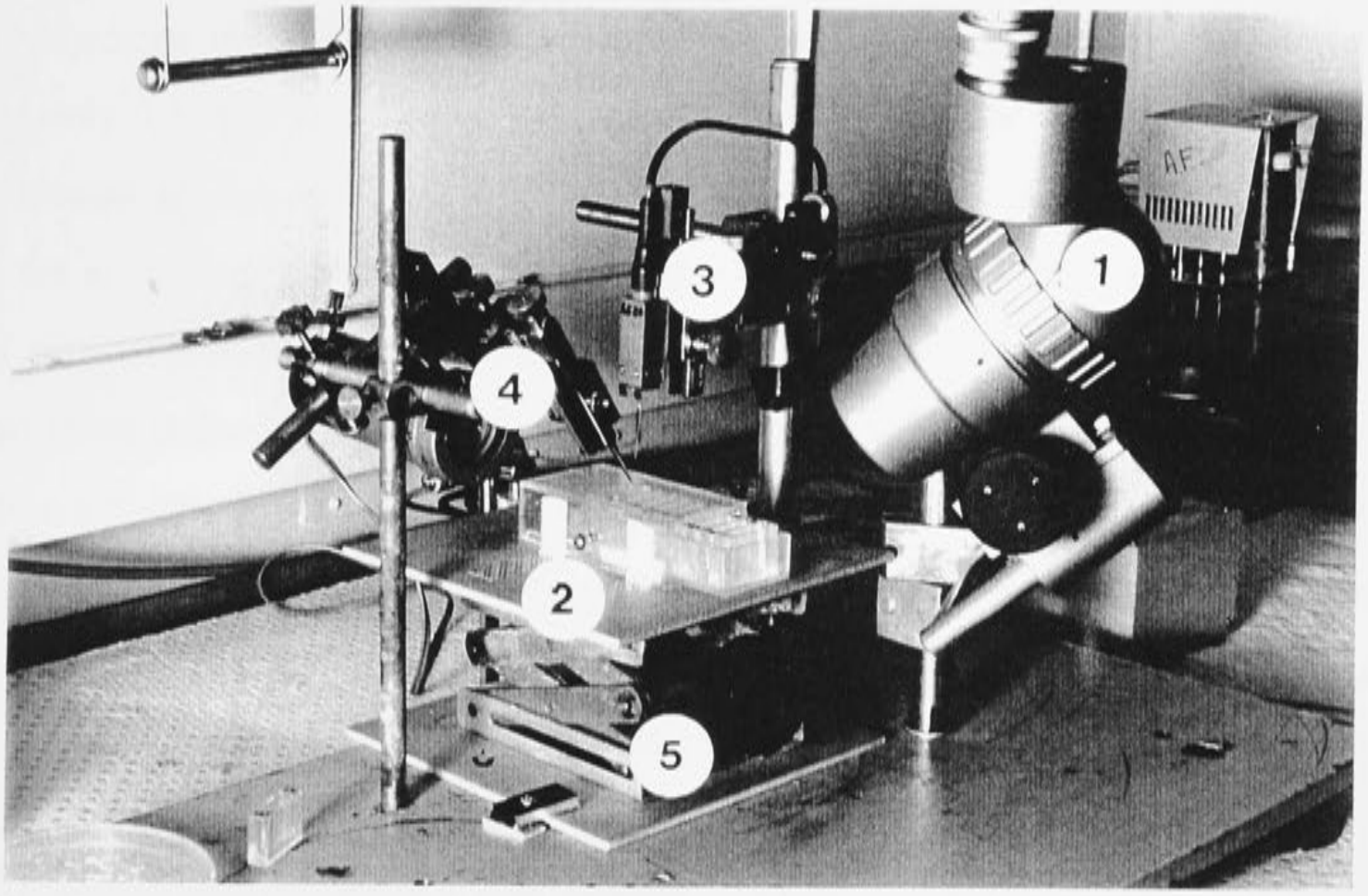


Figure 2.5

A photograph showing the apparatus used to record isometric tension in skinned fibres. The following features are numbered:

1. dissecting microscope (10 - 40 ×) used for the mounting of preparations
2. sliding perspex solution rack containing 10 individual 2 ml perspex baths
3. force transducer (attached to micromanipulators)
4. forceps (also attached to micromanipulators) for securing the non-transducer end of the fibre
5. laboratory jack for moving solutions up to and away from the skinned fibre

and force transducer were attached to micromanipulators (Narashige, Japan) which allowed them to be positioned correctly for attachment of the skinned fibre and were used for adjusting the fibre tension. Fibres were normally clamped with the forceps approximately 3-5 mm away from the end attached to the force transducer. A bigger solution change artefact was seen when preparations longer than 4 mm were transferred through the air -solution interface during solution changeover. This artefact arises as a result of surface tension-induced drag on the fibre. As a result, the longer preparations were also more prone to breakage. The mounted fibre was then cut free from the main preparation and the cut suture ends were trimmed as close to the fibre as possible. Fibre length was increased until all of the fibre slack was taken up but no force was produced. This was taken as the resting length of the fibre. The fibre diameter and resting length were then measured using an eyepiece micrometer. The fibre was then stretched a further 20 percent of the resting length which theoretically corresponds to a sarcomere length of approximately 3.1-3.2 μM (Owen, 1996)

The solution rack holding the petri dish containing the remainder of the preparation was then lowered and the petri dish was removed. The remaining skinned fibre (attached to the force transducer and forceps) was placed in the first bathing solution by elevating the solution rack until the fibre was immersed. Subsequent solution changes were made by rapidly lowering the solution rack, advancing the next solution cartridge into position and elevating the solution rack to reimmerse the fibre. Solution changeover was done as quickly as possible to prevent the fibre from drying out.

2.3.3 *Solution composition and preparation*

Two types of solutions were used in the skinned fibre experiments. Solution S which was the principal resting solution and solution B which contained 50 mM EGTA and was used to relax fibres following contraction in Ca^{2+} or Cd^{2+}. The composition of these two solutions is shown in Table 2.3. Ca^{2+} or Cd^{2+} activation solutions were made by adding appropriate amounts of $CaCl_2$ or $CdCl_2$ to solution S and the free cation concentration of each solution is shown as mM or as $-\log_{10}$[cation] ($\approx$ "pcation").

Solution	EGTA (mM)	K_2Succinate (mM)	MgO (mM)	MgCl2 (mM)	Hepes (mM)	NaN_3 (mM)	ATP (mM)	CP (mM)	caffeine (mM)
High relaxing (B)	50	0	10.3	0	90	1	8	10	0
Resting (S)	0	50	0	8.6	90	1	8	10	10

NB: *Each solution was adjusted to pH 7.4 ± 0.1 using 4 M KOH

*All concentrations shown are total concentrations (in mM)

Table 2.3 Composition of standard skinned fibre solutions used

A problem with the skinned fibre technique is that only 0.5 μM Ca^{2+} is required to activate the contractile proteins, a concentration easily reached by contaminating Ca^{2+} from various salt components. Normally, this problem is overcome by adding Ca^{2+} chelating agents such as EGTA. Unfortunately, EGTA is not suitable for use in Cd^{2+}-containing solutions since EGTA has a much higher affinity for Cd^{2+} than Ca^{2+}. See additional note below* To overcome this problem, Stephenson and Thieleczek, (1986) used succinate as a Ca^{2+} buffer. Since this compound neither buffers Ca^{2+} nor Cd^{2+} very strongly, contaminating Ca^{2+} was also removed by passing solutions through CHELEX -100 (BIO-RAD), a cation exchange resin (Blinks *et al.*, 1978), (see Appendix 3 for further details). Because CHELEX also binds Cd^{2+} and Mg^{2+}, these cations were added as Cl^- salts to the solution following the CHELEX treatment. As a result, some contaminating Ca^{2+} was present in the added $CdCl_2$ or $MgCl_2$. The concentration of contaminating Ca^{2+} was determined by Stephenson and Thieleczek (1986) using atomic absorption spectrophotometry to be less than 2.3×10^{-7} M for solutions containing a free $[Cd^{2+}]$ smaller than 3.2×10^{-5} M, which is below the contraction threshold. No contraction was observed in solution S alone in the present study indicating that the contaminating $[Ca^{2+}]$ was also sub threshold.

Since Ca^{2+}, Mg^{2+} and Cd^{2+} are all buffered by ATP and CP and succinate with varying affinities (Table 2.4), the computer software programme "BUFFA" (kindly provided by Dr Laver, this laboratory) was used to determine the relative amounts of these cations required in order to reach the desired free cation concentrations. A list of the added and resultant free concentrations of these cations in both the Ca^{2+} and Cd^{2+} activation solutions calculated using "BUFFA" is provided in Appendix 4. The added $[Mg^{2+}]$ was increased as required in the Cd^{2+} -containing solutions in order to maintain a constant [Mg-ATP] since the formation of Cd-ATP inhibits the contractile apparatus (Stephenson and Thieleczek, 1986).

2.3.4 *Contracture protocol*

Activation of the contractile proteins by Ca^{2+} and Cd^{2+} was tested using the following protocol (Fig. 2.6). Rat soleus fibres were freshly skinned in paraffin oil and

* HDTA, the primary anion used in skinned fibre solutions cannot be used in Cd^{2+} containing solutions because Cd^{2+} and HDTA form an insoluble complex.

Ligand	Ca^{2+} *K^{app} (M^{-1})	Mg^{2+} *K^{app} (M^{-1})	Cd^{2+} *K^{app} (M^{-1})
EGTA	$(5 \pm 0.5) \times 10^6$	46 ± 6	$(3.1 \pm 0.5) \times 10^{11}$
Succinate	16	16	124
ATP	$(3.4 \pm 0.3) \times 10^3$	$(6.5 \pm 0.5) \times 10^3$	$(4 \pm 0.5) \times 10^4$
CP	≤ 20	12 ± 1.5	50 ± 7

*Values obtained from Stephenson and Thieleczek (1986) for the following experimental conditions: total Na^+, 36 mM; total K^+, 100-200 mM; pH 7.10; temp, 23.0°C.

Table 2.4 Affinity constants used to calculate the free concentrations of Ca^{2+}, Mg^{2+} and Cd^{2+} in the skinned fibre solutions.

NB: The free concentrations of Ca^{2+}, Mg^{2+} and Cd^{2+} calculated using the above affinity constants may vary slightly from the true values since the skinned fibre solutions were adjusted to pH 7.4 not 7.1.

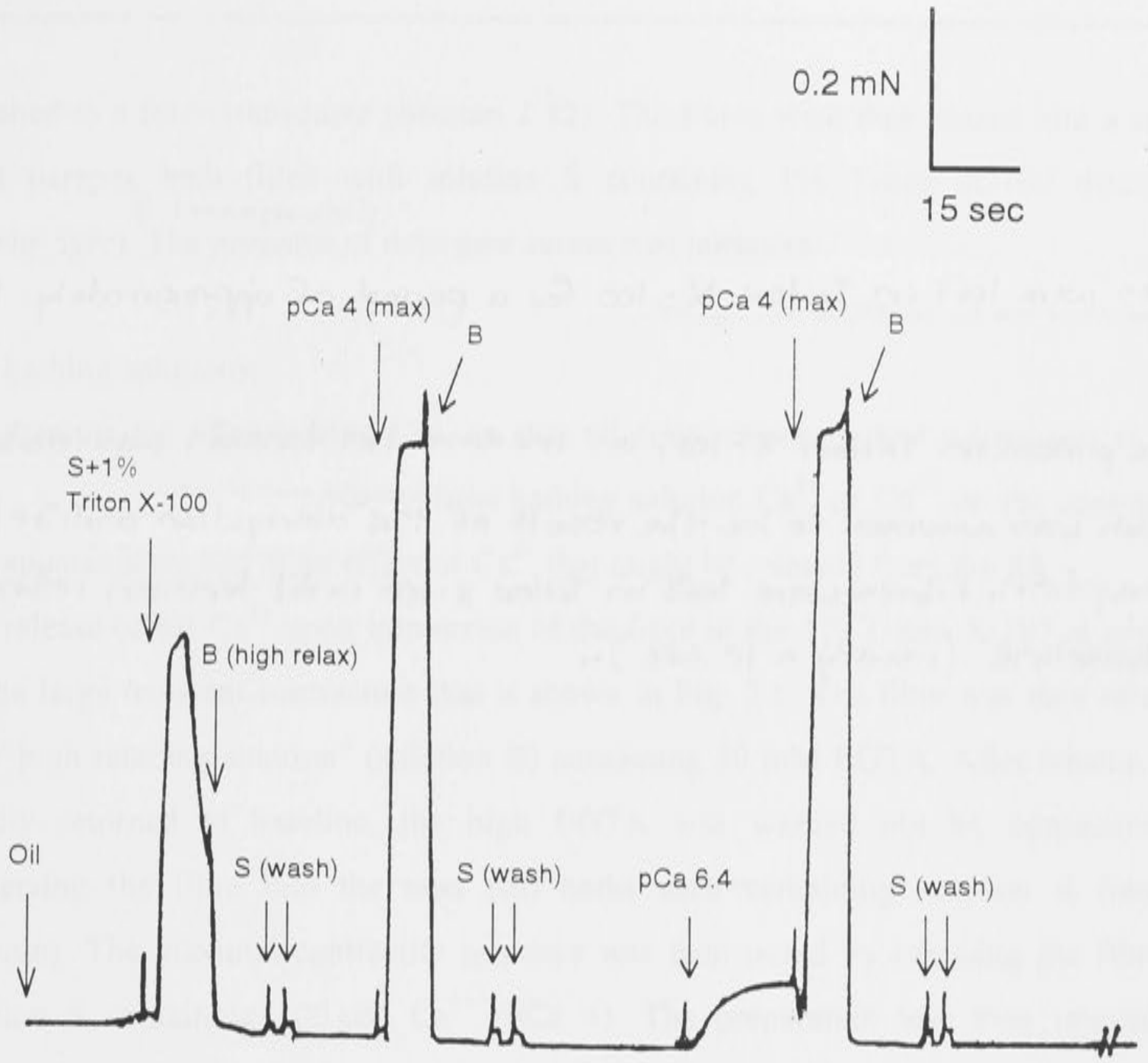


Figure 2.6 *Skinned fibre experiment protocol*

An example of the experimental protocol used to measure the response of the contractile proteins in a skinned fibre to elevated Ca^{2+} or Cd^{2+} solutions. Fibres were exposed to solutions containing a range of different Ca^{2+} or Cd^{2+} concentrations. One such response (to a solution of pCa 6.4) is shown above. Each response was then followed by exposure of the fibre to a solution containing 100μM Ca^{2+} (pCa 4) which induced maximal tension.

* Once placed in TRiton X-100, an increase in tension was observed which was assumed to be the result of Ca^{2+} released by SR disruption. Fibres were left in TritoN X-100 until tension returned to baseline (usually ~ 10 sec)..

attached to a force transducer (Section 2.12). The fibres were then placed into a small 2 ml perspex bath filled with solution S containing 1% Triton X-100 detergent (Boehringer). The presence of detergent serves two purposes:

1. removes the paraffin oil coating the fibre allowing even exposure of the fibre to the bathing solutions;
2. disrupts the SR releasing Ca^{2+} so that all contractures evoked subsequent to this treatment are a direct effect of the bathing solution Ca^{2+} or Cd^{2+} on the contractile apparatus instead of an effect of Ca^{2+} that might be released from the SR.

The release of SR Ca^{2+} upon immersion of the fibre in the 1% Triton X-100 is evident by the large transient contracture that is shown in Fig. 2.6. The fibre was then relaxed in a "high relaxing solution" (solution B) containing 50 mM EGTA. After tension had rapidly returned to baseline, the high EGTA was washed out by consecutively immersing the fibre into the next two baths each containing solution S (resting solution). The maximal contractile response was then tested by exposing the fibre to solution S containing 100 μM Ca^{2+} (pCa 4). The preparation was then relaxed in solution B and EGTA was removed by 2 consecutive washes in solution S as previously described. It is important to remove all of the EGTA so that no buffering occurs when the fibre is exposed to the Ca^{2+} or Cd^{2+} activating solutions. The fibre was then placed into the first of the Ca^{2+} or the Cd^{2+} activating solutions (Solution S + $CaCl_2$ or $CdCl_2$). Once a clear tension plateau was reached a maximal contractile response was evoked by placing the fibre into the maximum activating (pCa 4) solution. Thus, the contractile response in each Ca^{2+} or Cd^{2+} solution can be compared directly with the maximum tension response of the fibre. If the maximum tension was smaller than the first maximal response (<10%) the preparation was abandoned. Following relaxation of the fibre in the high relaxing solution and EGTA washout in solution S as before, the fibre was then exposed to the second Ca^{2+} or Cd^{2+} activating solution and the entire process was repeated until all of the activating solutions had been tested.

2.3.5 *Data presentation*

Contracture records presented throughout Chapter 6 are reproductions of the chart traces. Contractile tension produced by exposure of fibres to Ca^{2+} or Cd^{2+} -

activation solutions was normalised against maximal response to 100 μM Ca^{2+}. A contractile activation curve was then constructed by plotting the normalised tension averaged from 8 preparations against pCa or pCd using the software graphics program "Slidewrite plus" version 3. A sigmoidal curve was fitted to the data using the curve fit function in "slidewrite plus". The generalised form of the equation to the sigmoidal curve which is essentially the same as the equation used in Section 2.2.4E to construct voltage activation curves is as follows:

$$T_x = T_{max} / [1 + EXP (-\{p_x - p_{50\%}\} / k_x]$$

Where:

T_x = tension at Ca^{2+} or Cd^{2+} concentration p_x ($p = -\log_{10} [Ca^{2+}$ or $Cd^{2+}]$;

T_{max} = maximum tension ;

$p_{50\%}$ = pCa^{2+} or pCd^{2+} producing 50 % of T_{max};

k_x = slope factor ~ (slope $^{-1}$).

Varying values of T_{max} , $p_{50\%}$, and k_x were inserted into the above equation until the best fit of the data with the curve was obtained.

Chapter 3

The Cd^{2+}-withdrawal contracture

3.1 Introduction

As described briefly in Chapter 1, Dulhunty and Gage (1989) reported a transient contracture upon washout of 3 mM Cd^{2+} in rat soleus muscle. This "Cd^{2+}-withdrawal contracture" (CWC) has not been described elsewhere. The mechanism of the CWC is not known. There are many reports in the literature describing the effects of divalent cations on both excitable and non-excitable cells. The responses are varied and depend on:

1. the cation species;
2. [cation];
3. the target tissue;
4. whether the cation is being added or removed from the solution.

In general, divalent cations modulate skeletal muscle contraction. Metal cations in particular are thought to do this by interacting with sites on the surface membrane (Andersson and Edman 1974*a*&*b*; Parry *et al.*, 1974; Dorrscheidt-Kafer and Groki, 1978, Dorrscheidt-Kafer, 1979*a*&*b*), or by binding to the voltage sensor for E-C coupling (Luttgau *et al.*, 1987; Brum *et al.*, 1988; Dulhunty and Gage, 1989; Feldmeyer, 1989; Rios and Pizarro, 1991). The majority of metal cations are not believed to permeate the muscle fibre membrane since their effects occur rapidly and many of the cations are potent blockers of surface membrane channels. Indeed, the rapidity with which a Cd^{2+}-withdrawal contracture is evoked suggests that this effect of the cation is largely external. In this Chapter, the underlying mechanism of the CWC is investigated.

3.2 Aims

The aims of the experiments in this section are twofold:

1. to further characterise the CWC in rat soleus muscle;
2. to determine the mechanism underlying this contracture.

3.3 Results

NB: All information regarding solutions and methods used in this and subsequent chapters is provided in Chapter 2 (General Methods)

Part A: Further characterisation of the Cd^{2+} -withdrawal contracture

3.3.1 *General characteristics*

The CWC is a rapid transient increase in isometric tension triggered by the removal of Cd^{2+} from the bathing solution. An example of a CWC is shown in Fig. 3.1. Generally, after reaching a peak, withdrawal -contracture tension spontaneously decayed to a level approximately equal to that preceding Cd^{2+} washout. On average, CWC's evoked after 20 minutes in 3 mM Cd^{2+} reached a peak of 28.0 $\pm$ 3.2 % of control tetanic tension (n = 16). The duration of the contracture was on average, 3.6 $\pm$ 0.2 minutes.

Two other effects of Cd^{2+} can also be seen in Fig. 3.1:

1. a dramatic effect of Cd^{2+} on the amplitude of both twitch and tetanic tension;
2. a slow non-inactivating increase in tension shortly after the addition of Cd^{2+} to the bath "the cadmium-induced contracture" (CIC).

Both of these phenomena will be discussed separately in Chapters 4 and 5 respectively.

3.3.2 *Relationship of Cd^{2+}-withdrawal contracture amplitude to the time of Cd^{2+} exposure*

Dulhunty and Gage (1989) showed that the CWC is induced only after fibres have been exposed to 3 mM Cd^{2+} for a minimum period of approximately 4 minutes. The effect of Cd^{2+} exposure time on the amplitude of the CWC is shown in Fig. 3.2. A CWC was not induced following a 2.5 minute incubation of the preparation in 3 mM Cd^{2+}. A contracture did occur in preparations incubated in 3 mM Cd^{2+} for 5 minutes or more. The amplitude of the CWC, increased rapidly with the time of Cd^{2+} incubation from 5 to 20 minutes. After an incubation of 20 minutes, the CWC reached a maximum amplitude of approximately 30 percent of control tetanic tension. Incubation time in 3 mM Cd^{2+} was restricted to 30 minutes because the Cd^{2+}-induced contracture (Chapter 5) became very

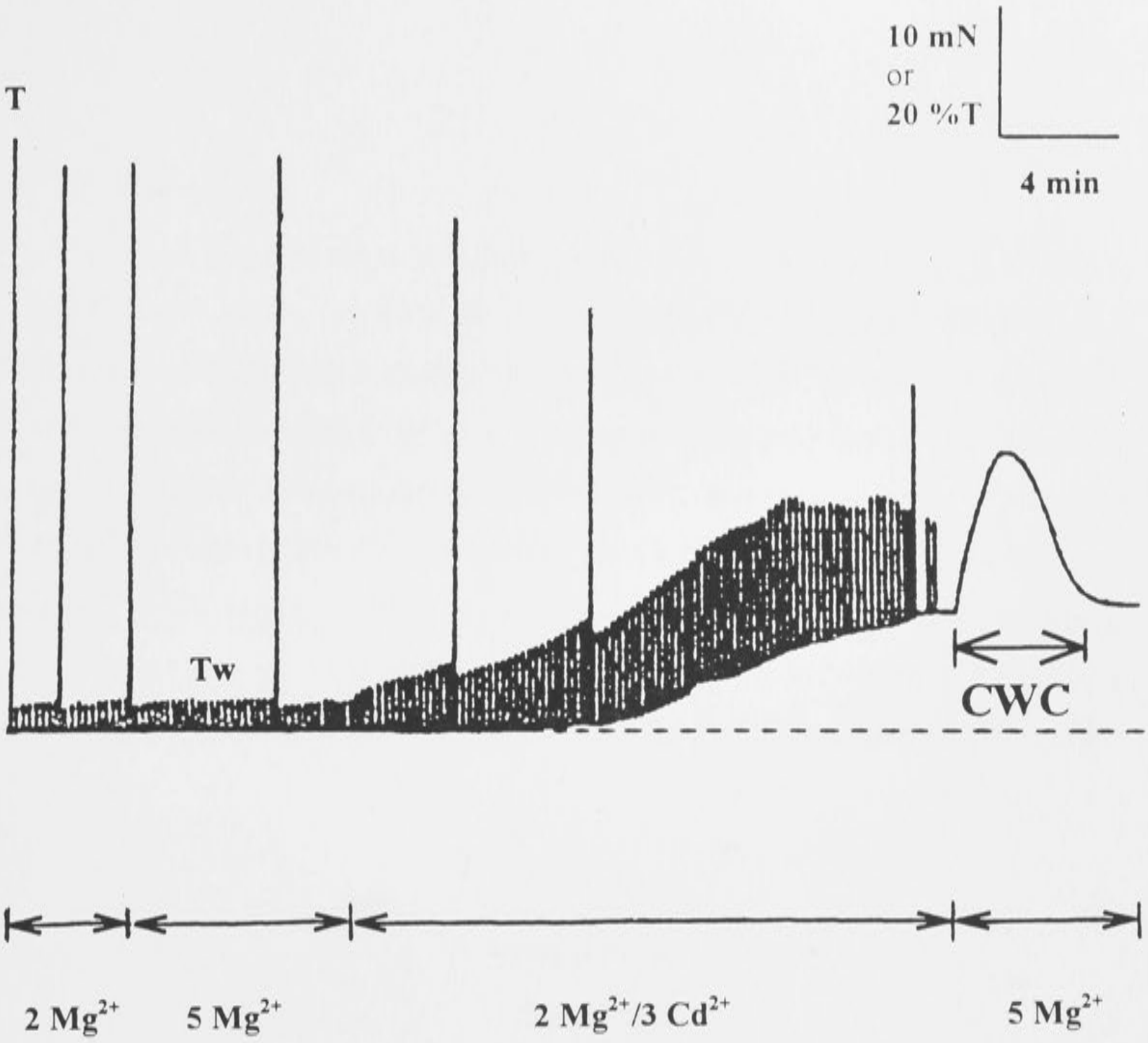


Figure 3.1 *The Cd^{2+}-withdrawal contracture*

A record illustrating the response of rat soleus muscle fibres during exposure to, and withdrawal of, Cd^{2+}. The CWC was produced by Cd^{2+} washout following a 20 minute incubation of the preparation in 3 mM Cd^{2+}. The large vertical deflections are tetani (**T**) and the smaller deflections are twitches (**Tw**). The dotted line indicates baseline tension prior to Cd^{2+} addition. Fibres were initially bathed in Krebs (solution 1A) containing (mM): 150 NaCl, 2 $MgCl_2$, 2 KCl, 2.5 $CaCl_2$, 11 glucose, 2 TES, pH 7.4 ± 0.1. The $[Mg^{2+}]$ of the bathing solution was then increased to 5 mM (solution 1B) for 10 minutes so that there would be no increase in divalent [cation] when 3 mM Cd^{2+} was added. Cd^{2+} was later introduced by exchanging the 5 mM Mg^{2+} solution with a solution containing 3 mM Cd^{2+}/2 mM Mg^{2+} (solution 1C). Changes to the bathing solution are indicated below the record (Divalent cation concentrations are given in mM). In this and subsequent records, CWC tension is indicated on the vertical scale bar as a percentage of the control tetanic tension immediately preceding Cd^{2+} addition, or as force (mN) and time is indicated on the horizontal scale bar in minutes (min).

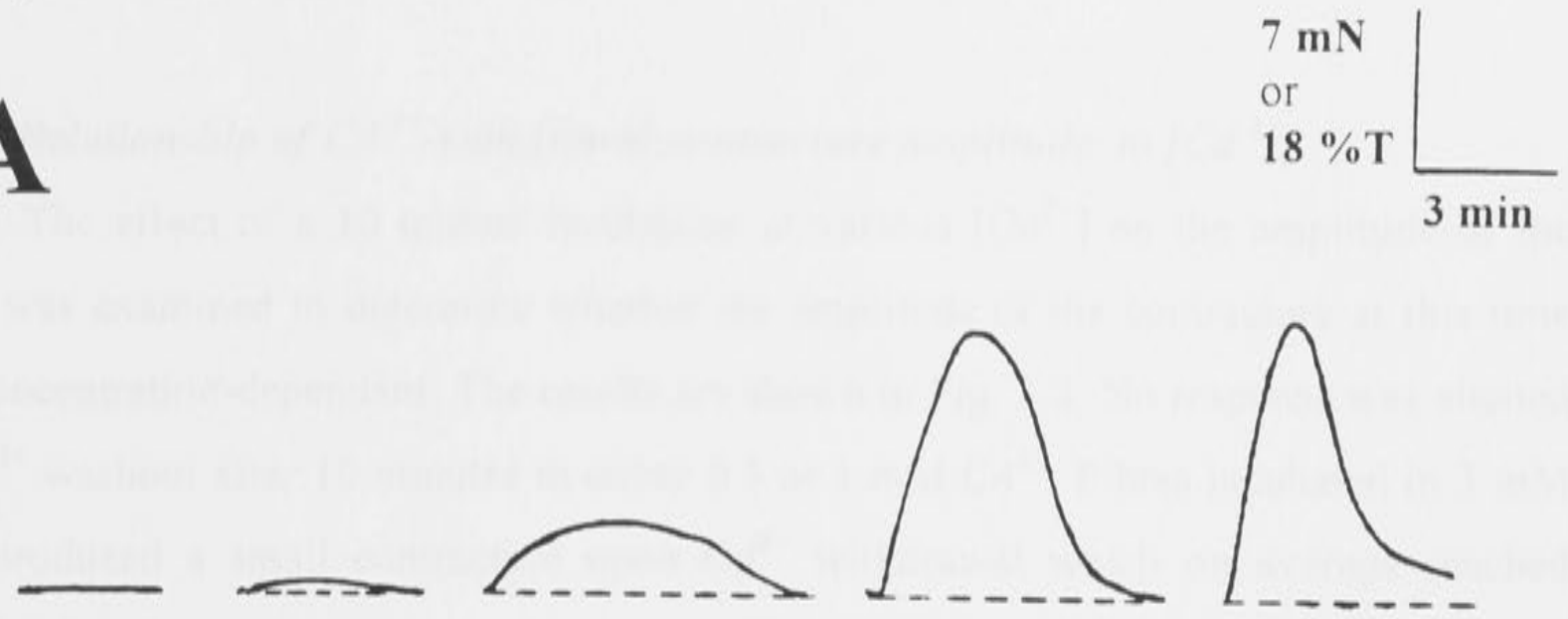
NB: Tw : T Ratio in the Above record is Low before addition of $3Cd^{2+}$. A more representAtive ratio can be seen in Fig. 3.5.

Figure 3.2 *Cd^{2+}-withdrawal contracture amplitude as a function of time*

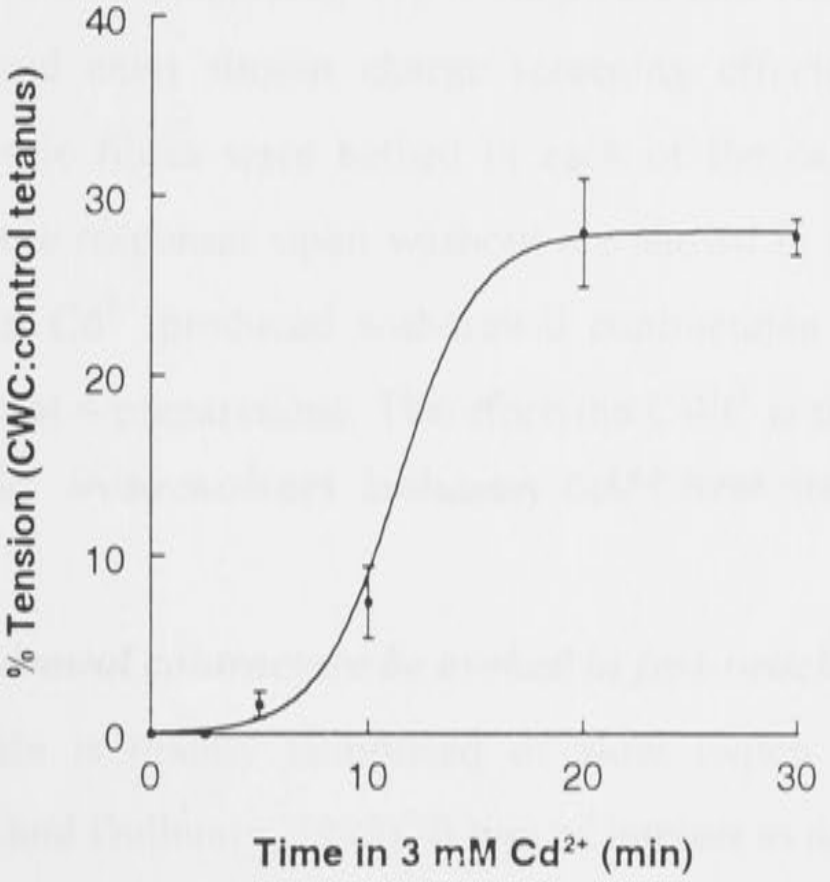
(A) Records showing CWC's evoked in 5 different rat soleus preparations, each exposed to 3 mM Cd^{2+} for a different time (which is indicated below each record, in minutes). The dotted line represents the level of tension immediately prior to Cd^{2+} withdrawal. **(B)** CWC tension (normalised to control tetanic tension) plotted as a function of time in 3 mM Cd^{2+}. Solid circles represent the average ($n \geq 4$) relative CWC amplitudes evoked following varying incubation times in 3 mM Cd^{2+}. Vertical bars represent $\pm$ 1 SEM. The data points have been fitted with a simple sigmoid function which has no physical significance.

Fig. 3.2

A


7 mN
or
18 %T
3 min
2.5
5
10
20
30
(min)

B


% Tension (CWC:control tetanus)
40
30
20
10
0
0
10
20
30
Time in 3 mM Cd2+ (min)

large (approaching maximum tetanic tension) beyond this point, making measurement of the CWC meaningless.

3.3.3 *Relationship of Cd^{2+}-withdrawal contracture amplitude to $[Cd^{2+}]$*

The effect of a 10 minute incubation at various $[Cd^{2+}]$ on the amplitude of the CWC was examined to determine whether the amplitude of the contracture at this time was concentration-dependant. The results are shown in Fig. 3.3. No response was elicited by Cd^{2+} washout after 10 minutes in either 0.5 or 1 mM Cd^{2+}. Fibres incubated in 3 mM Cd^{2+} produced a small contracture upon Cd^{2+} withdrawal which on average reached 5.3 ± 0.8 % of control tetanic tension, n = 4. The CWC amplitude increased rapidly with $[Cd^{2+}]$ above 3 mM, reaching a plateau of approximately 38 % of control tetanic tension at $[Cd^{2+}]$ between 6-10 mM. The results so far show that the induction and amplitude of CWC's depends both on $[Cd^{2+}]$ and Cd^{2+} exposure time.

3.3.4 *Is the withdrawal contracture Cd^{2+} specific?*

The ability of other metal cations to induce a withdrawal contracture upon washout was examined to determine whether the CWC was specific for Cd^{2+} or the result of a general effect of cations on the muscle fibre. Co^{2+}, Zn^{2+}, and La^{3+} were selected because there are reports that these cations have a similar potency to Cd^{2+}, as Ca^{2+} channel blockers (Hagiwara & Takahashi, 1967; Hagiwara and Kidokoro, 1971; Stefani & Chiarandini, 1982) and exert similar charge screening effects (Dorrscheidt-Kafer, 1981; Hille, 1992). Muscle fibres were bathed in each of the cations (3 mM) for 20 minutes and the contractile responses upon washout are shown in Fig. 3.4. None of the cations tested apart from Cd^{2+} produced withdrawal contractures upon washout. Each cation was tested in at least 4 preparations. Therefore, the CWC is the result of the removal of a specific interraction between Cd^{2+} and the muscle fibre.

3.3.5 *Can a Cd^{2+}-withdrawal contracture be evoked in fast-twitch fibres?*

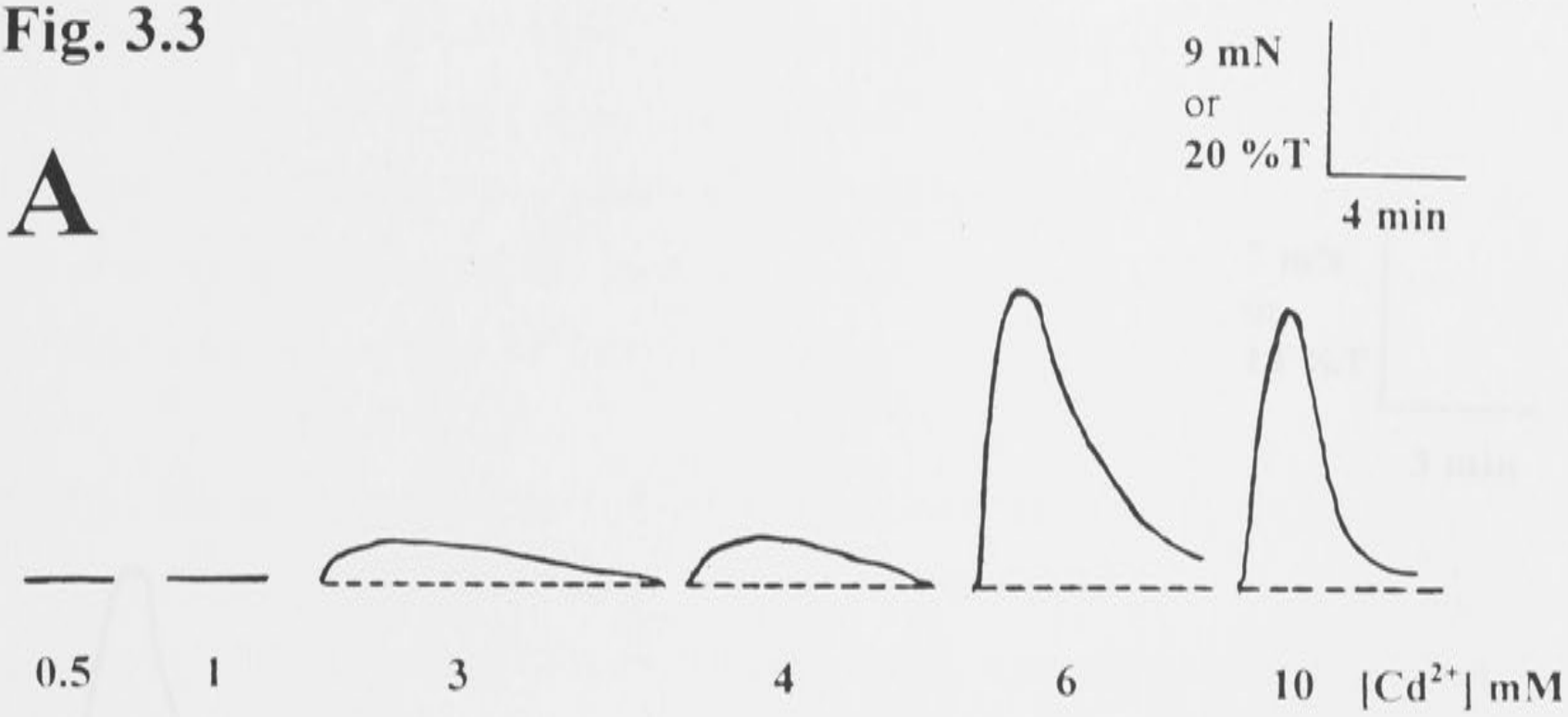
Rat soleus muscle is mainly comprised of slow twitch fibres (Fryer *et al.*, 1988; Chua and Dulhunty, 1992). It was of interest to determine whether fast twitch fibres could also produce a CWC. Therefore, the effect of Cd^{2+} withdrawal in rat

Figure 3.3 *Cd^{2+}-withdrawal contracture amplitude as a function of $[Cd^{2+}]$*

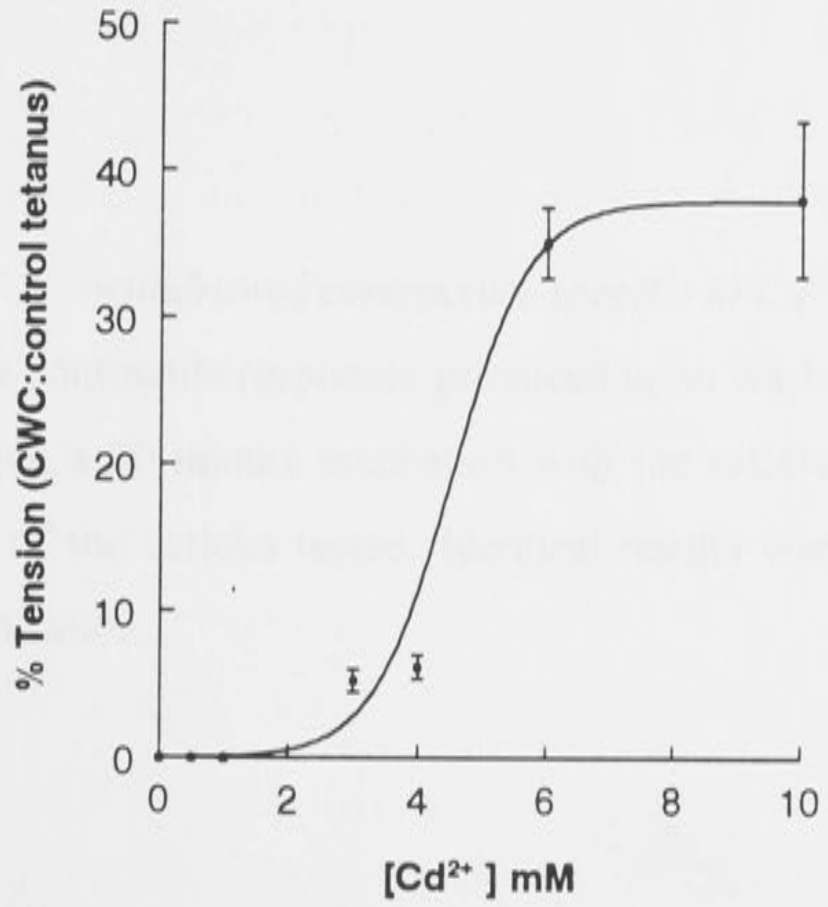
(A) Records showing CWC's evoked in six different preparations, after each was exposed to a different $[Cd^{2+}]$ for 10 minutes. The $[Cd^{2+}]$ is indicated below each record (in mM). Preparations were initially bathed in the control Krebs solution (Solution 1A, Table 2.1) before being exposed to either of the Cd^{2+} solutions (Solutions 1C and 1U-1Y, Table 2.1). Cd^{2+} was washed out after 10 minutes by replacing the Cd^{2+} containing solution with the control Krebs solution. No attempt was made to keep the total divalent cation concentration constant since it was shown in Fig. 3.1 that the CWC is not the result of changing [divalent cation] . In **(B)** the average results are shown. Solid circles represent average ($n \geq 4$) normalised amplitudes of CWC's evoked after 10 minutes at each $[Cd^{2+}]$. The vertical bars show $\pm$ 1 SEM. The data points have been fitted with a simple sigmoid function which has no physical significance.

Fig. 3.3

A


9 mN
or
20 %T
4 min
0.5
1
3
4
6
10
[Cd2+] mM

B


% Tension (CWC:control tetanus)
50
40
30
20
10
0
0
2
4
6
8
10
[Cd2+] mM

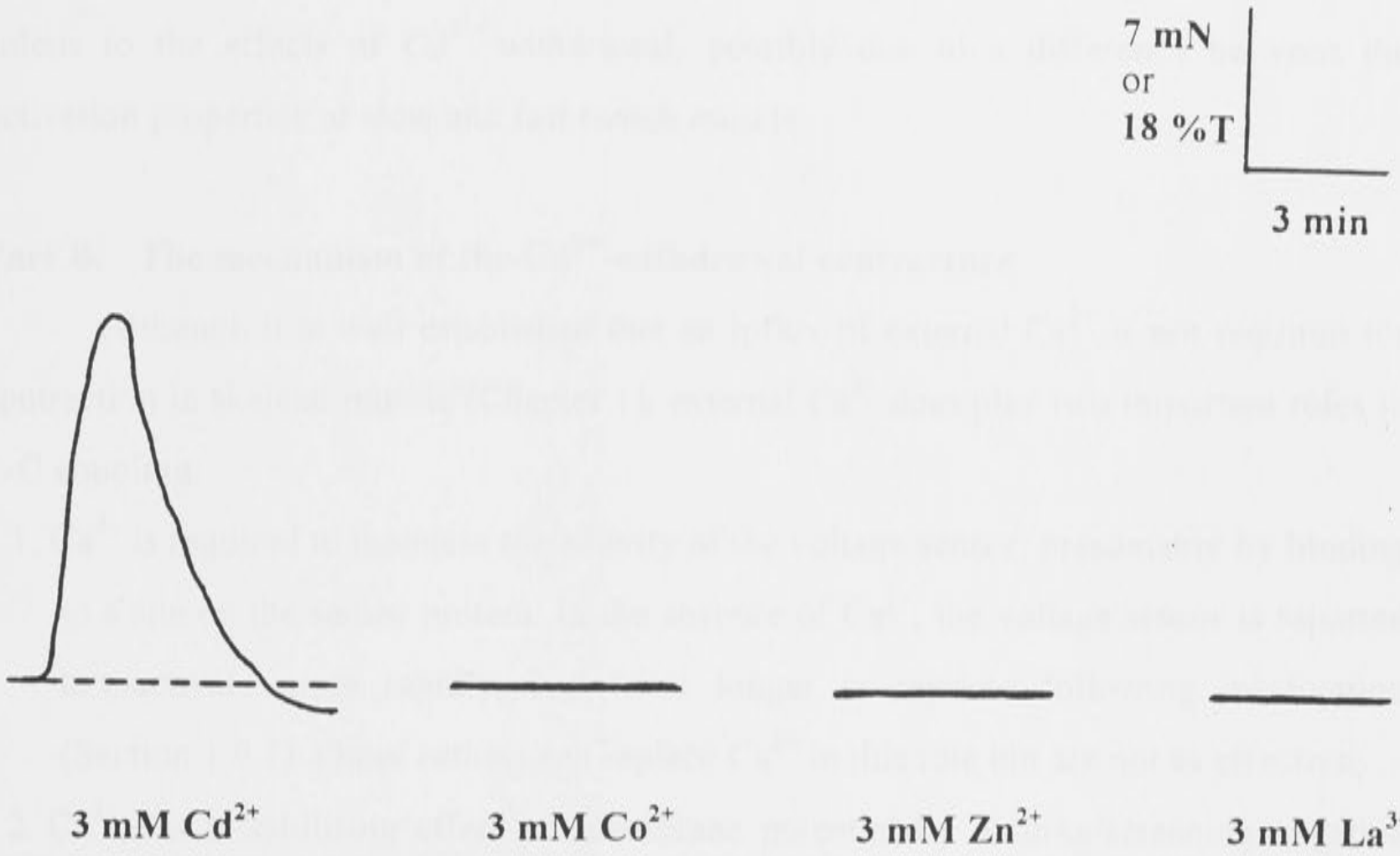


Figure 3.4 *Is the withdrawal contracture specific to Cd^{2+}*

Records showing the contractile responses produced upon washout of 3 mM Cd^{2+}, Co^{2+}, Zn^{2+} or La^{3+} following a 20 minute incubation with the cations. Different preparations were used for each of the cations tested. Identical results were obtained in at least 4 preparations for each cation.

EDL fibres was examined. Small bundles (10-15 fibres) of rat EDL muscle were exposed to 3 mM Cd^{2+} for 20 minutes before Cd^{2+} washout (Fig. 3.5). Cd^{2+}-withdrawal failed to produce a contractile response in rat EDL muscle in 3 separate preparations. Thus, either the mechanism for the CWC is absent from EDL fibres, or EDL is less sensitive than soleus to the effects of Cd^{2+} withdrawal, possibly due to a difference between the activation properties of slow and fast twitch muscle.

Part B: The mechanism of the Cd^{2+}-withdrawal contracture

Although it is well established that an influx of external Ca^{2+} is not required for contraction in skeletal muscle (Chapter 1), external Ca^{2+} does play two important roles in E-C coupling:

1. Ca^{2+} is required to maintain the activity of the voltage sensor, presumably by binding to a site on the sensor protein. In the absence of Ca^{2+}, the voltage sensor is reported to inactivate more rapidly, and takes longer to reprime following inactivation (Section 1.9.1). Other cations can replace Ca^{2+} in this role but are not as effective;
2. Ca^{2+} has a stabilising effect on membrane potential by binding/screening negative charges situated on the fibre surface membrane. This role is less specific for Ca^{2+} and can be carried out just as effectively by other cations. If all cations are removed, membrane depolarisation occurs.

The binding/screening of surface charges by cations also indirectly affects the voltage sensor, because surface charge contributes to the membrane electric field. Thus Cd^{2+} could affect the voltage sensor both directly by binding to the voltage sensor protein, or indirectly by an effect on membrane surface charge. The CWC could follow activation of the voltage sensor by removal of a charge screening / binding effect exerted during the presence of Cd^{2+}.

If it is assumed that the action of Cd^{2+} is external, there are a number of possible mechanisms for the withdrawal contracture:

1. activation of the voltage sensor for E-C coupling by surface membrane depolarisation upon Cd^{2+} washout;
2. activation of a calcium influx into the muscle upon Cd^{2+} withdrawal;

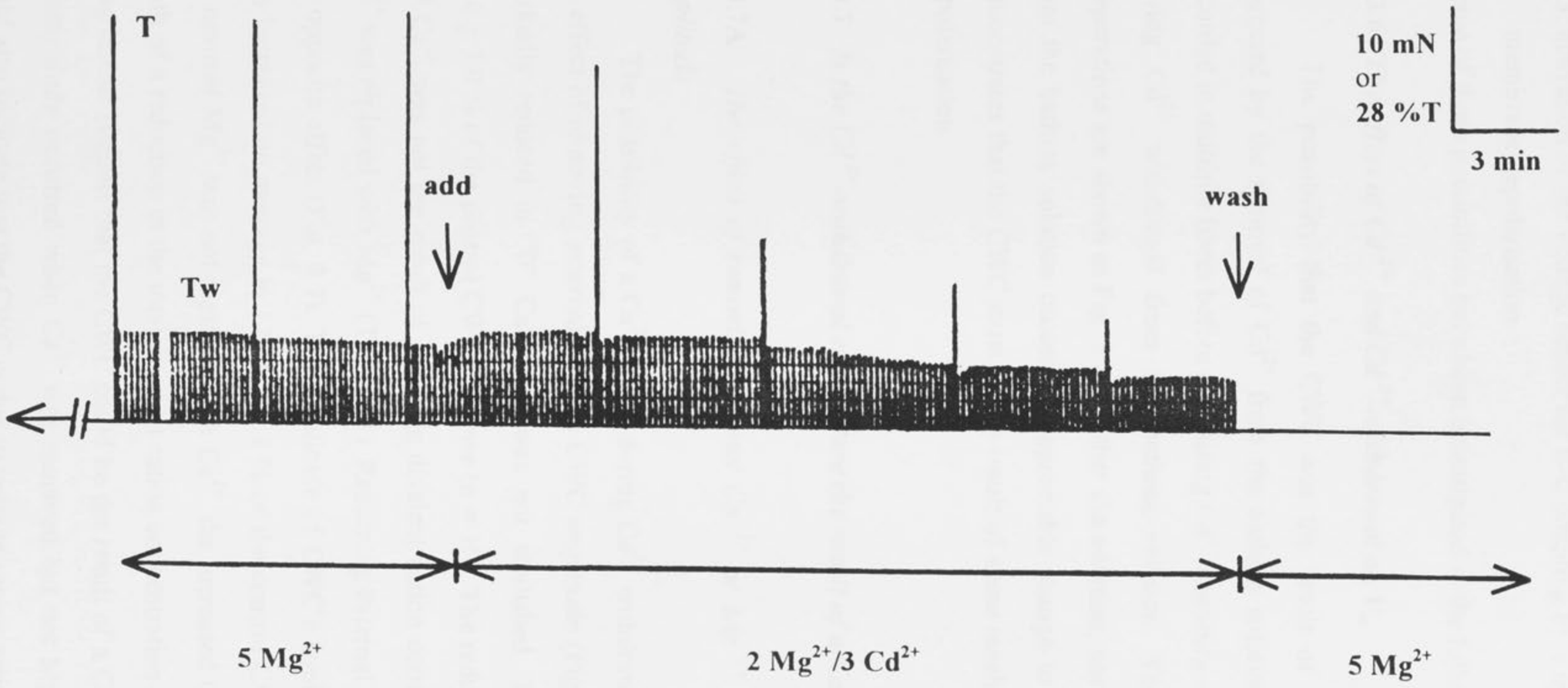


Figure 3.5 *Can the Cd^{2+}-withdrawal contracture be evoked in fast twitch muscle fibres?*

A record showing the effect of 3 mM Cd^{2+} and Cd^{2+} -withdrawal on rat EDL fibres. The experiment was identical to that described for rat soleus muscle in Fig. 3.1. Changes to the bathing solution are indicated below the record (all concentrations are in mM). Also shown are twitches (**Tw**) and tetanic contractions (**T**). Cd^{2+} addition and washout are indicated by the arrows above the record. No response to Cd^{2+} -withdrawal was obtained in 3 separate EDL preparations.

3. activation of the voltage sensor for E-C coupling by a mechanism other than surface membrane depolarisation.

Some of these possibilities have been investigated in the following experiments.

3.3.6 *The effect of Cd^{2+} and Cd^{2+}-withdrawal on V_m*

The possibility that the CWC was the result of sarcolemmal depolarisation triggered by the removal of Cd^{2+} from the bathing solution was investigated. V_m was recorded in multiple fibres before and during Cd^{2+} exposure and continuously in one fibre during Cd^{2+} withdrawal from the bathing solution. The average results from 3 preparations are shown in Fig. 3.6. Neither the addition, nor the removal of 3 mM Cd^{2+} from the bathing solution caused an appreciable change to the V_m. This result clearly demonstrates that the CWC must be the result of some mechanism other than membrane depolarisation.

3.3.7 *Is the Cd^{2+}-withdrawal contracture the result of a Ca^{2+} influx?*

3.3.7A *The effect of removing external Ca^{2+} or Mg^{2+} on withdrawal contracture amplitude*

The possibility of a Ca^{2+} influx during Cd^{2+} withdrawal was tested by examining the effect of removing external Ca^{2+} on CWC amplitude (Fig. 3.7). CWC amplitude was markedly reduced in "0" Ca^{2+}, but was not abolished. The CWC was reduced to 20.6 ± 7.0 % of the control CWC response (n = 10). The reduction of CWC amplitude in "0" Ca^{2+} was not the result of reducing divalent cation concentration since the omitted Ca^{2+} was replaced with Mg^{2+} (Table 2.1). Removing external Mg^{2+} on the other hand had the opposite effect (Fig. 3.7). The amplitude of CWC's evoked in the absence of Mg^{2+} was increased on average to 129.4 ± 3.3 % of the control CWC response (n = 4). Since the omitted Mg^{2+} was not replaced with Ca^{2+}, the increased CWC amplitude may be the result of a reduction in the total divalent cation concentration. The results from these two experiments suggest that the CWC could be the result of a Ca^{2+} influx since a reduction of amplitude occurred when Ca^{2+} was removed but not Mg^{2+}. Alternately, the results could also indicate that the CWC is due to voltage sensor activation since this would also

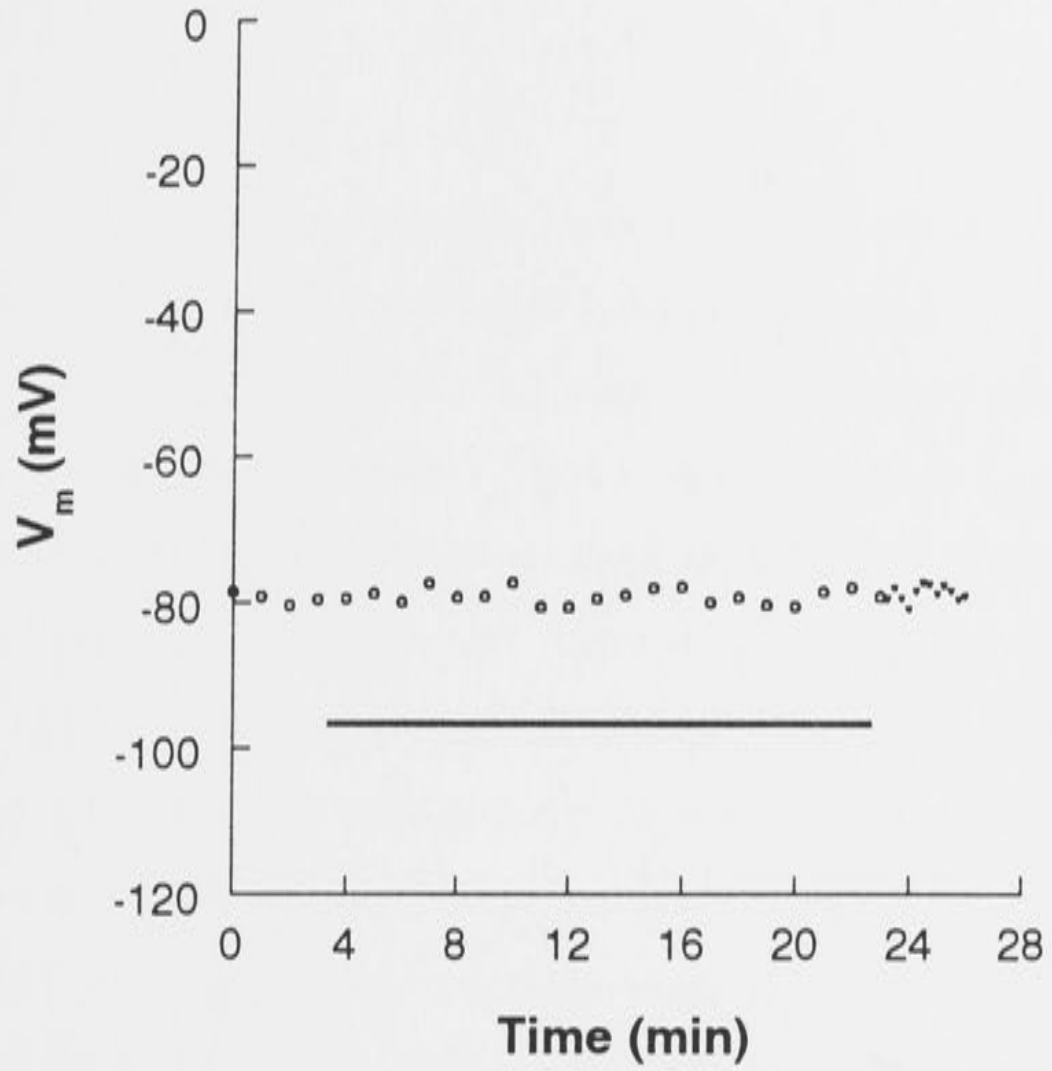


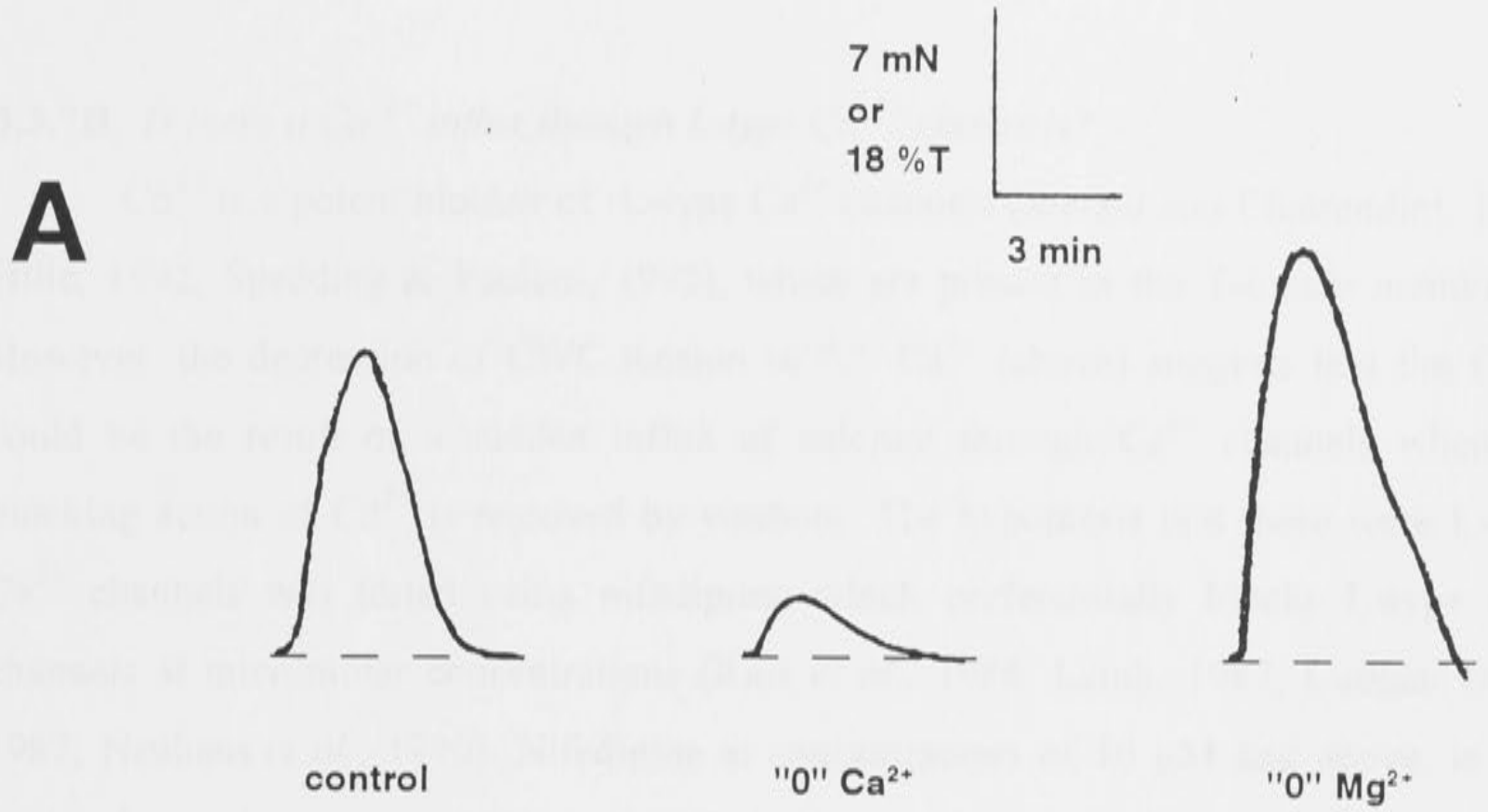
Figure 3.6 *The effect of Cd^{2+} and Cd^{2+} -withdrawal on V_m*

Resting membrane potential (V_m) recorded in rat soleus muscle fibres before the addition of 3 mM Cd^{2+} to the bathing solution, in 3 mM Cd^{2+} and during Cd^{2+} withdrawal. Data points represent the V_m averaged from 3 separate experiments, each from a different animal, performed on different days. Measurements of V_m before and during Cd^{2+} addition were made by impaling different fibres at one minute intervals (open circles). During Cd^{2+} withdrawal, V_m was recorded at 15 second intervals in one fibre (solid triangles). Initially fibres were bathed in a control Krebs solution containing 5 mM Mg^{2+} (solution 1B). Cd^{2+} was later introduced into the bath by replacing the 5 mM Mg^{2+} solution with another containing 3 mM Cd^{2+}/2 mM Mg^{2+} (solution 1C). Cd^{2+} was washed out of the bath by replacing the Cd^{2+} solution with the 5 mM Mg^{2+} control solution. The solid bar below the data points indicates the presence of 3 mM Cd^{2+} in the bathing solution.

Figure 3.7 *The Cd^{2+} -withdrawal contracture depends on external Ca^{2+}*

(A) Records of CWC's evoked in three different preparations of rat soleus fibres following a 20 minute incubation in 3 mM Cd^{2+}. The first record on the left is a "control" CWC evoked in the presence of Mg^{2+} and Ca^{2+}. The other records (middle and right) show CWC's evoked in the absence of Ca^{2+} or Mg^{2+}, respectively. Fibres were exposed to the "Ca^{2+} free" (solution 1O, Table 2.1) or "Mg^{2+} free" (solution 1I) solutions for 20 minutes prior to the addition of Cd^{2+} (solutions 1P or 1J, respectively). Cd^{2+} was washed out after 20 minutes by replacing the Cd^{2+} containing solutions with the solutions in which the preparation was bathed immediately preceding Cd^{2+} addition. The "Ca^{2+} or Mg^{2+} free" solutions were made by omitting Ca^{2+} or Mg^{2+} from the standard Krebs solution. EGTA and other Ca^{2+} buffers could not be used as they have a higher affinity for Cd^{2+} (Stephenson and Thieleczek, 1986). Consequently, it was expected that a small amount of Ca^{2+} or Mg^{2+} arising from contaminants or remaining in the T-tubules would have been present. In the "Ca^{2+} free" solution, extra Mg^{2+} was added in place of the omitted Ca^{2+} to prevent depolarisation of the preparation and to maintain a constant divalent cation concentration. However, in the "Mg^{2+} free" solution, the omitted Mg^{2+} was not replaced by any cation. **(B)** Vertical columns represent the average CWC tension (normalised to control tetanic tension) obtained in the control, "0" Ca^{2+} , and "0" Mg^{2+} solutions. The vertical bars show ± 1 SEM. The number of preparations in each experiment is indicated above the error bars. *** indicates a value significantly different to the control ($P \leq 0.05$).

Fig. 3.7


A
7 mN
or
18 %T
3 min
control
"0" Ca2+
"0" Mg2+
B
% tension (CWC:control tetanus)
50
40
30
20
10
0
16
***
10
4
control
"0" Ca2+
"0" Mg2+

be depressed in the absence of external Ca^{2+} and potentiated by decreasing the total divalent cation concentration through a reduction in screening of surface charge.

3.3.7B *Is there a Ca^{2+} influx through L-type Ca^{2+} channels?*

Cd^{2+} is a potent blocker of L-type Ca^{2+} channels (Stefani and Chiarandini, 1982; Hille, 1992; Spedding & Paoletti, 1992), which are present in the T-tubule membrane. However, the depression of CWC tension in "0" Ca^{2+} (above) suggests that the CWC could be the result of a sudden influx of calcium through Ca^{2+} channels when the blocking action of Cd^{2+} is removed by washout. The hypothesis that these were L-type Ca^{2+} channels was tested using nifedipine, which preferentially blocks L-type Ca^{2+} channels at micromolar concentrations (Rios *et al.*, 1986; Lamb, 1987; Luttgau *et al.*, 1987; Neuhaus *et al.*, 1990). Nifedipine at concentrations of 10 μM and above, is also reported to depress contraction in skeletal muscle (Dulhunty and Gage, 1988, Neuhaus *et al.*, 1990), presumably via an effect on the voltage sensor since this drug has similar effects on asymmetric charge movement (Lamb, 1986). 1 μM nifedipine was used in this experiment as a compromise between providing an effective block of L-type Ca^{2+} channels whilst minimising effects on the voltage sensor. Nifedipine (1 μM) was added to the bathing solution 10 minutes prior to the addition of 3 mM Cd^{2+} and was present during Cd^{2+} withdrawal. The results of this experiment are shown in Fig. 3.8. Nifedipine (1μM) caused no appreciable change to the amplitude of the CWC compared to a control response (102.1 ± 20.5 % of control CWC tension, n = 6). This result suggests that the CWC is not due to an influx of Ca^{2+} through L-type Ca^{2+} channels.

3.3.7C *Through other types of Ca^{2+} channels?*

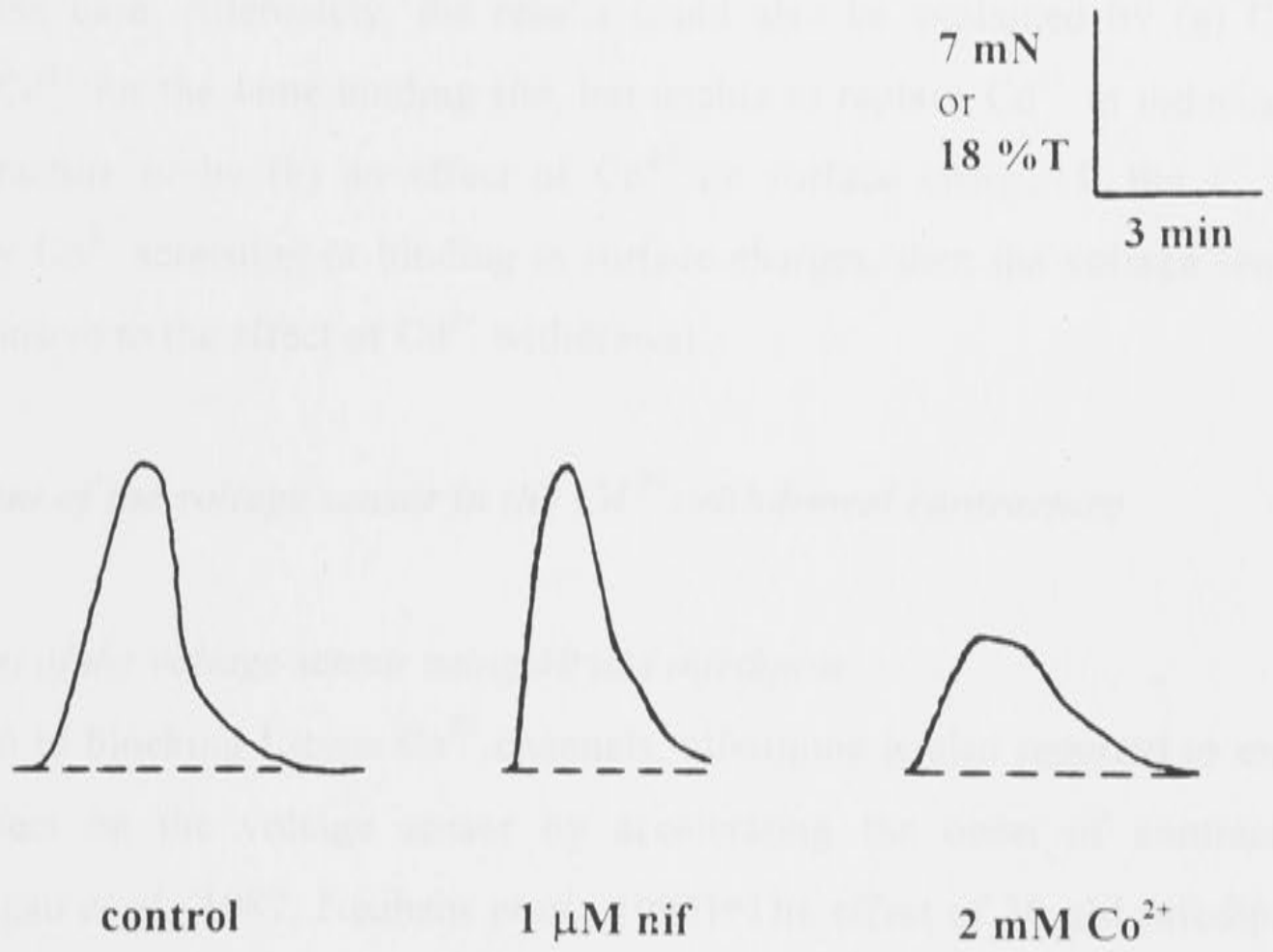
Another possibility is that the CWC could be the result of a Ca^{2+} influx through nifedipine-insensitive Ca^{2+} channels. To test this, the effects of a non-specific Ca^{2+} channel blocker on the CWC was examined. The divalent cation Co^{2+} is reported to block a wide variety of Ca^{2+} channel types (Lorkovic & Rudel, 1983; Beaty *et al.*, 1987; Hille, 1992). The effect of 2 mM Co^{2+} on the CWC is shown in Fig. 3.8. Co^{2+} caused a reduction of the CWC amplitude to 56.2 ± 16.1 % of control CWC tension, (n = 6), suggesting that the contracture could be induced by a non-specific Ca^{2+} influx during

Figure 3.8 *The effect of Ca^{2+} channel blockage on the Cd^{2+} - withdrawal contracture*

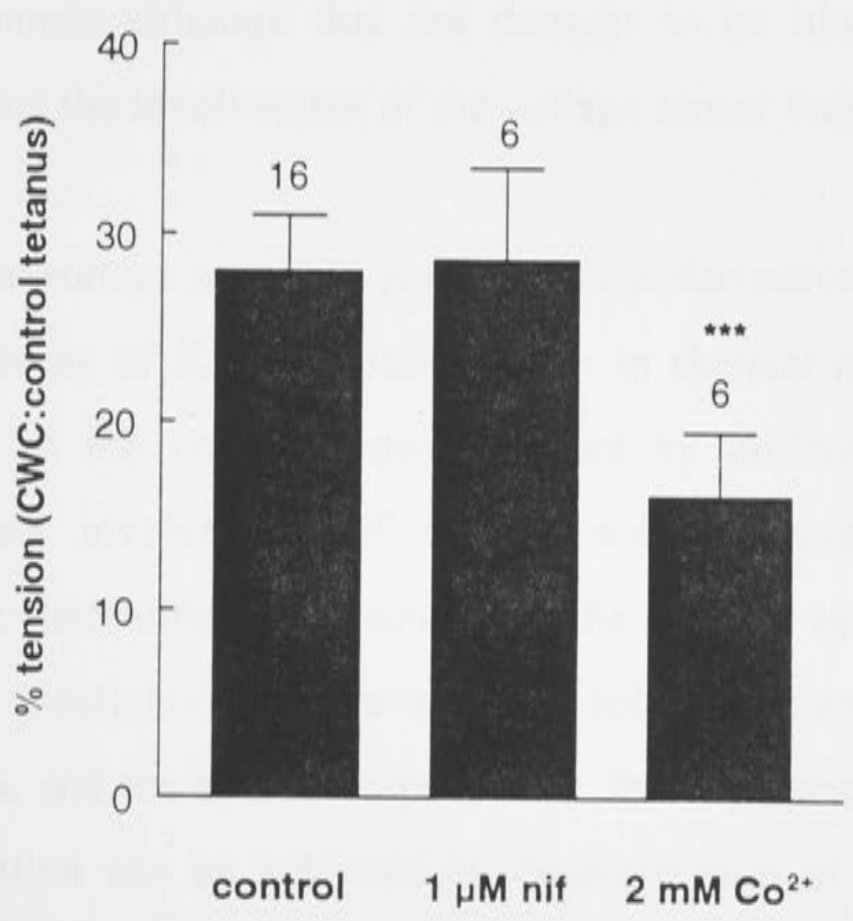
(A) Records showing CWC's evoked in the presence of 1 μM nifedipine or 2 mM Co^{2+}. A "control" CWC record is provided for comparison and the 3 records are from different preparations. Preparations were bathed in 1μM nifedipine (made by adding stock nifedipine to solution 1B, Table 2.1) or 2 mM Co^{2+} (solution 1G) for 10 minutes prior to the addition of 3 mM Cd^{2+}. The Cd^{2+} solutions also contained nifedipine (stock added to solution C) or Co^{2+} (solution 1H). Cd^{2+} was washed out after 20 minutes by replacing the Cd^{2+} solutions with the nifedipine or Co^{2+} containing, Cd^{2+} free solutions. The average results for this experiment are summarised in **(B).** Normalised amplitudes of CWC's evoked in the presence of 1 μM nifedipine or 2 mM Co^{2+} are shown compared to the control response. Vertical bars represent ± 1 SEM. The number of preparations in each experiment is indicated above each error bar. *** indicates a significant difference to the control ($P \leq 0.05$).

Fig. 3.8

A


7 mN
or
18 %T
3 min
control
1 μM nif
2 mM Co^{2+}

B


% tension (CWC:control tetanus)
40
30
20
10
0
16
6
***
6
control
1 μM nif
2 mM Co^{2+}

Cd^{2+} withdrawal. However, the fact that a significant contracture still remained suggests that this is not the case. Alternately, the results could also be explained by (a) Co^{2+} competing with Cd^{2+} for the same binding site, but unable to replace Cd^{2+} in inducing a withdrawal contracture or by (b) an effect of Co^{2+} on surface charge. If the V_m is hyperpolarised by Co^{2+} screening or binding to surface charges, then the voltage sensor may be less responsive to the effect of Cd^{2+} withdrawal.

3.3.8 *Involvement of the voltage sensor in the Cd^{2+}-withdrawal contracture*

3.3.8A *Inhibition of the voltage sensor using 50 μM nifedipine*

In addition to blocking L-type Ca^{2+} channels, nifedipine is also reported to exert an inhibitory effect on the voltage sensor by accelerating the onset of contractile inactivation (Luttgau *et al.*, 1987; Neuhaus *et al.*, 1990). The effect of 50 μM nifedipine on CWC amplitude was examined to determine whether voltage sensor activation is involved in the development of this contracture. The results are shown in Fig. 3.9. The amplitude of CWC's evoked in the presence of 50 μM nifedipine was severely depressed to 19.6 ± 11.4 % of control CWC tension, (n = 6).* This result suggests that activation of the skeletal muscle voltage sensor is required for a CWC. The result might also indicate that nifedipine was more effective at this higher concentration in blocking a Ca^{2+} influx through L-type Ca^{2+} channels although this not thought to be likely. Another more specific treatment for testing the involvement of the voltage sensor was therefore needed.

3.3.8B *Inactivation of the voltage sensor by prolonged depolarisation in 40 mM K^+*

The spontaneous decay of K contracture tension in skeletal muscle is generally attributed to inactivation of the voltage sensor induced by prolonged depolarisation (Chapter 1). The possible involvement of voltage sensor activation during Cd^{2+} withdrawal could thus be determined by inactivating the voltage sensor prior to Cd^{2+} washout. The degree of inactivation in elevated K^+ solutions depends both on the concentration of the K^+ in, and the time of exposure to, the depolarising solution. Thus strong contractile inactivation can be achieved by depolarisation in 40 mM K^+ (40K) provided the preparation is exposed to this solution for a suitable length of time. 40K was

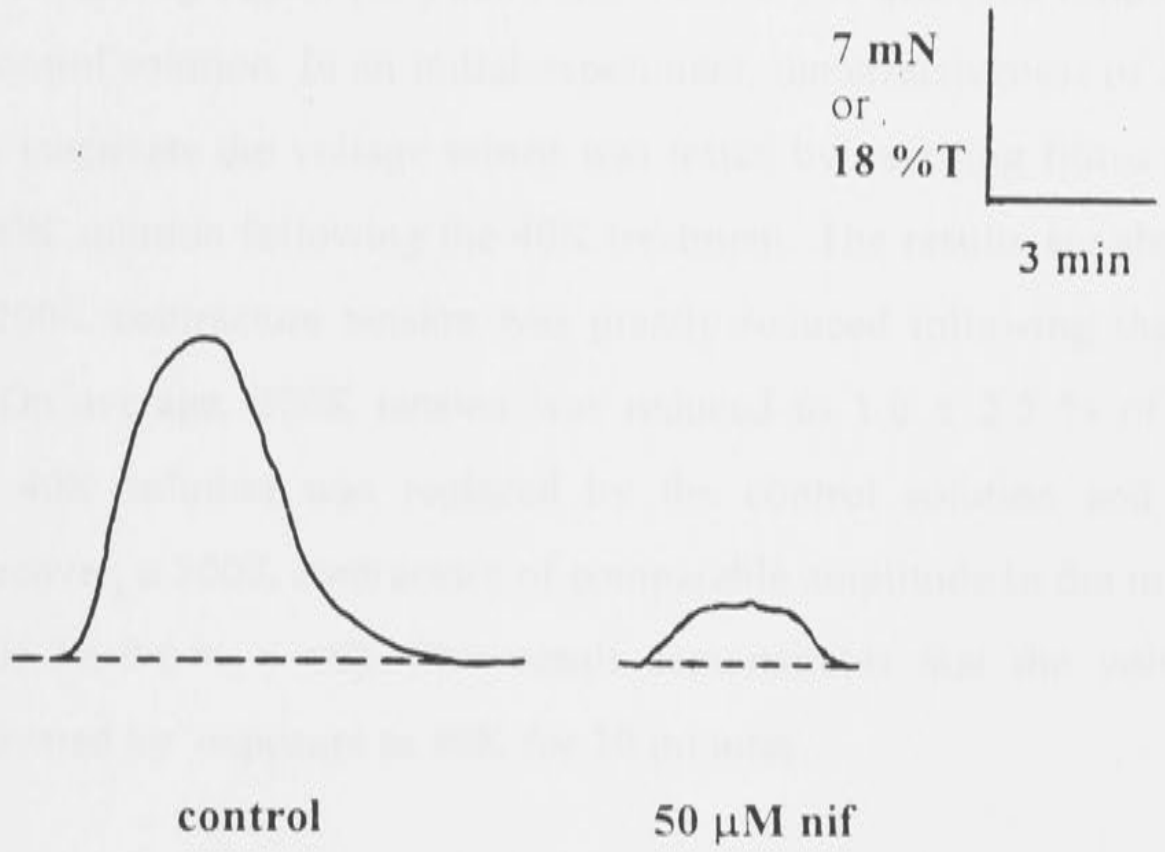
* Tetanic tension was also depressed in 50μM nifedipine to 68.5 ± 8.2% of control tetanic tension (n=4) after 10 min, prior to addition of 3mM Cd^{2+}.

Figure 3.9 *The effect of 50 μM nifedipine on the Cd^{2+}-withdrawal contracture*

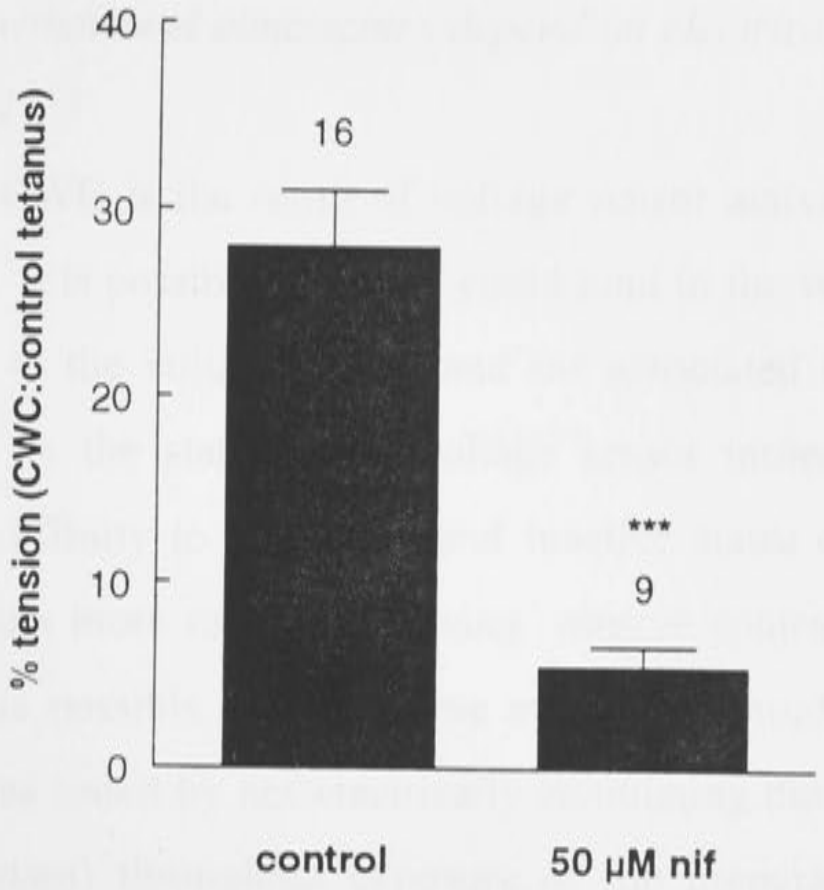
(A) Records showing CWC's evoked in the presence and absence of 50 μM nifedipine. Nifedipine (50 μM) was added directly to the bathing solution (solution 1B, Table 2.1) 10 minutes prior to the addition of 3 mM Cd^{2+}. Nifedipine was also added to the 3 mM Cd^{2+} solution (solution 1C) and was present during Cd^{2+}-withdrawal. **(B)** The average normalised amplitude of CWC's evoked in the presence of 50 μM nifedipine are shown compared to the average control response. Vertical bars represent ± 1 SEM. The number of preparations used in each part of the experiment is indicated above the error bars. *** represents a significant difference from the control value ($P \leq 0.05$).

Fig. 3.9

A


7 mN
or
18 %T
3 min
control
50 μM nif

B


% tension (CWC:control tetanus)
40
30
20
10
0
16
***
9
control
50 μM nif

preferred as the voltage sensor inactivating solution since it is not as harsh on a preparation as solutions containing higher [K^+] and better recovery is achieved following repriming in a low K^+ control solution. In an initial experiment, the effectiveness of a 20 minute 40K exposure to inactivate the voltage sensor was tested by exposing fibres to a maximally activating 200K solution following the 40K treatment. The results are shown in Fig. 3.10. Maximal 200K contracture tension was greatly reduced following the 20 minute 40K treatment. On average, 200K tension was reduced to 1.6 ± 2.5 % of the control, (n = 7). If the 40K solution was replaced by the control solution and the preparation allowed to recover, a 200K contracture of comparable amplitude to the initial control was evoked (105.3 ± 9.6 %, n = 6). This result demonstrates that the voltage sensor is reversibly inactivated by exposure to 40K for 20 minutes.

3.3.8C *The effect of voltage sensor inactivation on Cd^{2+}-withdrawal contracture amplitude*

The effect of voltage sensor inactivation on CWC tension (instead of 200K contracture tension) was examined (Fig. 3.11). The CWC was reversibly abolished by the 40K treatment in each of 5 different preparations.* The results, therefore, strongly suggest that the CWC depends on the activation of the voltage sensor by Cd^{2+}-withdrawal.

3.3.8D *Does the Cd^{2+}-withdrawal contracture depend on electrical stimulation of the fibre during exposure to Cd^{2+}?*

It appears that the CWC is the result of voltage sensor activation when Cd^{2+} is removed from the solution. It is possible that Cd^{2+} could bind to the voltage sensor itself. The binding of nifedipine to the voltage sensor and the associated muscle paralysis is reported to be dependant on the state of the voltage sensor molecule. Nifedipine is thought to bind with high affinity to the active and inactive states of the sensor since paralysis in nifedipine occurs more rapidly following muscle contraction (Bean, 1984; Rios and Brum, 1987). It is possible that the same mechanism might occur with Cd^{2+} binding. This hypothesis was tested by not electrically stimulating the preparation (ie not generating twitches and tetani) throughout exposure of the preparation to Cd^{2+} (Fig. 3.12). Lack of muscle stimulation had no effect on the CWC amplitude. On average the

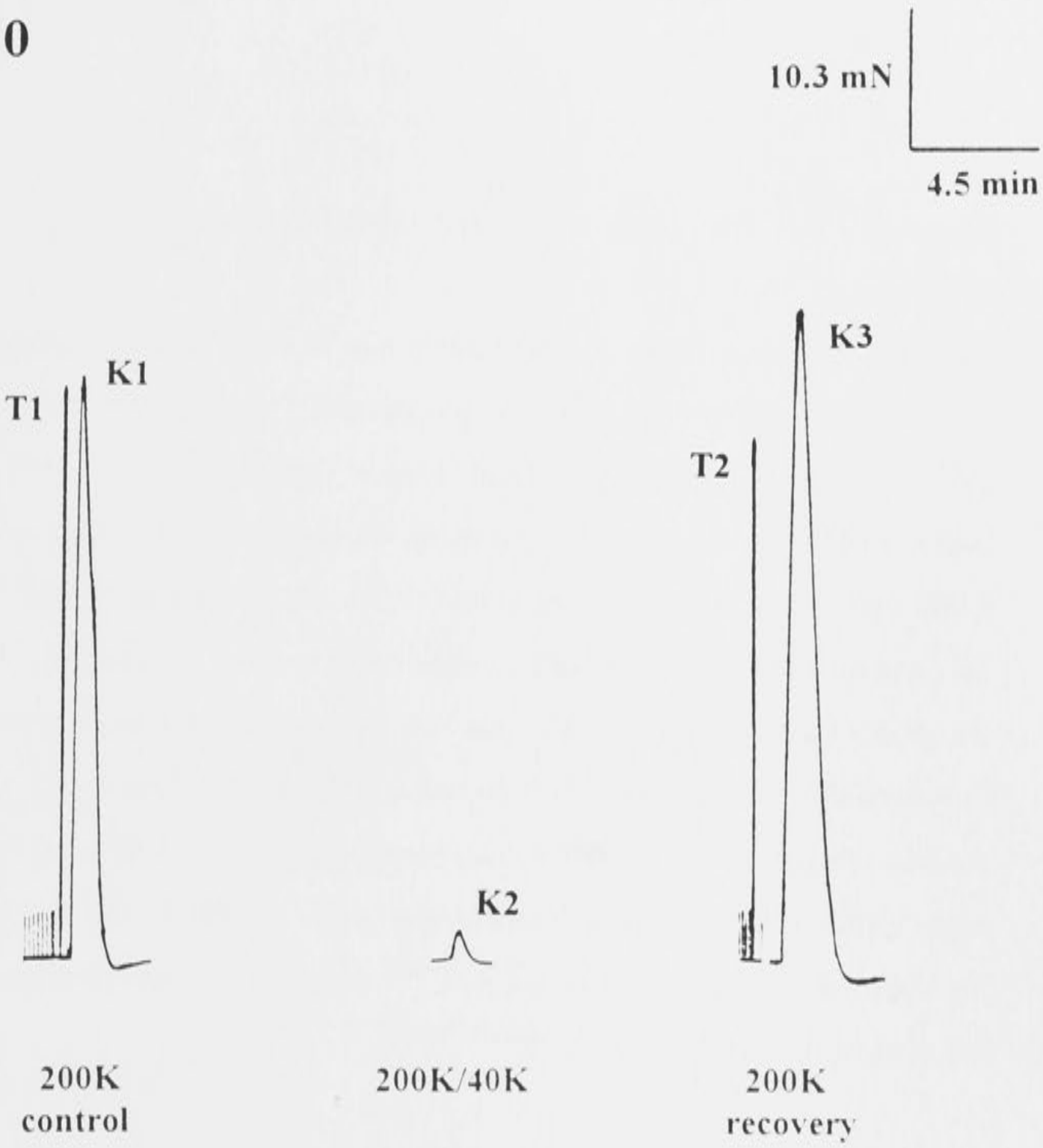
NB: * The amplitude of the recovered CWC evoked in the Soy^{2-} solution is substantial even though the free [Cd^{2+}] in the Soy^{2-} solution was calculated to be only 0·2–0·4nM.

Fig 3.10 *Voltage sensor inactivation by 40K*

(A) The effectiveness of a 20 minute incubation in a 40 mM K^+ (40K) solution in causing voltage sensor inactivation was tested by measuring the effect of this treatment on the amplitude of maximal 200K contractures. Preparations were initially bathed in a control SO_4^{2-}-containing solution (solution 2A, Table 2.1). A 200K contracture was then evoked by rapidly replacing the control solution with another containing 200 mM K^+ (solution 2M). Tetanic tension was then left to recover in the control solution. Following preparation recovery, preparations were exposed to a solution containing 40 mM K^+ (solution 2E) which produced a small contracture. After preparations had been incubated in the 40K solution a second 200K contracture was evoked. Following the 200K contracture, the preparation was bathed in the control solution until tetanic tension recovered. Following recovery, a third 200K contracture was evoked. Shown are 200K contractures elicited in the same preparation before the 40K treatment (**K1**), after 20 minutes in 40K (**K2**), and following a 20 minute recovery after washout of 40K (**K3**). Neither twitches nor tetanic contractions could be evoked during the 40K treatment. The tetanus amplitude in this particular preparation (**T2**) did not fully recover after washout of 40K, even though the 200K contracture was larger than the control. **(B)** Average 200K amplitude (normalised to the control tetanus elicited prior to the 40K treatment) is shown before, during and 20 minutes after the 40K treatment. Vertical bars represent ± 1 SEM. The number of preparations are indicated above each error bar and *** depicts a value significantly different from the initial control 200K contracture ($P \leq 0.05$).

Fig. 3.10

A


10.3 mN
4.5 min
T1
K1
K2
T2
K3
200K
control
200K/40K
200K
recovery

B

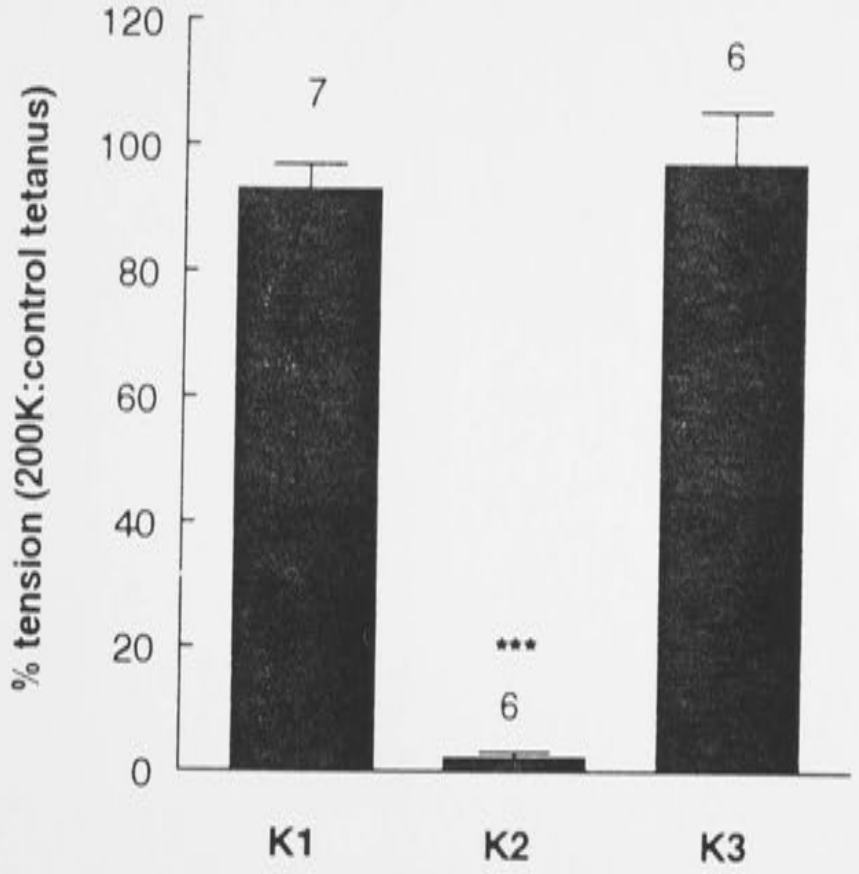
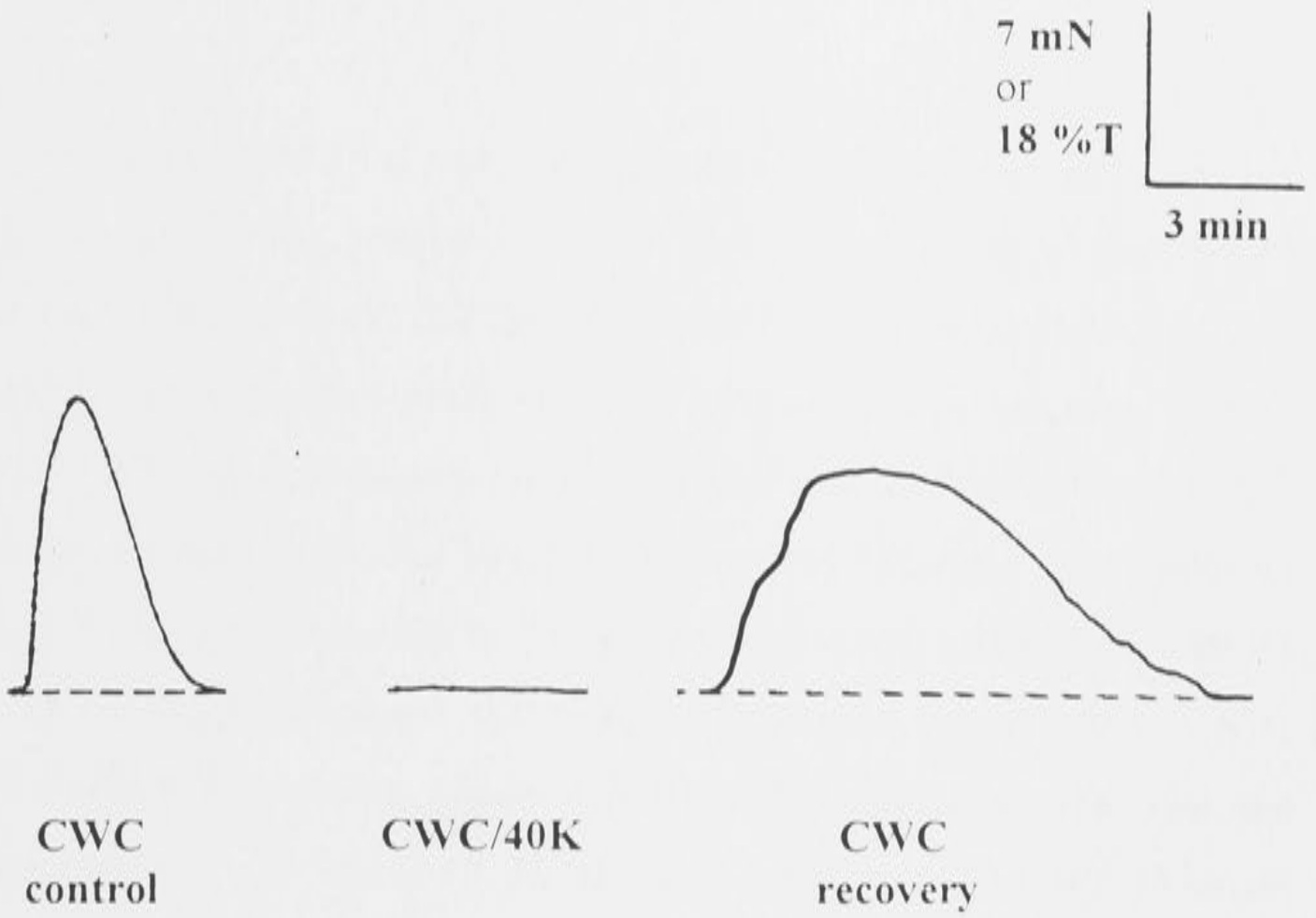

% tension (200K:control tetanus)
120
100
80
60
40
20
0
7
***
6
6
K1
K2
K3

Figure 3.11 *The effect of voltage sensor inactivation on the Cd $^{2+}$-withdrawal contracture.*

(A) The first trace (left) is a representative "control" CWC contracture. The second trace obtained from a different preparation, shows the contractile response to Cd^{2+}-withdrawal following voltage sensor inactivation in a 40K/3 Cd^{2+} solution (solution 2F, Table 2.2). The third trace, obtained from the same preparation shows a CWC evoked following voltage sensor repriming in the control $S0_4^{2-}$ solution (solution 2A) and an additional 20 minute incubation in 3 mM Cd^{2+} (solution 2B). The increased duration of the recovered CWC was not investigated but was attributed to one of the "irreversible" effects of Cd^{2+} on the muscle fibres (see Chapter 2). **(B)** Average CWC tension is shown for the control response, during the 40K treatment and following recovery after 40K washout. Vertical bars show ± 1 SEM. The number of preparations are indicated above each error bar and *** indicates a value significantly different from the control ($P \leq 0.05$).

Fig. 3.11

A


7 mN
or
18 %T
3 min
CWC
control
CWC/40K
CWC
recovery

B

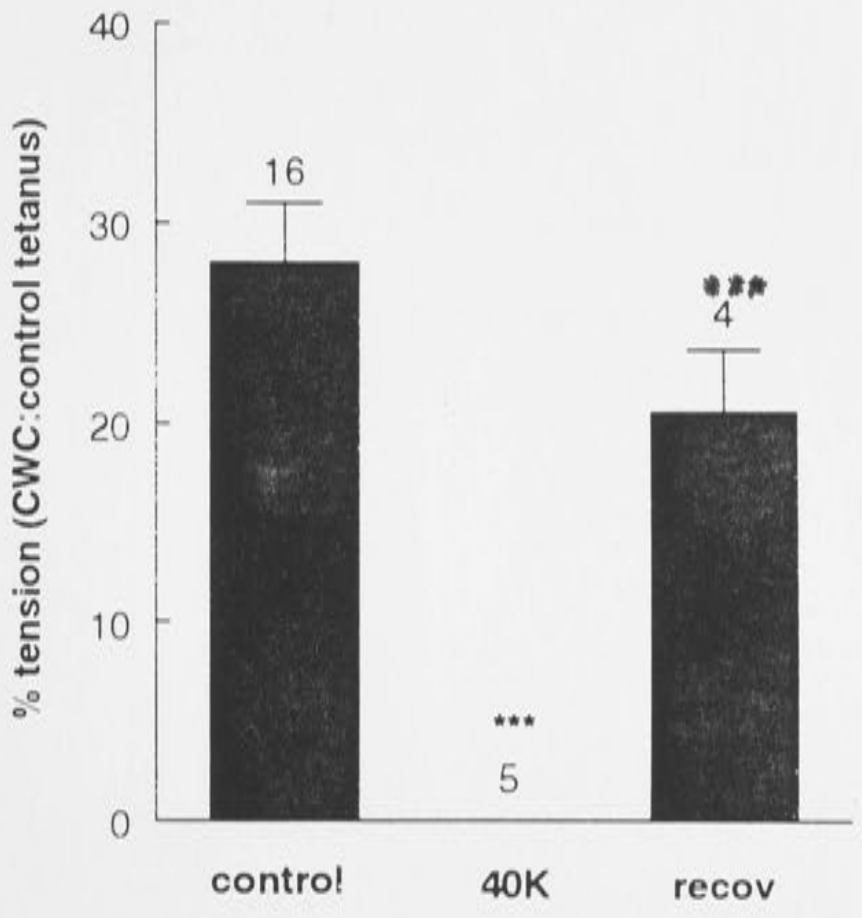
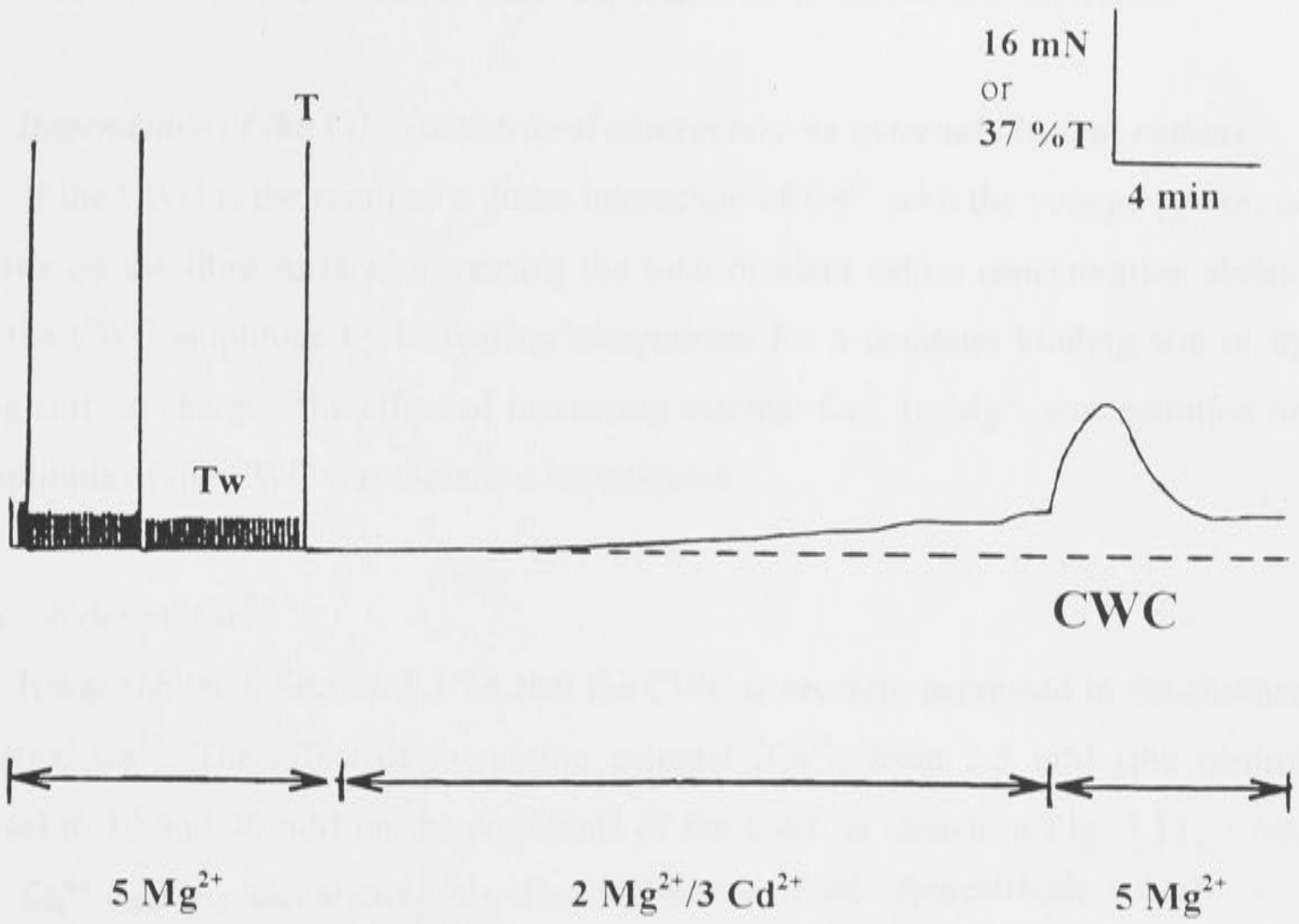

% tension (CWC:control tetanus)
40
30
20
10
0
16
***
5
***
4
control
40K
recov

Figure 3.12 *The effect of no stimulation on the Cd^{2+} -withdrawal contracture*

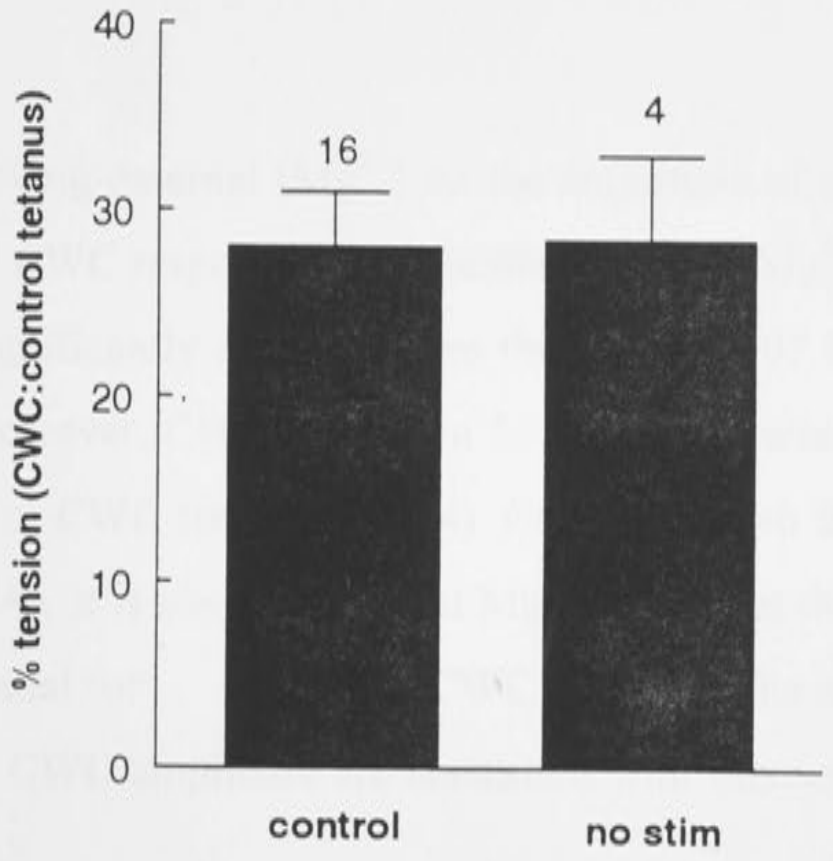
(A) A Record showing the effect of not stimulating the muscle during the period of Cd^{2+} incubation, on the amplitude of the CWC. Twitches (**Tw**) and tetani (**T**) were elicited prior to the addition of Cd^{2+} to the bath. Following the addition of Cd^{2+}, the preparation was left for 20 minutes without stimulation before Cd^{2+} washout. Solution changeover is indicated below the trace (all concentrations are in mM). The slow increase in baseline tension following the addition of 3 mM Cd^{2+} to the bath is the "CIC" (see Chapter 5). **(B)** Average CWC tension (normalised to control tetanic tension) obtained after no stimulation is shown compared to the control (stimulated) response. Vertical bars represent ± 1 SEM. The number of preparations used in each experiment is indicated above the error bars.

Fig. 3.12

A


16 mN
or
37 %T
4 min
T
Tw
CWC
5 Mg2+
2 Mg2+/3 Cd2+
5 Mg2+

B


% tension (CWC:control tetanus)
40
30
20
10
0
16
4
control
no stim

contracture amplitude when fibres were not stimulated during Cd^{2+} exposure was 101 ± 19.3 % of control CWC tension (n = 4). This result suggests that the interaction of Cd^{2+} with the fibre prior to washout is not dependent on voltage sensor activation.

3.3.9 *Dependence of the Cd^{2+}-withdrawal contracture on external divalent cations*

If the CWC is the result of a direct interaction of Cd^{2+} with the voltage sensor, or some site on the fibre surface, increasing the total divalent cation concentration should affect the CWC amplitude by increasing competition for a common binding site or by altering surface charge. The effect of increasing external Ca^{2+} or Mg^{2+} concentration on the amplitude of the CWC was therefore investigated.

3.3.9A *External Ca^{2+}*

It was shown in Section 3.3.7A that the CWC is severely depressed in the absence of external Ca^{2+}. The effect of increasing external [Ca^{2+}] from 2.5 mM (the control response) to 10 and 30 mM on the amplitude of the CWC is shown in Fig. 3.13. 10mM Ca^{2+} had no statistically significant effect on CWC amplitude which was 93.3 ± 12.6% of control CWC Amplitude (n=4). However, CWC amplitude was reduced in 30 mM Ca^{2+} to 46.4 ± 14.4 % of the control response, (n = 6). After combining these results with those from Section 3.3.7A, it is clear that CWC amplitude is dramatically affected by extreme changes in external [Ca^{2+}] but is not sensitive to changes between 2.5 and 10 mM.

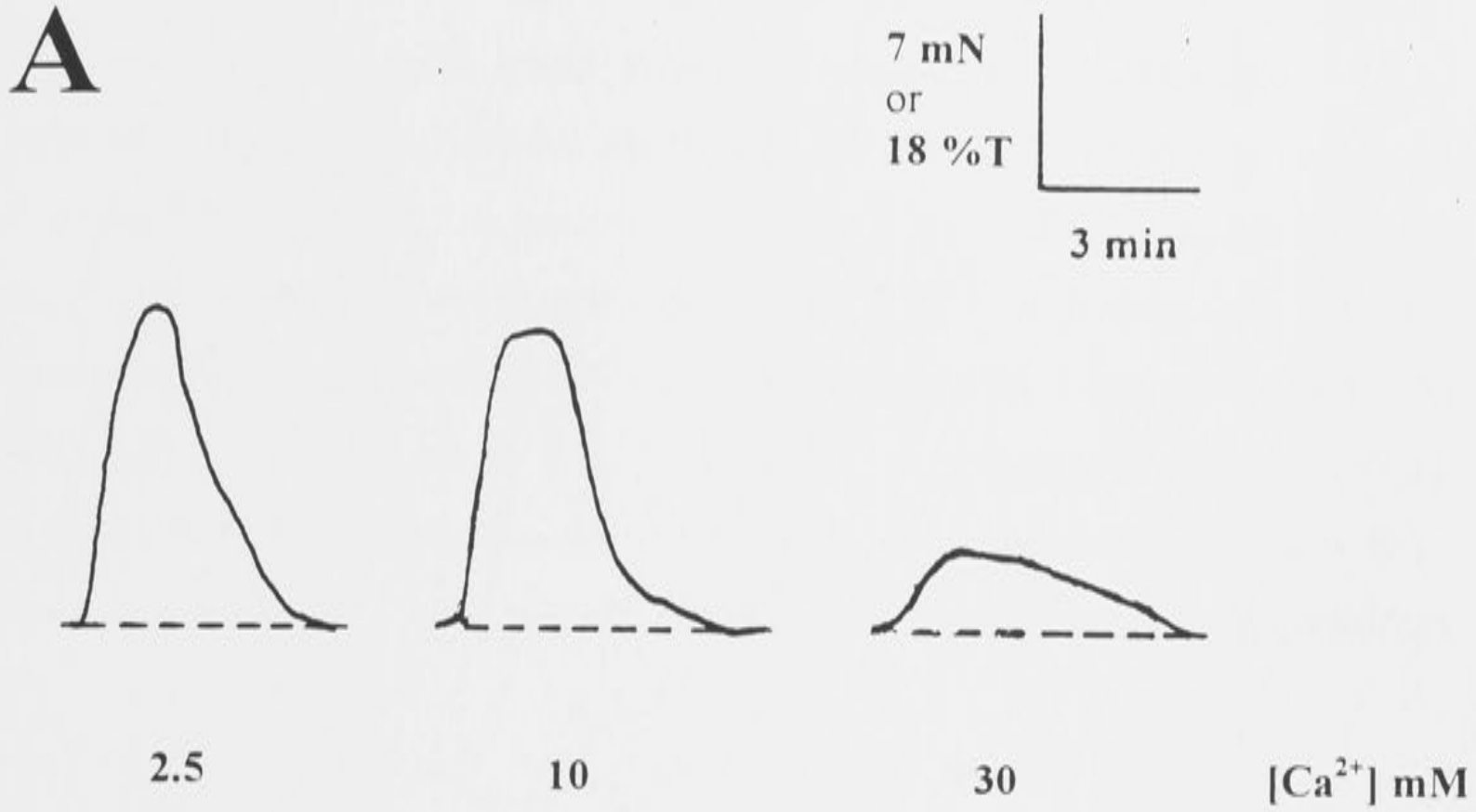
3.3.9B *External Mg^{2+}*

The effect of varying external [Mg^{2+}] on the amplitude of the CWC is shown in Fig. 3.14. The "control" CWC response was obtained in 2 mM Mg^{2+}. CWC amplitude in 10 mM Mg^{2+} was not significantly different from the control (107.8 ± 11.1 % of control CWC tension, n = 4). However, CWC tension in 30 mM Mg^{2+} was markedly reduced to 27.97 ± 3.27 % of control CWC tension, (n = 4). Combined with the results obtained in "0" Mg^{2+} (Section 3.3.7A), it is clear that whilst Mg^{2+} modulates the CWC, the presence of this cation is not essential for a CWC to occur. The similarity of effects of high Mg^{2+}, and Ca^{2+} on CWC amplitude are consistent with increased screening/binding

Figure 3.13 *The Cd^{2+}-withdrawal contracture is inhibited at high [Ca^{2+}]*

(A) Records showing CWC's evoked following a 20 minute incubation of preparations in 3 mM Cd^{2+} at varying [Ca^{2+}]. Preparations were equilibrated in Krebs solution containing 2.5, 10 or 30 mM Ca^{2+} (Solutions 1A, 1Q or 1S respectively, Table 2.1). After 10 minutes in the elevated Ca^{2+} solution, preparations were exposed to another solution containing 3 mM Cd^{2+} in addition to the elevated Ca^{2+}. 20 minutes later a CWC was evoked by replacing the Cd^{2+} containing solution with the same elevated Ca^{2+} solution in which the preparation was bathed preceding Cd^{2+} addition. The CWC evoked in 2.5 mM Ca^{2+} is the control response. The average results for this experiment are shown in **(B)**. The columns represent the average CWC tension (normalised to control tetanic tension) evoked in the presence of 2.5, 10 or 30 mM Ca^{2+}. Vertical bars show $\pm$ 1 SEM. The number of preparations are indicated above each error bar and *** indicates a value significantly different from the control ($P \leq 0.05$).

Fig. 3.13


A
7 mN
or
18 %T
3 min
2.5
10
30
[Ca2+] mM

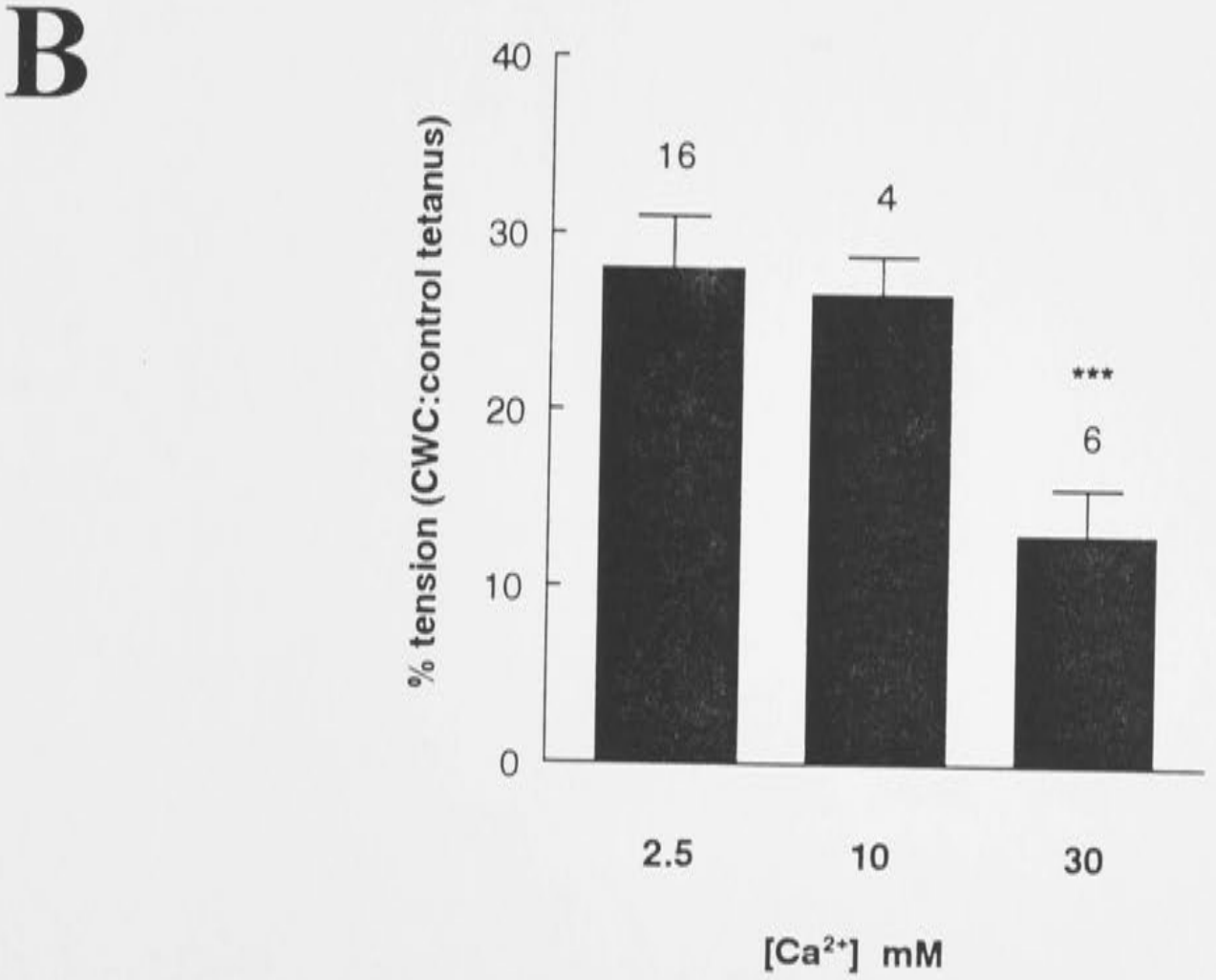
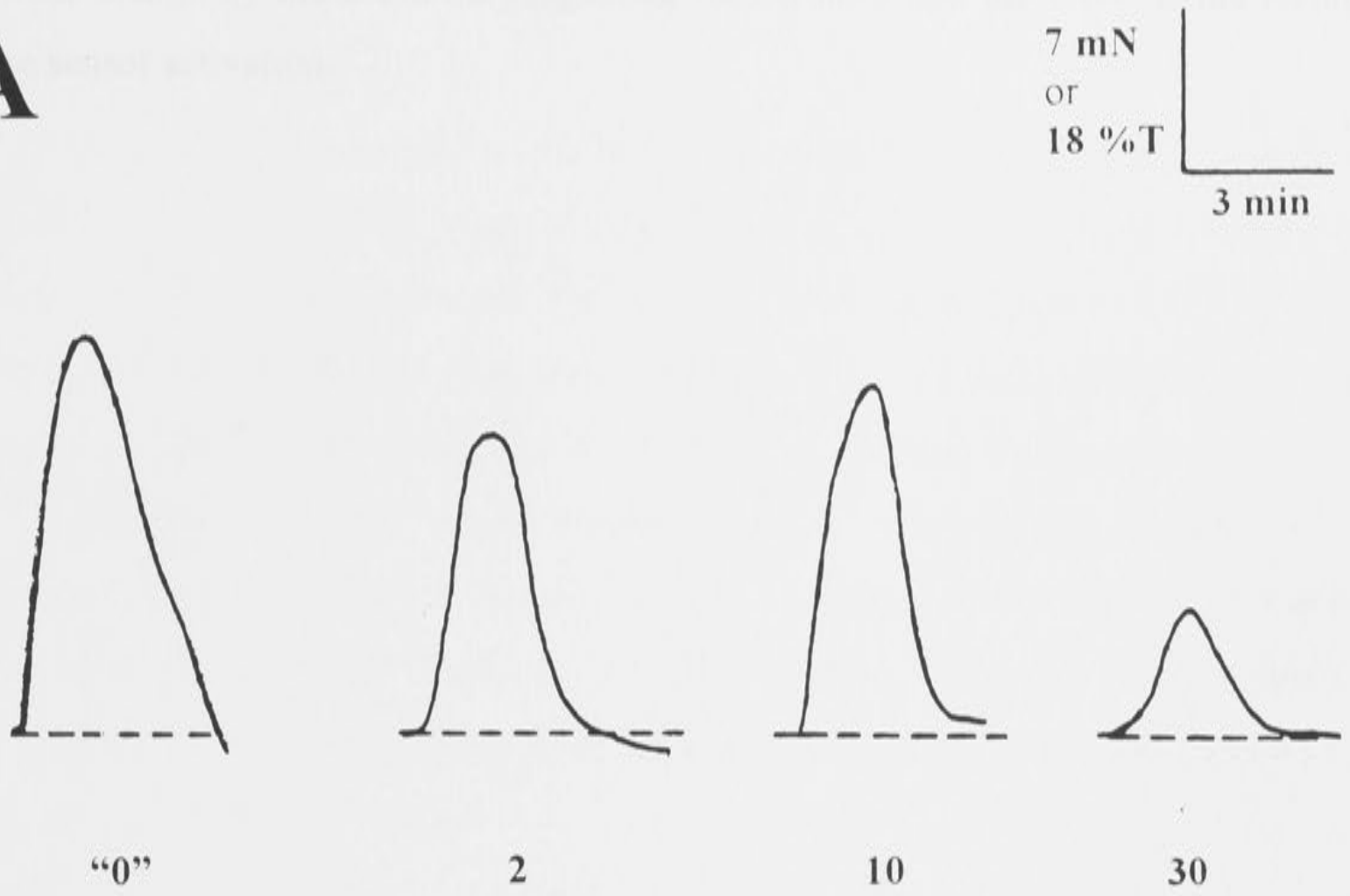

B
% tension (CWC:control tetanus)
40
30
20
10
0
16
4
***
6
2.5
10
30
[Ca2+] mM

Figure 3.14 *Mg^{2+} weakly modulates the Cd^{2+}-withdrawal contracture*

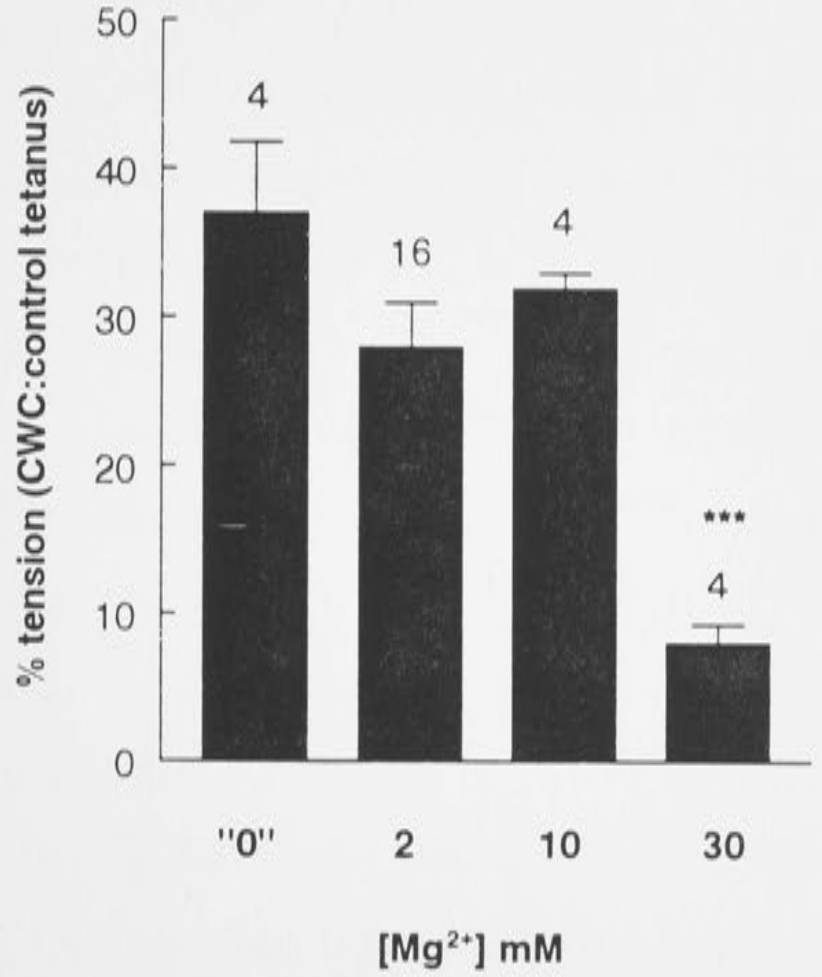
(A) Records showing CWC's evoked in rat soleus fibres following a 20 minute incubation in 3 mM Cd^{2+} at varying [Mg^{2+}]. The procedure is essentially the same as in Fig. 3.13 except that the [Ca^{2+}] was kept constant at 2.5 mM. The "control" CWC response was evoked in 2 mM Mg^{2+}. The records are from different preparations. **(B)** The columns represent average CWC tension (normalised to control tetanic tension) evoked in the presence of 2, 10 or 30 mM Mg^{2+}. Vertical bars show ± 1 SEM. The number of preparations are indicated above each error bar, and *** indicates a value significantly different from the control ($P \leq 0.05$).

Fig. 3.14

A


7 mN
or
18 %T
3 min
"0"
2
10
30
[Mg2+] mM

B


% tension (CWC:control tetanus)
50
40
30
20
10
0
4
16
4
***
4
"0"
2
10
30
[Mg2+] mM

of surface charge by these cations suggesting furthermore that the CWC is the result of voltage sensor activation.

3.4 Discussion

Rat soleus fibres exposed to a solution containing 3 mM Cd^{2+} for a minimum of 5 minutes produced a CWC upon washout of the cation. The amplitude of this contracture was strongly dependent on both the incubation time in Cd^{2+} and Cd^{2+} concentration. The withdrawal contracture appears to be a specific effect of Cd^{2+} since the washout of Zn^{2+}, Co^{2+} and La^{3+} did not produce a response, when present at the same concentration.

Again, since the CWC occurs rapidly upon Cd^{2+} washout, it is assumed that the mechanism of this contracture is the result of Cd^{2+} acting on an external site on the fibre surface membrane. At the beginning of this Chapter, some possible mechanisms underlying the CWC were proposed. Each of these mechanisms are now discussed below in context with the results obtained.

Membrane potential

The CWC is not the result of surface membrane depolarisation since neither the addition nor washout of 3 mM Cd^{2+} affected V_m. Similar results were also obtained by Dulhunty and Gage (1989).

Calcium influx

The failure of low Ca^{2+}, 50 μM nifedipine and 2 mM Co^{2+} to block the CWC suggests that a Ca^{2+} current is not the underlying mechanism of this contracture.

The possibility that the CWC was induced by a Ca^{2+} influx upon Cd^{2+} washout was suggested when the CWC was depressed in the absence of external Ca^{2+} but not in the absence of Mg^{2+}. The possibility that an L-type Ca^{2+} channel was involved was investigated initially. 1μM nifedipine had no effect on CWC amplitude. However, recent evidence suggests that 1μM nifedipine may have been ineffective in blocking L-type Ca^{2+} channels in the presence of Cd^{2+}. Peterson and Catterall (1995) reported that the binding of dihydropyridines to L-type Ca^{2+} channels is inhibited by 50 percent in the presence of 6.5 μM Cd^{2+} and fully inhibited by 100 μM Cd^{2+}. The L-type Ca^{2+} channel dihydropyridine binding site is thought to be allosterically coupled to a Ca^{2+} binding site located within the pore of the channel. Binding of Ca^{2+} to this site theoretically stabilises

the dihydropyridine binding site in a high affinity conformation. Cd^{2+} probably causes a reduction of dihydropyridine binding by interacting with the Ca^{2+} binding site. Based on these observations, it is highly likely that the effectiveness of 1μM nifedipine in blocking a Ca^{2+} influx through L-type Ca^{2+} channels in this study with 3 mM Cd^{2+} would have been severely reduced. Indeed, the failure of 50 μM nifedipine to block the CWC might also be attributed to inhibition of nifedipine binding by Cd^{2+}. The co-application of 3 mM Cd^{2+} with 2 mM Co^{2+}, a non-specific Ca^{2+} channel blocker, once again significantly reduced but did not abolish the CWC. Thus whilst the failure of nifedipine to block the CWC could be explained by a reduction of nifedipine binding in the presence of Cd^{2+}, the depression rather than the abolition of the CWC by all of the above treatments suggests that the contracture is not the result of a Ca^{2+} influx.

Activation of the voltage sensor

Voltage sensor activation during Cd^{2+}-withdrawal was strongly indicated when CWC's were reversibly abolished by inactivating the voltage sensor in a solution containing 40 mM K^+ prior to Cd^{2+} washout. In addition, the reduction of CWC amplitude in low Ca^{2+}, 50 μM nifedipine and 2 mM Co^{2+} can also be explained by a CWC induced by voltage sensor activation, since the voltage sensor would be depressed by each of these treatments (see below)..

Activation of the skeletal muscle voltage sensor is strongly dependent on the presence of extracellular Ca^{2+}. In the absence of Ca^{2+}, E-C coupling is depressed due to a more rapid onset of contractile inactivation (see Chapter 1, Section 1.8.1). An effect of low Ca^{2+} on the voltage sensor could explain why the CWC was depressed but not abolished when Ca^{2+} was removed from the bathing solution. Treatment of fibres with 50 μM nifedipine also caused a reduction but did not abolish the CWC. Since voltage sensor activation/inactivation is accelerated by 50μM nifedipine (Dulhunty & Gage, 1988), this result also implies that the CWC is dependent on voltage sensor activation. The reduction of CWC amplitude in the presence of 2 mM Co^{2+} can also be explained by a CWC mechanism based on voltage sensor activation since Co^{2+} has been shown to have a stronger effect than Ca^{2+} or Mg^{2+} on charge screening/binding at the fibre surface and

to cause a shift in the voltage dependence of contractile activation towards more positive membrane potentials (Dulhunty and Gage, 1989).

Results from experiments in which the effect of increasing or decreasing the concentrations of other cations on the CWC further support a CWC mechanism based on voltage sensor activation. High (30 mM) Ca^{2+} or Mg^{2+} severely depressed CWC amplitude. This result does not support a Ca^{2+} influx mechanism for the CWC since one would expect the contracture amplitude to be larger in elevated Ca^{2+} solutions as a result of increased Ca^{2+} driving force, or unaffected if the Ca^{2+} conductance became saturated at high Ca^{2+} concentrations. On the other hand, both Mg^{2+} and Ca^{2+} at 30 mM would exert a considerable screening effect on membrane surface charge (as previously described for Co^{2+}) which would hyperpolarise the membrane field and shift the threshold for voltage sensor activation and contraction towards more positive potentials. Conversely, the CWC was potentiated in the absence of external Mg^{2+}. Since the omitted Mg^{2+} was not replaced by another divalent cation, this effect can be explained by increased activation of the voltage sensor due to a reduction of charge screening by Mg^{2+}.

Thus the results strongly suggest that the CWC is the result of voltage sensor activation and not a Ca^{2+} influx. However, the site of Cd^{2+} action and the mechanism by which the voltage sensor becomes activated during Cd^{2+}-withdrawal are not known. The CWC cannot be explained by the removal of a charge screening effect exerted by Cd^{2+} at the membrane surface since the total divalent cation concentration was kept constant during washout by replacing Cd^{2+} with Mg^{2+}. Furthermore, the washout of Co^{2+}, Zn^{2+} and La^{3+} which are reported to exert equal if not greater charge screening effects than Cd^{2+} (Blaustein and Goldman, 1968; Dorrscheidt-Kafer, 1981) did not result in a contracture. The differential effect of Cd^{2+} to other cations can be explained if Cd^{2+} binds to a site with a much higher affinity than other cations, or if the site is only accessible to Cd^{2+}.

Cd^{2+} binding sites

Oba and colleagues (1992), proposed that the contractile activating effects of Ag^{+} are the result of an interaction between Ag^{+} and critical sulfhydryl groups present on the voltage sensor protein. The importance of sulfhydryl groups in E-C coupling is realized

in experiments by Caputo and coworkers (1993) who found that voltage activated contraction, but not caffeine contractures are inhibited by the sulfhydryl oxidants para-chloromercuribenzoic acid (PCMB) and para-hydroxymercuriphenylsulfonic acid (PHMPS) in frog skeletal fibres. The depressing effects of these compounds could be prevented or reversed by dithiothreitol (DTT) which reduces oxidised sulfhydryl groups. The observation that charge movement is also affected by PCMB (Gonzalez *et al.*, 1993) suggests that the critical sulfhydryl groups are located on the voltage sensor. Cd^{2+} which is also known to react potently with sulfhydryl groups (Begenisich and Lynch, 1974; Jacobson and Turner, 1980), protected fibres against the charge movement depressing effects of PCMB (Gonzalez *et al.*, 1993), which suggested that Cd^{2+} may also bind to the voltage sensor. A direct interaction between Cd^{2+} and the voltage sensor is supported by evidence given in Chapter 4 and observations that Cd^{2+} alters the kinetics of charge movement (Hui , 1991). It is possible that occupation of sites on the voltage sensor by Ca^{2+} stabilises the protein in its resting conformation just as Ca^{2+} is reported to stabilise the DHPR in a high affinity conformation for binding DHP'S (Peterson and Catterall, 1995). Since La^{3+}, and probably also Cd^{2+}, displace Ca^{2+} from membrane sites (Weiss, 1970; Langer and Frank, 1972), it seems reasonable to suggest that Cd^{2+} might displace Ca^{2+} from putative binding sites on the voltage sensor. Interestingly, Cd^{2+} is reported to have an ionic crystal radius similar to Ca^{2+} (Trosper and Philipson, 1983), a factor that might allow Cd^{2+} to interact with Ca^{2+} binding sites which are inaccessible to other cations. As Cd^{2+} exerts a greater effect on charge screening/binding than Ca^{2+} (Blaustein and Goldman, 1968; Dorrscheidt-Kafer, 1981), it is suggested that the binding of Cd^{2+} to these charged sites causes a hyperpolarisation of the membrane field in the vicinity of the voltage sensor. A hyperpolarising effect of Cd^{2+} on the membrane field is indicated in Chapter 4 (below) where Cd^{2+} is shown to cause a positive shift in the curve relating K contracture tension to membrane potential.

A hypothetical model to explain the Cd^{2+} -withdrawal contracture

A hypothesis to explain the CWC is that the sudden removal of Cd^{2+} from the binding sites both on the membrane and voltage sensor causes a transient depolarisation of the membrane field to values more positive than the threshold for voltage sensor

activation and contraction. A model summarising this hypothesis for the CWC is shown in Fig. 3.15

The primary assumption in the hypothesis (Dulhunty and Gage, 1988), is that depolarisation of the T-tubule membrane, or an equivalent reduction in the membrane field, beyond a threshold level causes activation of the voltage sensor for E-C coupling. Activation of the voltage sensor causes Ca^{2+} release from the SR and tension is generated when myoplasmic [Ca^{2+}] exceeds the threshold for activation of the contractile proteins.

The second assumption is that Cd^{2+} binds with low affinity to a relatively inaccessible, but Cd^{2+}-specific, site on the voltage sensor. The high concentrations (>3mM) and long exposure times (> 4 mins) required before a contracture is induced, may in part be due to restricted Cd^{2+} diffusion through the T-tubules, but also suggests that the binding site is relatively inaccessible and/or of low affinity. The rapid onset of the contracture when Cd^{2+} is removed from the solution is again indicative of a low affinity site, while the length of the contracture (~ 3 mins) suggests that Cd^{2+} may be removed slowly.

The third assumption is that the Cd^{2+} binding site is negatively charged and contributes significantly to the electrical field "seen" by the voltage sensor. The negative charge is neutralised when Cd^{2+} binds to the site, so that there is an increase (hyperpolarisation) in the field across the membrane (Fig. 3.15). A specific effect of Cd^{2+} increasing the membrane field has been reported previously (Dulhunty and Gage, 1989) and is indicated by a shift to more positive potentials in the tension vs membrane potential curve derived from K contracture experiments in Chapter 4.

The final assumption is that Cd^{2+} unbinding from the site causes a gross conformational change in the voltage sensor which results in a higher than normal concentration of negative charges being exposed on the fibre surface. Therefore, the membrane field is reduced beyond the threshold for activation of the voltage sensor and Ca^{2+} release from the SR and hence a withdrawal contracture develops (Fig. 3.15). The conformational change relaxes with time and the membrane field returns to its resting value. As a consequence, the voltage sensor is deactivated and the withdrawal contracture tension falls to zero when the membrane field increases to potentials more negative than the threshold for contraction (Fig. 3.15). It is necessary to postulate that there is a

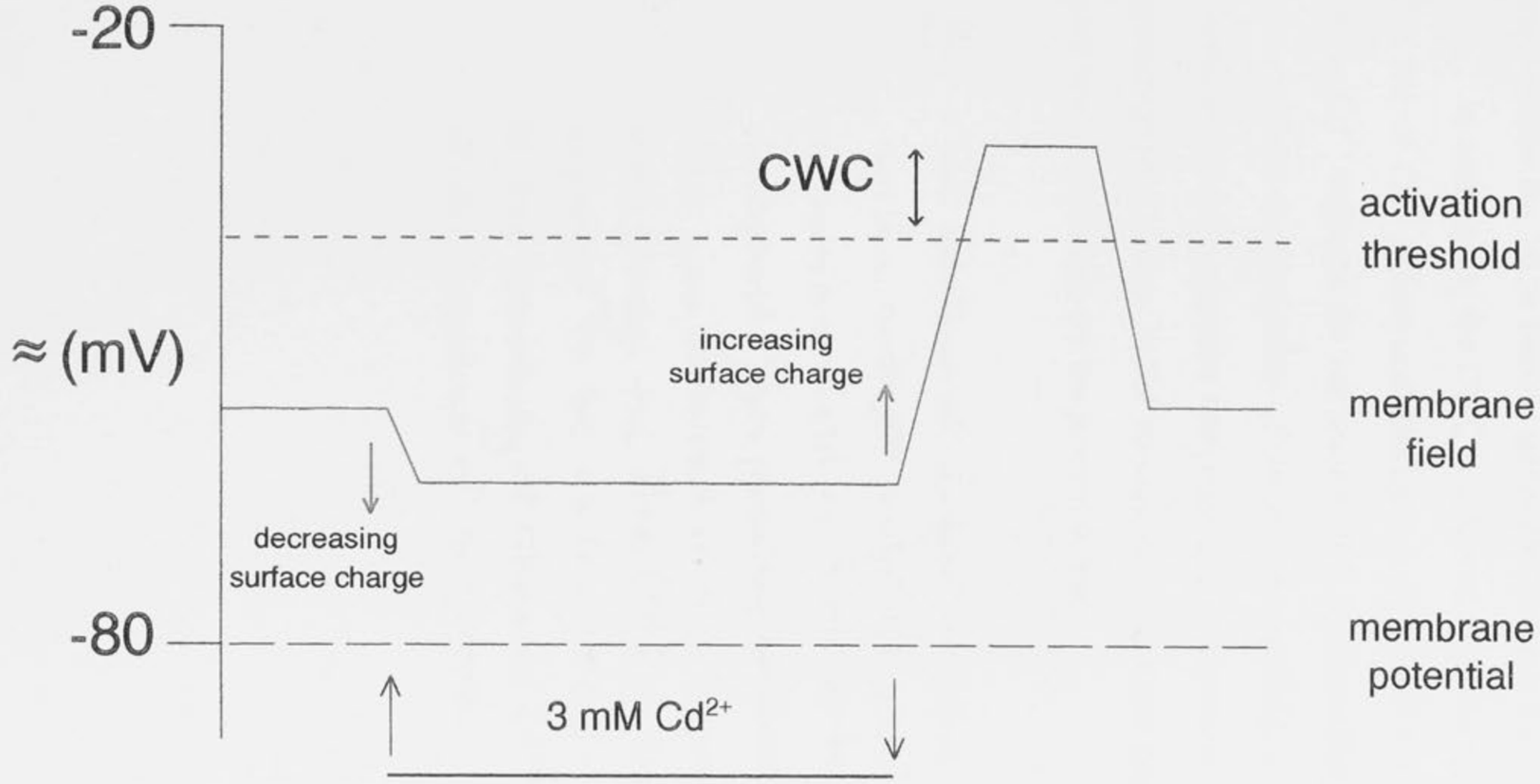


Figure 3.15 *A hypothetical model to explain the Cd^{2+}-withdrawal contracture in rat soleus fibres*

Shown in the model are the threshold potential for voltage sensor activation (dotted purple line), the membrane field (solid blue line), and membrane potential (dotted black line). Approximate potential is shown by the scale on the left in mV. The presence of 3 mM Cd^{2+} is indicated at the bottom of the diagram by the solid black line and the blue arrows show the changes in surface potential which accompany Cd^{2+} addition and removal. Also shown is the depolarisation of the membrane field upon Cd^{2+}-washout to a level more negative than the threshold for activation which is proposed to cause the CWC.

conformational change upon Cd^{2+}-removal in order to reduce the membrane field below contraction threshold. Without this conformational change, the field would simply return to its normal value at which the voltage sensor is resting so that there would be no Ca^{2+} release from the SR or tension generated through the E-C coupling process. *

In conclusion, the CWC is believed to be the result of voltage sensor activation. The site of Cd^{2+} action and mechanism by which the voltage sensor becomes activated during Cd^{2+} washout are not known. It is hypothesised that the CWC could be the result of a transient depolarisation of the membrane field, to potentials more positive than the threshold for voltage sensor activation and contraction. The depolarisation occurs due to a conformational change in the voltage sensor protein during Cd^{2+} washout, which exposes extra negative charges on the protein surface.

* NB:- The addition of further negative charge to the membrane surface such as sulphate would tend to shift the membrane field even closer towards the contraction threshold. This possibly explains why a substantial CWC was obtained in the Solphate containing solutions even though the free [Cd] in these solutions was expected to be as low as 0·2 – 0·4 mM. (see Appendix 1). ie. The sensitivity of fibres to Cd^{2+} appears to be increased in the presence of sulphate.

Chapter 4

The Effect of Cd^{2+} on Twitches, Tetani and K Contractures

4.1 Introduction

In addition to the Cd^{2+} -induced and Cd^{2+} -withdrawal contractures, Cd^{2+} is also reported to affect twitch, tetanus and potassium (K) contracture tension in the following manner (Dulhunty and Gage, 1989):

1. twitches are initially potentiated in 3 mM Cd^{2+} and then depressed;
2. tetanic contractions are depressed;
3. K contracture tension is reduced.

The literature concerning the effects of cations on these contractile parameters is vast. It is generally accepted that cations modulate contraction by a number of different mechanisms including modulation of the voltage sensor for E-C coupling, charge screening and binding effects and blockade of channels involved in the propagation of action potentials (Chapter 1).

In this Chapter, the effect of Cd^{2+} on twitches, tetani and K contracture tension in rat soleus muscle is examined further.

4.2 Aims

1. To characterise the effects of Cd^{2+} on twitch, tetanic and K contracture tension in rat soleus muscle.
2. To determine the mechanisms by which Cd^{2+} produces these effects.

4.3 Results

Part A: Studies on twitches and tetani

4.3.1 *The effect of Cd^{2+} on twitches and tetani*

The effect of 3 mM Cd^{2+} on twitch and tetanic tension in small bundles of rat soleus fibres is shown in Fig. 4.1. Cd^{2+} caused an immediate potentiation of twitches and twitch amplitude increased steadily, reaching an average maximum amplitude of 294.0 ± 35.0 % of control twitch tension (n = 12) after 10 minutes. After longer periods, twitch tension decreased, and after 30 minutes in Cd^{2+}, the average twitch height was reduced to 80.0 ± 6.6 % of control twitch tension (n = 12). Tetanic tension, on the other hand, was gradually depressed in the Cd^{2+} solution reaching an average of only 18.1 ± 1.1 % of control tetanic tension (n = 12) after 30 minutes.

The effect of Cd^{2+} in SO_4^{2-} containing solutions was also examined because such solutions were used during the K contracture experiments presented later in this Chapter. If the bathing solution contained SO_4^{2-} as the principal anion instead of Cl^-, the depressing effects of Cd^{2+} were more severe (Fig. 4.2). Incubation of preparations in a SO_4^{2-} solution also containing 3 mM Cd^{2+} resulted in a brief period of twitch potentiation, followed by a rapid depression of both twitches and tetani. After 10 minutes, twitches were depressed to 50.0 ± 4.4 % of controls (n = 28) and tetanic contractions were reduced to 24.6 ± 1.3 % of control tetanic tension (n = 21).

4.3.2 *Are the effects of Cd^{2+} reversible?*

Since sufficient data concerning the reversibility of the effects of Cd^{2+} on twitches and tetani was obtained from the K contracture experiments (Section 4.3.6), the effects of Cd^{2+} washout on twitches and tetani was not examined specifically. Consequently, the results which are shown in Fig. 4.2 are for Cd^{2+} reversibility in SO_4^{2-} - containing solutions only. In addition, the reversal of the effects on twitch and tetanus of Cd^{2+} applied in Krebs solution were variable and incomplete (see methods, Chapter 2). A 10 minute washout of Cd^{2+} in SO_4^{2-} solutions led to a recovery of peak tetanic tension to 81.8 ± 2.7 % of control (n = 23). Twitches on the other hand

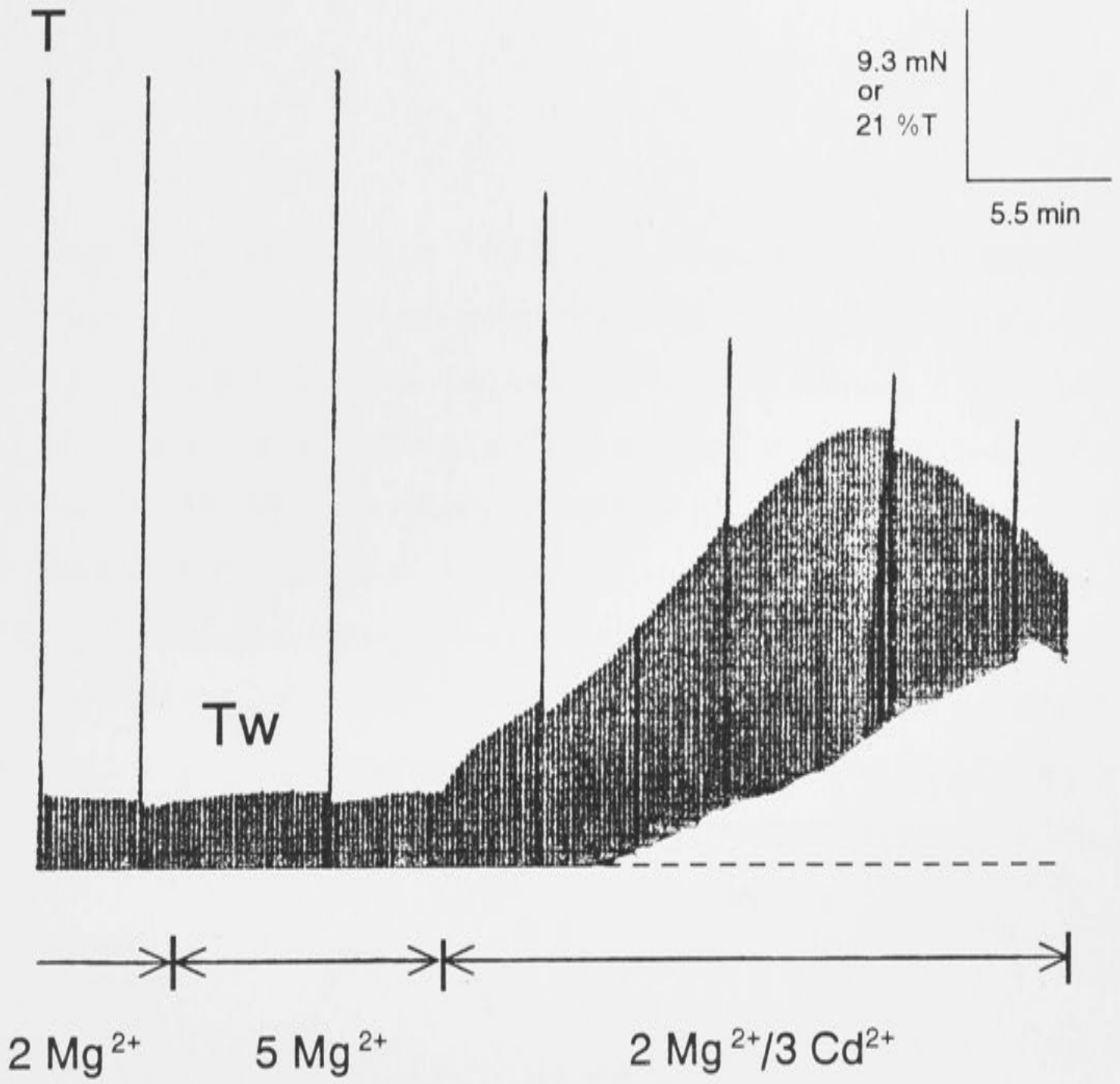


Figure 4.1(1) *The effect of Cd^{2+} on twitch and tetanus*

A record showing the effect of 3 mM Cd^{2+} on twitches (**Tw**) and tetani (**T**) evoked in a small bundle of 10-15 rat soleus fibres. The preparation was initially bathed in a Cl^- Krebs solution (solution 1A, Chapter 2) containing (mM): 150 NaCl, 2 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 2 TES, 11 glucose, pH 7.4 ± 0.1. As described previously, the [Mg^{2+}] was then increased to 5 mM so that there would be no change to the divalent cation concentration when 3 mM Cd^{2+} was substituted for 3 mM Mg^{2+}. Cd^{2+} was then added by replacing the 5 mM Mg^{2+} solution with another containing 3 Cd^{2+}/2 Mg^{2+} (solution 1C). Changes to the bathing solution are indicated below the trace, (all concentrations are in mM). The dotted line represents baseline tension. Note also the Cd^{2+}-induced contracture appearing shortly after Cd^{2+} addition (see Chapter 5). Isometric tension is indicated on the vertical scale bar as force (mN) or as a percentage of the control tetanic tension. Time is indicated on the horizontal scale bar in minutes (min).

Figure 4.1(2) Records showing the effect of Cd^{2+} on individual twitches and tetani Selected twitches (**A**) and tetani (**B**) evoked before and after a 10 or 30 minute exposure to 3 mM Cd^{2+}. All records are from the same preparation. The tension obtained from several preparations is shown below each of the records as a percentage of the control twitch or tetanus tension. Each average is shown ± 1SEM and the number of preparations is indicated in brackets. Dotted lines have been drawn at baseline and control peak tension levels to allow comparison between records easier. The vertical scale bar shows isometric tension as a percentage of the control contraction amplitude (%t). Time is indicated on the horizontal scale bar in seconds (sec). Please note that the force scales for the twitches and tetani records are different.

Fig. 4.1(2)

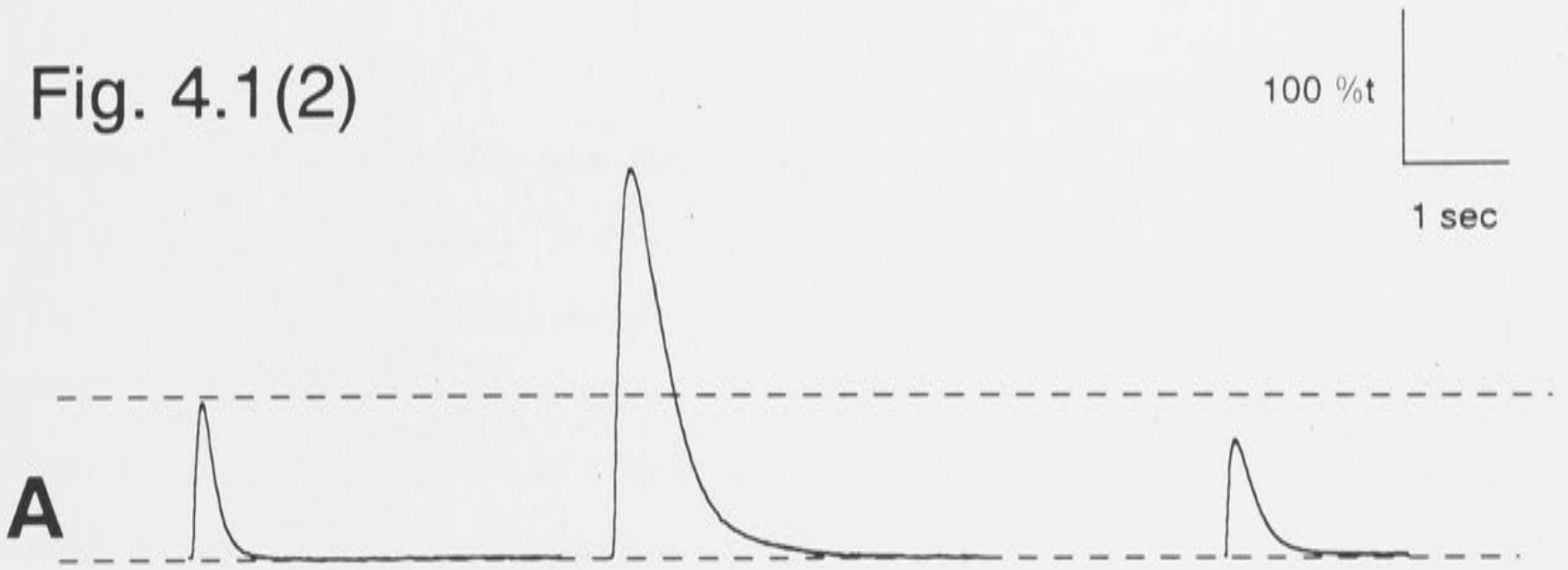
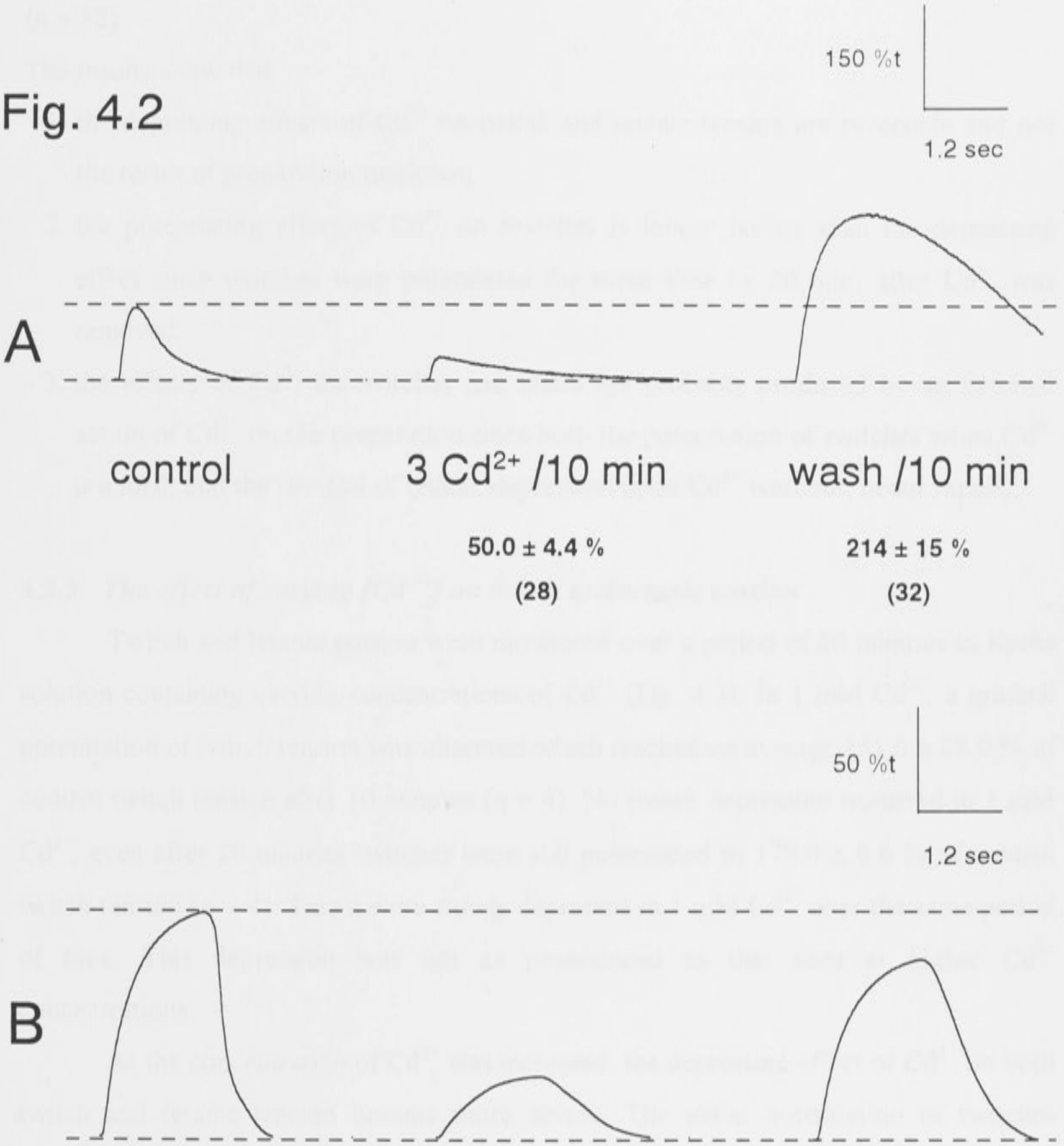

100 %t
1 sec
A
control
3 Cd2+/10 min
294 ± 35 %
(12)
3 Cd2+/30 min
80 ± 6.6 %
(12)
50 %t
1 sec
B
control
3 Cd2+/10 min
90.2 ± 8.02 %
(12)
3 Cd2+/30 min
18.1 ± 1.1 %
(12)

Figure 4.2 *Are the effects of Cd^{2+} reversible?*

Records of twitches (**A**) and tetani (**B**) elicited in control SO_4^{2-} solution (solution 2A, Table 2.2), 10 minutes after the addition of 3 mM Cd^{2+} (solution 2B), and 10 minutes after Cd^{2+} washout into the control solution. All records are from the same preparation. The average twitch or tetanus tension (shown as a percentage of the control contraction) obtained from numerous experiments is shown below each of the records ± 1 SEM. The number of preparations is shown in brackets. Dotted lines have been drawn to represent the baseline and control contraction peak tension levels. Tension is shown on the vertical scale as a percentage of the control contraction amplitude (%t), and time is shown on the horizontal scale in seconds (sec). Note that the tension scales for the twitch and tetanus records are different.

Fig. 4.2


150 %t
1.2 sec
A
control
3 Cd2+ /10 min
wash /10 min
50.0 ± 4.4 %
(28)
214 ± 15 %
(32)
50 %t
1.2 sec
B
control
3 Cd2+ /10 min
wash /10 min
24.6 ± 1.3 %
(21)
81.8 ± 2.7 %
(23)

were potentiated after Cd^{2+} washout to 214.0 ± 15.8 % of control twitch tension (n = 32).

The results show that:

1. the depressing effects of Cd^{2+} on twitch and tetanic tension are reversible and not the result of preparation rundown;
2. the potentiating effect of Cd^{2+} on twitches is longer lasting than the depressing effect since twitches were potentiated for some time (> 20 min) after Cd^{2+} was removed;
3. the effects of Cd^{2+} on twitches and tetani are probably produced by an external action of Cd^{2+} on the preparation since both the potentiation of twitches when Cd^{2+} is added, and the reversal of tetanic depression upon Cd^{2+} washout, occur rapidly.

4.3.3 *The effect of varying [Cd^{2+}] on twitch and tetanic tension*

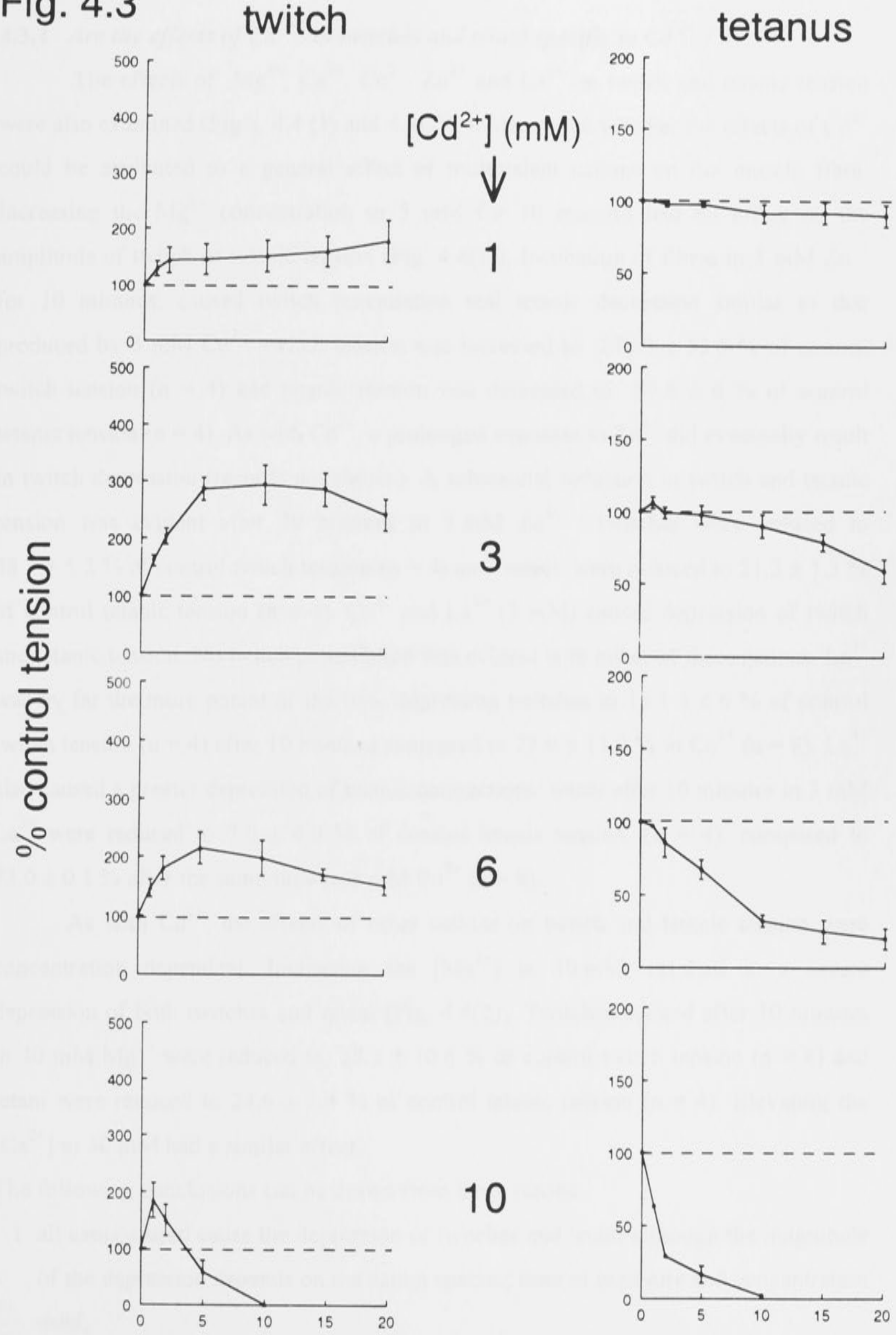
Twitch and tetanic tension were monitored over a period of 20 minutes in Krebs solution containing varying concentrations of Cd^{2+} (Fig. 4.3). In 1 mM Cd^{2+}, a gradual potentiation of twitch tension was observed which reached on average 151.0 ± 28.0 % of control twitch tension after 10 minutes (n = 4). No twitch depression occurred in 1 mM Cd^{2+}, even after 20 minutes twitches were still potentiated to 179.0 ± 6.6 % of control twitch tension (n = 4). Tetani were slowly depressed in 1 mM Cd^{2+} over the same period of time. This depression was not as pronounced as that seen at higher Cd^{2+} concentrations.

As the concentration of Cd^{2+} was increased, the depressing effect of Cd^{2+} on both twitch and tetanic tension became more severe. The initial potentiation of twitches became smaller in magnitude and the duration of potentiation became shorter. In 10 mM Cd^{2+}, both twitches and tetani were almost abolished after 10 minutes: twitch tension was reduced to 0.8 ± 0.9 % of control twitch tension (n = 4), and tetanic tension was reduced to 2.1 ± 0.8 % of control tetanic tension (n = 4). The results show that the time dependant effects of Cd^{2+} on twitch and tetanic tension are related to Cd^{2+} concentration such that the effects occur more rapidly as the concentration of Cd^{2+} is increased.

Figure 4.3 *The effects of Cd^{2+} on twitch and tetanus are dependent on $[Cd^{2+}]$*

The average twitch (left panel) and tetanic (right panel) tension measured at different times during a 20 minute incubation of different preparations in varying $[Cd^{2+}]$. Each point represents the average tension (expressed as a percentage of the control). The vertical bars show ± 1SEM for ≥ 4 preparations. Tension is represented on the vertical axis as a % of the control twitch or tetanus tension. Time in Cd^{2+} is indicated on the X axis (in minutes). Zero time is the point at which Cd^{2+} was added to the bath. The Cd^{2+} concentration (mM) used in each experiment is the number given in the middle of the figure.

Fig. 4.3


twitch
tetanus
[Cd2+] (mM)
1
3
6
10
% control tension
time (min)
time (min)

4.3.4 *Are the effects of Cd^{2+} on twitches and tetani specific to Cd^{2+} ?*

The effects of Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and La^{3+} on twitch and tetanic tension were also examined (Fig's. 4.4 (1) and 4.4(2)), to determine whether the effects of Cd^{2+} could be attributed to a general effect of multivalent cations on the muscle fibre. Increasing the Mg^{2+} concentration to 5 mM for 10 minutes had no effect on the amplitude of twitch or tetanic tension (Fig. 4.4(1)). Incubation of fibres in 3 mM Zn^{2+} for 10 minutes, caused twitch potentiation and tetanic depression similar to that produced by 3 mM Cd^{2+}: twitch tension was increased to 276.0 ± 52.0 % of control twitch tension (n = 4) and tetanic tension was decreased to 89.6 ± 6 % of control tetanic tension (n = 4). As with Cd^{2+}, a prolonged exposure to Zn^{2+} did eventually result in twitch depression (records not shown). A substantial reduction in twitch and tetanic tension was evident after 30 minutes in 3 mM Zn^{2+} : twitches were reduced to 48.7 ± 5.2 % of control twitch tension (n = 4) and tetani were reduced to 21.2 ± 1.3 % of control tetanic tension (n = 4). Co^{2+} and La^{3+} (3 mM) caused depression of twitch and tetanic tension. No twitch potentiation was evident with either of these cations. La^{3+} was by far the more potent of the two, depressing twitches to 15.1 ± 6.6 % of control twitch tension (n = 4) after 10 minutes compared to 73.0 ± 11.0 % in Co^{2+} (n = 8). La^{3+} also caused a greater depression of tetanic contractions: tetani after 10 minutes in 3 mM La^{3+} were reduced to 7.0 ± 4.0 % of control tetanic tension, (n = 4), compared to 73.0 ± 0.1 % after the same time in 3 mM Co^{2+} (n = 8).

As with Cd^{2+}, the effects of other cations on twitch and tetanic tension were concentration dependant. Increasing the $[Mg^{2+}]$ to 30 mM, resulted in a severe depression of both twitches and tetani (Fig. 4.4(2)). Twitches evoked after 10 minutes in 30 mM Mg^{2+} were reduced to 28.2 ± 10.6 % of control twitch tension (n = 4) and tetani were reduced to 24.6 ± 6.4 % of control tetanic tension (n = 4). Elevating the $[Ca^{2+}]$ to 30 mM had a similar effect.

The following conclusions can be drawn from these results:

1. all cations used cause the depression of twitches and tetani although the magnitude of the depression depends on the cation species, time of exposure and concentration used;

Figure 4.4(1) *The effect of Cd^{2+} on twitches and tetani is non-specific*

Selected records of twitches (**A**) and tetani (**B**) evoked following a 10 minute exposure of preparations to 5 mM Mg^{2+}, or 3 mM Cd^{2+}, Co^{2+}, Zn^{2+} or La^{3+}. Tension is shown on the vertical scale bar as a percentage of the control contraction (%t). Time is indicated on the horizontal scale bar in seconds (sec). Please note that the force scales for the twitch and tetanus records are different. Each record is from a different preparation, and the amplitude has been normalised against the control response obtained within the same preparation. A representative control record has been provided for comparison. A dotted line has been drawn at baseline and peak control tension to make the comparison between records easier. The average tension obtained from several preparations is shown below each of the records. The values are shown expressed as a percentage of the control contraction tension ± 1SEM. The number of preparations is indicated in brackets.

Fig. 4.4(1)


250 %t
0.7 sec
A
control
5 Mg^{2+}
3 Cd^{2+}
3 Co^{2+}
3 Zn^{2+}
3 La^{3+}
100 ± 4.1 %
(12)
294 ± 35 %
(12)
73 ± 11 %
(8)
276 ± 52 %
(4)
15.05 ± 6.6 %
(4)

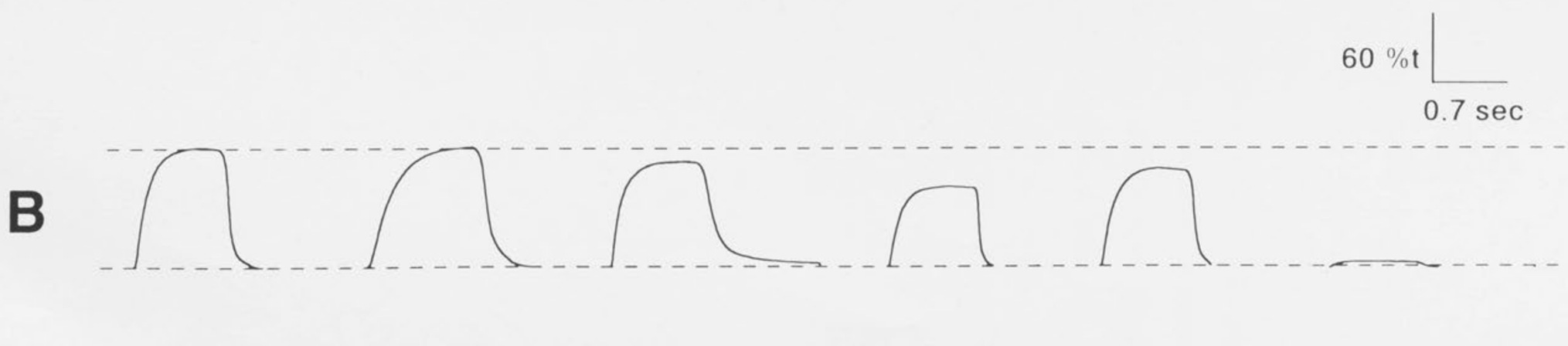
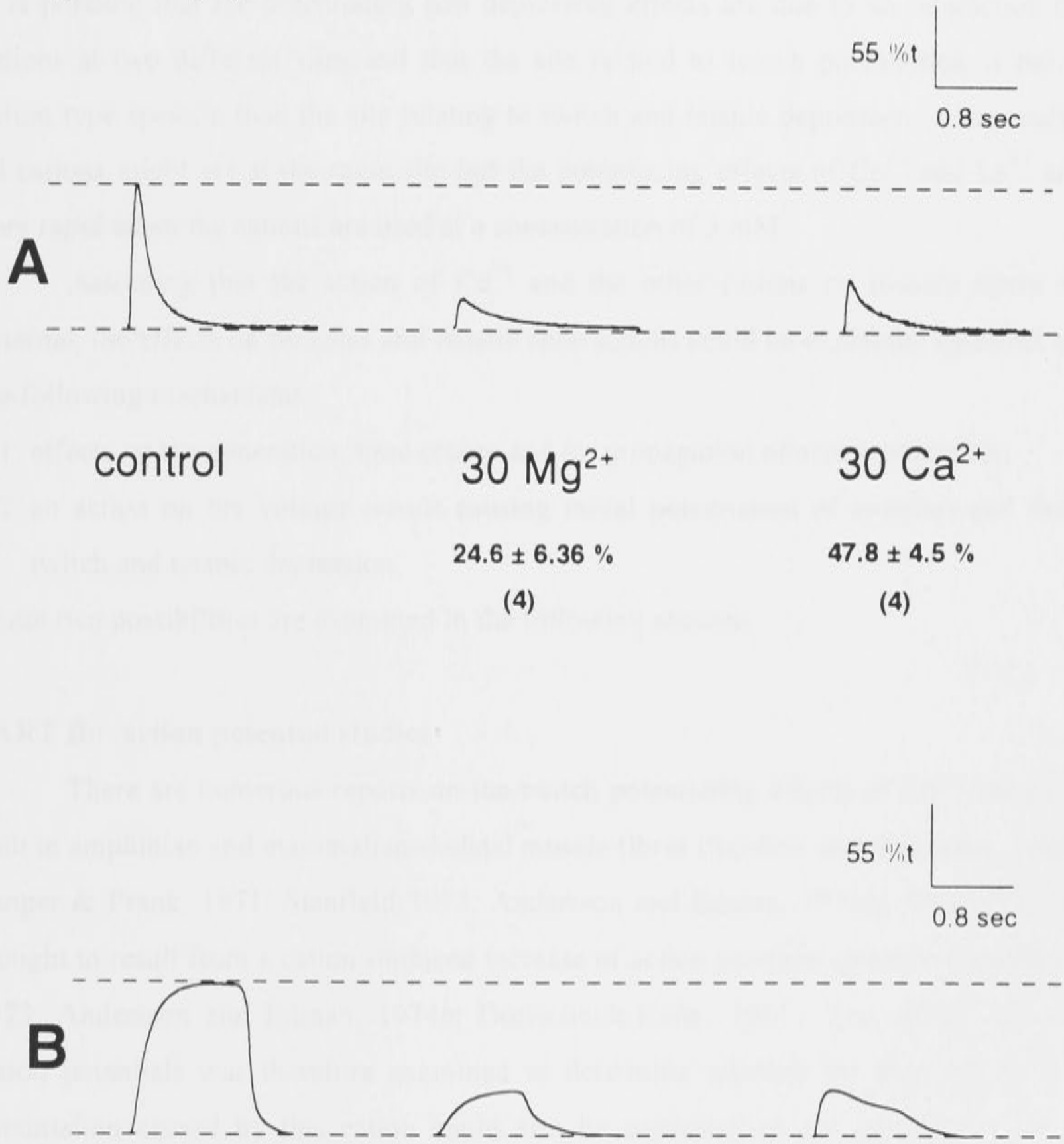

60 %t
0.7 sec
B
control
5 Mg^{2+}
3 Cd^{2+}
3 Co^{2+}
3 Zn^{2+}
3 La^{3+}
98 ± 5.8 %
(12)
90 ± 8.0 %
(12)
73 ± 2.2 %
(8)
89.6 ± 6.0 %
(4)
7.02 ± 4.0 %
(4)

Figure 4.4(2) *The effect of 30 mM Mg^{2+} or Ca^{2+} on twitch and tetanus amplitude*

Selected records of twitches (**A**) and tetani (**B**) evoked before and after a 10 minute exposure of preparations to 30 mM Mg^{2+} or 30 mM Ca^{2+} (Solutions 1M or 1S respectively, Table 2.1). Each record was obtained from a different preparation and the contractile amplitudes have been normalised against the control response obtained from within the same preparation. Representative control twitch and tetanus records obtained from a different preparation are provided for comparison. Dotted lines have been drawn at baseline and peak control contraction tension to make the comparison between records easier. Below each trace, the average tension obtained from 4 preparations is shown ± 1SEM. Each value is expressed as a percentage of the control tension. The number of preparations used in each experiment are shown in brackets.

Fig. 4.4(2)


55 %t
0.8 sec
A
control
30 Mg^{2+}
24.6 ± 6.36 %
(4)
30 Ca^{2+}
47.8 ± 4.5 %
(4)
55 %t
0.8 sec
B
control
30 Mg^{2+}
28.15 ± 10.6 %
(4)
30 Ca^{2+}
32.96 ± 6.7 %
(4)

2. some species of cations (namely Cd^{2+} and Zn^{2+}) also cause initial twitch potentiation suggesting that the two cations may have a similar efficacy and perhaps mechanism of action.

It is possible that the potentiating and depressing effects are due to an interaction of cations at two different sites and that the site related to twitch potentiation is more cation type specific than the site relating to twitch and tetanic depression. Alternately, all cations might act at the same site but the potentiating effects of Co^{2+} and La^{3+} are very rapid when the cations are used at a concentration of 3 mM.

Assuming that the action of Cd^{2+} and the other cations on muscle fibres is external, the effects on twitches and tetanic contractions could be explained by either of the following mechanisms:

1. effects on the generation, time course and /or propagation of action potentials;
2. an action on the voltage sensor causing initial potentiation of twitches and then twitch and tetanic depression.

These two possibilities are examined in the following sections.

PART B: action potential studies

There are numerous reports on the twitch potentiating effects of Zn^{2+} and La^{3+} both in amphibian and mammalian skeletal muscle fibres (Sandow and Isaaccson, 1966; Langer & Frank, 1971; Stanfield 1973; Andersson and Edman, 1974*b*). This effect is thought to result from a cation -induced increase of action potential duration (Stanfield, 1973; Andersson and Edman, 1974*b*; Dorrscheidt-Kafer, 1981). The effect Cd^{2+} on action potentials was therefore examined to determine whether the observed twitch potentiation caused by this cation could also be explained by an increase in action potential duration.

4.3.5 *The effect of Cd^{2+} on action potentials*

Control action potentials were recorded in Krebs solution (solution 1A; Chapter 2) in 2-3 fibres before the 3 mM Cd^{2+} solution (solution 1C) was added to the bath. After 10 minutes in Cd^{2+}, action potentials were recorded in 4-6 different fibres. The results of this experiment are shown in Fig 4.5(1). Usually, only 2-3 action potentials

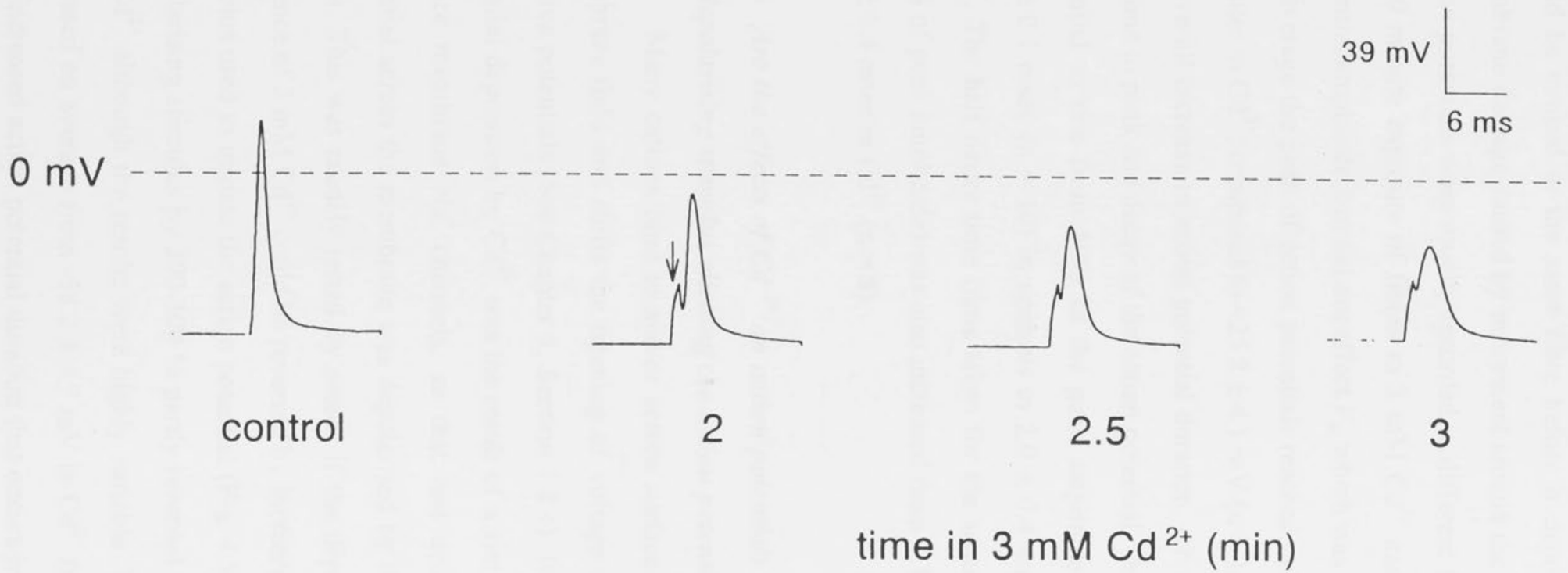


Figure 4.5(1) *The effect of Cd^{2+} on action potentials*

Selected records of action potentials evoked in rat soleus fibres in the presence and absence of 3 mM Cd^{2+}. The record on the left is a control action potential evoked in a Cl^- Krebs solution (solution 1A). The other records show action potentials evoked consecutively in the same fibre following exposure to 3 mM Cd^{2+} (solution 1C). The vertical scale shows membrane potential (mV) and the horizontal scale is time (ms). The dotted line is drawn at a membrane potential of 0 mV. The arrow in the second record shows the depolarisation resulting from the electrotonic spread of the stimulating pulse from the stimulating electrode.

could be evoked in the same fibre before it depolarised, presumably as a result of membrane damage caused by movement around the electrodes. This meant that control action potentials were usually recorded in different fibres from those recorded in Cd^{2+}. A 10 minute exposure of fibres to 3 mM Cd^{2+} caused a marked reduction of action potential amplitude, but did not affect V_m which was on average -78.0 ± 2.6 mV (n = 8). On average the peak of action potentials reached only -58.2 ± 4.7 mV (n = 8) after 10 minutes in Cd^{2+}, compared to +25.2 ± 4.1 mV (n = 10) in the controls. Cd^{2+} also caused an overall increase in action potential duration, which appears to be an effect both on the time to peak and decay of the action potential. On average, time taken for the action potential to rise from 50% of the peak amplitude to the peak was increased from 0.7 ± 0.1 msec (n = 10) in controls to 2.0 ± 0.4 msec (n = 8) after 10 min in 3 mM Cd^{2+}. The half decay time (time taken for the action potential to decay from peak to 50 % of peak amplitude) was also increased from 1.1 ± 0.1 msec (n = 10) in controls to 2.8 ± 0.4 msec in Cd^{2+} (n = 8).

4.3.6 *Are the effects of Cd^{2+} on action potentials reversed by increasing the size of the depolarising stimulus eliciting the action potential*

Many cations bind to and/or screen surface charges which hyperpolarises the membrane field and shifts the opening of voltage dependant channels towards more positive potentials (See Chapter 1, Section 1.8.4). It was therefore possible that action potential depression by Cd^{2+} was the result of a similar shift in the opening/closing of surface membrane Na^+ channels, so that less activation occurred when the gross potential across the membrane was depolarised by the same amount as that in control fibres. This was crudely tested by seeing if the depression of action potentials in the presence of 3 mM Cd^{2+} could be reversed by increasing the strength of the depolarising stimulus used to initiate the action potential (Fig. 4.5(2)). Increasing the strength of the depolarising stimulus by 200-300 % partly reversed the depression of action potentials by Cd^{2+} although the results were highly variable: The peak of action potentials was increased on average from -58.2 ± 4.7 mV in Cd^{2+} (n = 8) to -19.7 ± 15.3 mV (n = 6). The increased action potential duration that occurs in the presence of Cd^{2+} on the other hand was not reversed by this treatment.

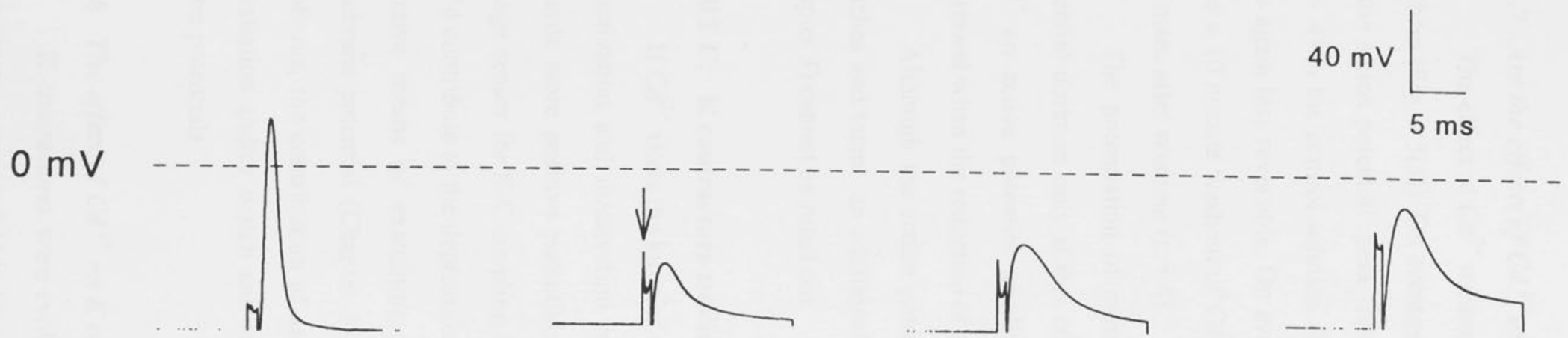


Figure 4.5(2) *Can the effects of Cd^{2+} on action potentials be overcome by increasing the stimulus strength?*

The effects of increasing the strength of the depolarising pulse eliciting the action potential on the amplitude of action potentials depressed by exposure to 3 mM Cd^{2+}. The record on the left is a control action potential recorded in the Cl^- Krebs control solution (solution 1A). The other records show action potentials evoked consecutively (left to right) in a different fibre following a 10 minute exposure to 3 mM Cd^{2+}. The size of the depolarising stimulus has been increased in each record. The increase in stimulus strength is seen in the increased amplitude of the electrotonically spread depolarisation preceding each action potential (marked by an arrow in the second record). Membrane potential (mV) and time (ms) are shown on the scale bars, and the dotted line has been drawn at a membrane potential of 0 mV.

4.3.7 *Are the effects of Cd^{2+} on action potentials reversed by Cd^{2+} washout?*

The effect of Cd^{2+} washout on the recovery of action potentials was examined in 4 fibres (Fig. 4.5(3)). On average, a 10 minute washout of Cd^{2+} led to a partial recovery of the action potential peak from -58.23 ± 4.66 mV in 3 mM Cd^{2+} to -10.7 ± 8.9 mV (n = 4) in the control solution. The increased duration of the action potential by Cd^{2+} was again less reversible. On average, the half decay time of action potentials evoked after a 10 minute washout of Cd^{2+} had decreased from 2.8 ± 0.4 msec in Cd^{2+} to 2.3 ± 0.5 msec after washout (n = 4).

The potentiation of twitches by Cd^{2+} can be explained by increased action potential duration only if this effect on twitches overwhelms the depressing effect of Cd^{2+} on action potential amplitude. Thus, it is likely that twitches are eventually depressed when the reduction of action potential amplitude exerts a stronger effect.

Although the action potential observations can explain the effects of Cd^{2+} on twitches and tetani, an additional effect of Cd^{2+} on the voltage sensor (as suggested in Chapter 3) cannot be ruled out.

PART C: K contracture studies

If Cd^{2+} alters the kinetics of action potentials by shifting the voltage dependence of activation and inactivation of channels involved in action potential propagation towards more positive potentials, the effective hyperpolarisation could influence the voltage sensor for E-C coupling in a similar way. Such an effect on the voltage sensor could contribute to the depression of twitches and tetani by Cd^{2+}. K contractures are an effective means of examining the response of the voltage sensor to changes in membrane potential (Chapter 1). By examining the effect of Cd^{2+} on K contracture amplitude, the contribution of an effect of Cd^{2+} on the voltage sensor towards twitch potentiation and/or twitch and tetanic depression can be isolated from its effects on action potentials.

4.3.8 *The effect of Cd^{2+} on K contracture tension*

K contractures were evoked using [K^+] ranging from 30-200 mM (Chapter 2). Contractures were evoked in fibres before and 20 minutes after Cd^{2+} addition, and after

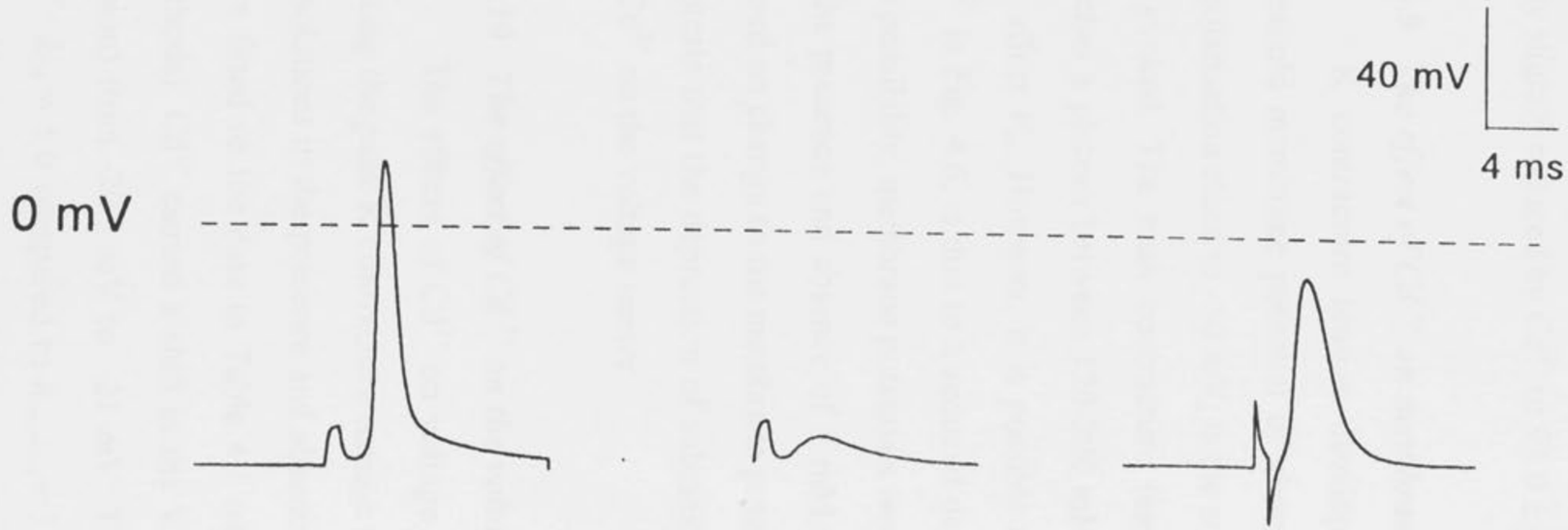


Figure 4.5(3) *Are the effects of Cd^{2+} on action potentials reversed by Cd^{2+} washout?*

Selected records of action potentials recorded in control Krebs, after 10 minutes in 3 mM Cd^{2+} and 10 minutes after Cd^{2+} washout in the control solution. Records were obtained from different fibres. Membrane potential (mV) and time (ms) are shown on the scale bars. The dotted line is drawn at a membrane potential of 0 mV.

Cd^{2+} washout. The effects of 3 mM Cd^{2+} on 40, 80 and 200K contracture tension is shown in Fig. 4.6. The average results obtained with 30, 40, 60, 80, 120 and 200K are shown in Table 4.1. Cd^{2+}* (3 mM) caused a depression of submaximal K contracture tension in rat soleus fibres. Maximal 200K contracture tension on the other hand was only slightly reduced by Cd^{2+} to 93.0 ± 4.5 % of control 200K tension (n = 12).

4.3.9 *The effect of Cd^{2+} on membrane depolarisation in high K^+ solutions*

K contracture tension develops only when fibres are depolarised beyond a threshold membrane potential of about -50 mV. Thus, 20 mM K^+ which produces a depolarisation close to -50 mV, is the smallest [K^+] with which a contractile response can be evoked. The peak contracture tension is graded with [K^+] beyond 20 mM, and reaches a plateau between 120-200 mM K^+. In Chapter 3 it was shown that Cd^{2+} does not affect V_m. However, it is possible that the depression of K contracture tension by Cd^{2+} in Fig. 4.6, is due to a reduced depolarisation in the elevated K^+ solutions. To test this possibility, membrane potentials were measured in each of the high K^+ solutions both in the presence and absence of 3 mM Cd^{2+}. The results are shown in Table 4.1. Cd^{2+} caused no change to the membrane potential measured in any of the solutions. This result suggests that the depression of submaximal K contracture tension is therefore an effect of Cd^{2+} on the voltage sensor.

4.3.10 *The effect of Cd^{2+} on the voltage sensor*

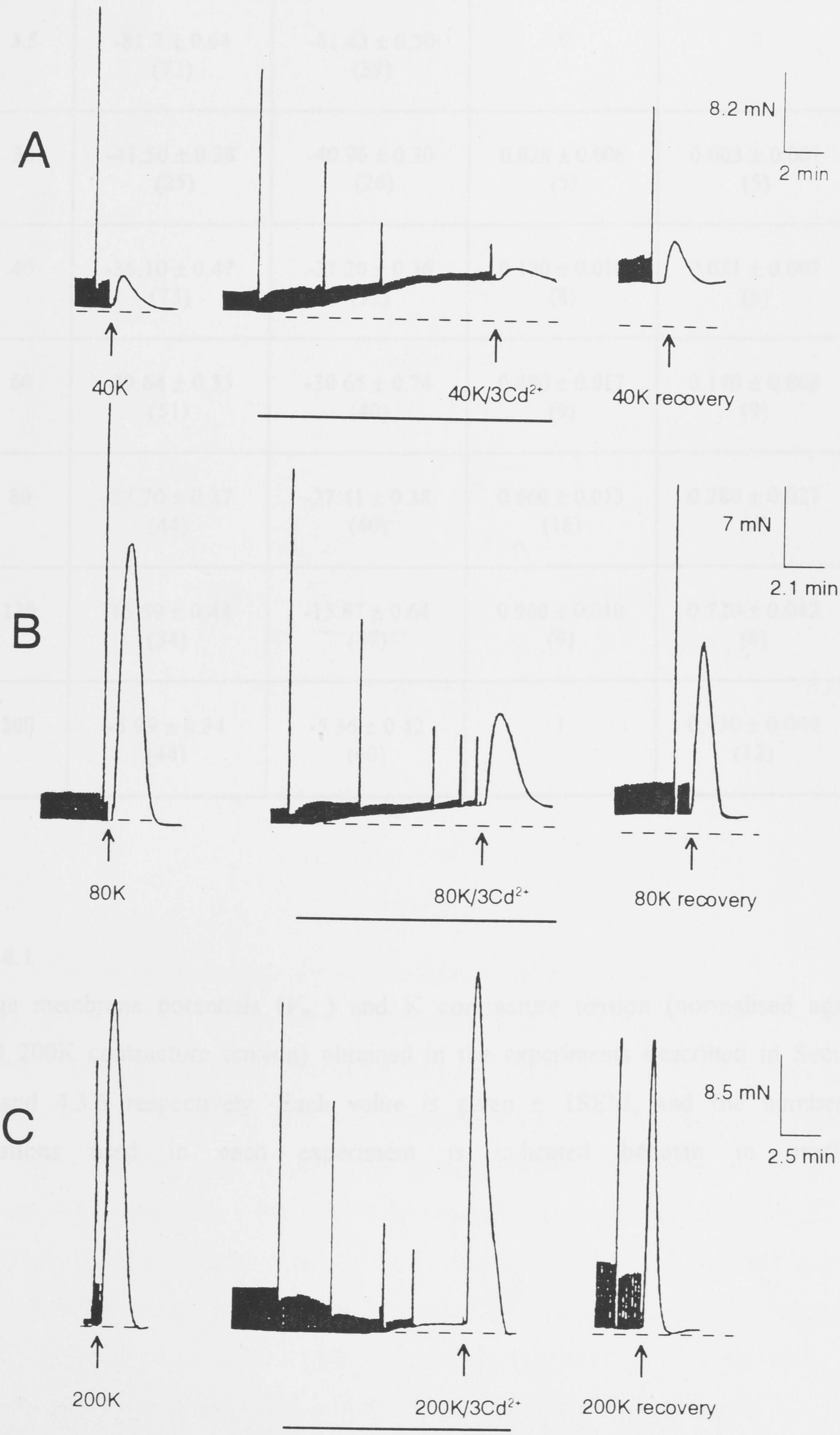
The effects of Cd^{2+} on voltage sensor activation were determined from curves relating the peak K contracture tension to membrane potential in the corresponding high K^+ solutions in the presence and absence of 3 mM Cd^{2+} (Fig. 4.7). Boltzmann equations were fitted to the data in Table 4.1 using the method outlined in Chapter 2 (General Methods). Cd^{2+} caused a shift in the $V_{0.5}$ (membrane potential relating to half maximal tension) from -29.3 mV to -21 mV. The slope of the curve (k) was also less steep in Cd^{2+}: k_{Cd} = 5.0 compared to $k_{control}$ = 3.7. V_{max} on the other hand was not substantially altered by Cd^{2+}. This result shows that Cd^{2+} causes a shift in the voltage dependence of contractile activation towards more positive membrane potentials. Such an effect could contribute to the depression of twitches and tetani by this cation, but would be expected

* NB: Although referred to as 3mM Cd^{2+}, the free [Cd^{2+}] in SO_4^{2-} containing solutions is expected to be somewhat less than 3mM. See Appendix 1 for the approximate free [cation] of the SO_4^{2-} solutions.

Figure 4.6 *The effect of Cd^{2+} on K contracture tension*

Records showing the effect of 3 mM Cd^{2+} on 40, 80 and 200K contracture tension. Each trace (A, B, and C) shows a control potassium contracture, a contracture evoked in the same $[K^+]$ after 10 min exposure to 3 mM Cd^{2+} and another control contracture evoked following Cd^{2+} washout. All 3 contractures at each $[K^+]$ were evoked in the same preparation, but different preparations were used for each $[K^+]$. The large vertical deflections are tetanic contractions and the smaller deflections are twitches. Fibres were bathed in the SO_4^{2-} solution identical to that described in Fig 4.2. Potassium contractures were evoked by rapidly replacing the control SO_4^{2-} solution for another containing elevated potassium, (indicated below each record by the arrows). Following each contracture, the elevated potassium solution was replaced by the control solution and the tetanic tension was allowed to recover. The solid bar below each trace represents the presence of 3 mM Cd^{2+} in the bathing solution whilst the dotted line indicates baseline tension. Contractile force (mN) and time (min) are indicated on the vertical and horizontal scale bars respectively.

Fig. 4.6


A
8.2 mN
2 min
40K
40K/3Cd2+
40K recovery
B
7 mN
2.1 min
80K
80K/3Cd2+
80K recovery
C
8.5 mN
2.5 min
200K
200K/3Cd2+
200K recovery

$[K^+]$ mM	V_m (mV)		Relative tension	
	Control	3 mM Cd^{2+}	Control	3 mM Cd^{2+}
3.5	-81.7 ± 0.64 (72)	-81.43 ± 0.50 (39)	0	0
30	-41.50 ± 0.38 (25)	-40.96 ± 0.30 (26)	0.028 ± 0.006 (5)	0.003 ± 0.001 (5)
40	-36.10 ± 0.47 (73)	-37.20 ± 0.36 (32)	0.130 ± 0.010 (8)	0.031 ± 0.007 (6)
60	-29.64 ± 0.33 (51)	-30.65 ± 0.74 (40)	0.480 ± 0.017 (9)	0.140 ± 0.008 (9)
80	-27.70 ± 0.37 (44)	-27.41 ± 0.38 (40)	0.660 ± 0.013 (16)	0.280 ± 0.027 (6)
120	-16.59 ± 0.44 (34)	-15.87 ± 0.64 (47)	0.960 ± 0.010 (9)	0.720 ± 0.042 (8)
200	-5.99 ± 0.34 (44)	-5.36 ± 0.42 (60)	1	0.930 ± 0.049 (12)

Table 4.1

Average membrane potentials (V_m) and K contracture tension (normalised against control 200K contracture tension) obtained in the experiments described in Sections 4.3.7 and 4.3.6 respectively. Each value is given ± 1SEM, and the number of preparations used in each experiment is indicated beneath in brackets.

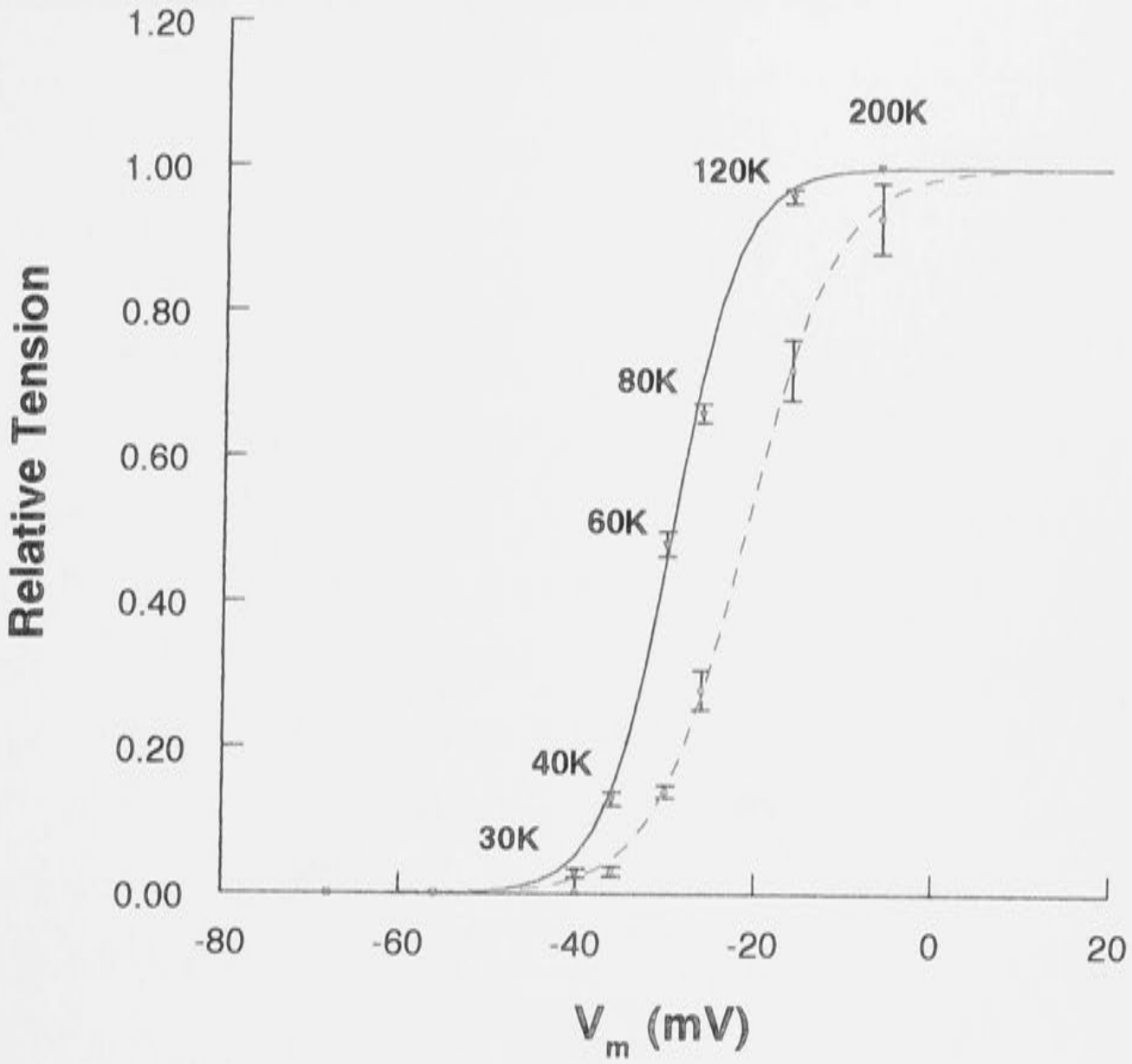


Figure 4.7 *Cd^{2+} shifts the voltage dependence of contractile activation*

The effects of 3 mM Cd^{2+} on the voltage dependence of contractile activation in rat soleus fibres. Contractile activation curves for preparations bathed in control (solid blue line) and 3 mM Cd^{2+} (dotted green line) solutions are shown. Average normalised (Chapter 2) K contracture tension obtained in each of the elevated potassium solutions listed in Table 4.1 is plotted as a function of the membrane potentials measured in these solutions. The solid blue triangles represent the average control data and the solid green circles represent the average data obtained in 3 mM Cd^{2+}. Vertical bars show ± 1SEM. The $[K^+]$ corresponding to each data point is shown (in mM). Curves generated using a Boltzmann equation were fitted to the data by a "least squares method" (Chapter 2).

to have a greater effect on twitches (submaximal contractions) than on tetani (near maximal contractions) according to the results shown in Fig. 4.7.

4.4 Discussion

The results demonstrate that Cd^{2+} has a very strong effect on twitch, tetanic and K contracture tension in rat soleus fibres. Some possible mechanisms underlying the observed effects are discussed below.

Part A: The effect of Cd^{2+} on twitches and tetani

Application of 3 mM Cd^{2+} to rat soleus fibres caused an immediate potentiation of twitch tension which was later followed by twitch depression. Tetanic contractions on the other hand were steadily depressed by Cd^{2+} although the depression did not always occur immediately. Since control peak tetanic tension represents the maximum force output of the preparation, potentiation of the tetanus by Cd^{2+}, cannot occur. Thus an initial period of potentiated SR Ca^{2+} release could explain the delay that was observed in some preparations before the onset of tetanic depression.

The depressing effect of Cd^{2+} on twitches and tetani became more severe and occurred more rapidly as the $[Cd^{2+}]$ increased. The magnitude of the initial twitch potentiation, on the other hand, became smaller and more brief with increasing $[Cd^{2+}]$ indicating that the depressing effect of Cd^{2+} dominated its potentiating effects.

The increase of twitch tension was not restricted to Cd^{2+} since potentiation of a similar magnitude followed by subsequent depression was also observed in preparations exposed to 3 mM Zn^{2+}. As with Cd^{2+}, tetanic contractions were steadily depressed by Zn^{2+}. Similar potentiating effects of Cd^{2+} and Zn^{2+} on twitch tension have been reported in frog sartorius (Sandow and Isaaccson, 1966) and frog semitendinosis fibres (Stanfield, 1973) although the concentrations used in these studies were much lower (0.1-0.5 mM). In contrast to Cd^{2+} and Zn^{2+}, 3 mM Co^{2+} and La^{3+} caused depression of both twitches and tetani with no potentiating effects. Other studies show the effects of La^{3+} to be concentration dependent, with twitch potentiation occurring at a $[La^{3+}]$ of 0.3 mM (Andersson and Edman, 1974*b*) and depression leading to eventual loss of muscle excitability in $[La^{3+}] > 0.5$ mM (Parry *et al.*, 1974; Andersson and Edman, 1974*b*). Thus, a potentiating effect of Co^{2+} and La^{3+} may have been seen in the present study if lower concentrations of Co^{2+} and La^{3+} had been used.

The depression of twitch and tetanic tension by 3 mM Cd^{2+} was largely reversible upon washout, provided the incubation period was no longer than ≈ 30 minutes. Twitch potentiation on the other hand remained after Cd^{2+} washout and did not appear to be reversible. This observation suggests that the mechanism for twitch potentiation by Cd^{2+} differs from the mechanism of twitch and tetanic depression.

Part B: The effect of Cd^{2+} on action potentials

It was thought that twitch potentiation by Zn^{2+} and La^{3+} in other studies, was the result of these cations increasing action potential duration (Stanfield, 1973; Dorrscheidt-Kafer, 1981). The results from the present study show that Cd^{2+} has similar effects, because action potentials evoked after 2 minutes or more in 3 mM Cd^{2+} were substantially longer both in time to peak and decay than control action potentials. The mechanism by which Cd^{2+} increases action potential duration is not known. Zn^{2+} and La^{3+} are thought to slow action potentials by reducing the activation of the delayed rectifier K^+ channels which are involved in membrane repolarisation during the decay phase of an action potential (Stanfield, 1970; Ildefonse and Rougier, 1971; Dorrscheidt-Kafer, 1981). A slowing of Na^+ channel inactivation by Cd^{2+} binding to sites on the channel protein which affect channel kinetics might also contribute to increased action potential duration. Another possibility, is that the increase of action potential duration is due to a reduction of Cl^- conductance since both Zn^{2+} and Cd^{2+} are both reported to block surface membrane Cl^- channels (Stanfield, 1970 ; Bretag, 1987) and Cl^- movement is important to fibre repolarisation during action potentials in twitch muscles (Heiny *et al.*, 1990; Hille, 1992).

In addition to the effects on action potential duration, Cd^{2+} caused a severe depression of action potential amplitude. This result might explain the depression of twitches and tetani in the presence of this cation. Similar depressing effects on action potential amplitude have been reported for Cd^{2+} in lobster giant axon (Takata, 1966b) and La^{3+} in frog sartorius fibres (Dorrsheidt-Kafer, 1981). The literature shows that the depression of action potential amplitude only occurs when divalent cations are used at concentrations >1 mM whereas the effect of divalent cations to increase action potential duration is generally observed at concentrations < 0.5 mM. This could explain why

twitch and tetanic depression occurred more rapidly as [Cd^{2+}] was increased, whereas twitch potentiation without depression was observed at lower [Cd^{2+}].

Cations are reported to affect action potentials by shifting the voltage dependence of ion channel opening and closing towards more positive potentials by a screening binding effect on surface charge (Blaustein and Goldman, 1968; D'Arrigo, 1973; Dorrscheidt-Kafer, 1981; Hille, 1992). Charge binding rather than screening by Cd^{2+} was considered to be the most likely mechanism for changes in twitch and tetanic tension because although all of the divalent cations tested in the present study caused twitch and tetanic depression, there was a definite order of efficacy. For example, 5 mM Mg^{2+} had no effect, on the amplitude of twitches or tetani but increasing the [Mg^{2+}] to 30 mM led to twitch and tetanic depression of a magnitude similar to that produced by 6 mM Cd^{2+} or 3 mM La^{3+} over the same time. The order of efficacy is probably related to different binding affinities for the various cations to sites on the fibre surface membrane. Benitah and colleagues (1996) list a number of factors which might influence the affinity with which cations bind to a particular site.

These factors are:

1. the electrostatic potential of the binding site;
2. p*ka* of the coordinating groups;
3. steric accessibility of the binding site;
4. the degree of strain introduced into the protein to accommodate the metal ion.

Subtle differences in the physical properties of the ion, such as ionic radius and overall geometry would be sufficient to determine how well the ion would bind.

Since action potential amplitude is largely governed by opening of voltage dependant Na^{+} channels, it was considered that depression of twitch and tetanic contractions by Cd^{2+} and the other cations may be the result of the cations binding to surface charges, causing a shift of the voltage dependence of Na^{+} channel opening towards more positive potentials. Various authors have proposed orders of efficacy between different cations to neutralise surface charge and cause voltage shifts in channel gating. The gating of Na^{+} channels in particular is reported to be highly sensitive to changes in external cation concentration (see Fig. 1.11, Chapter 1). Blaustein, and Goldman (1968) report the following order of potency by which cations

cause a positive shift in the activation of Na^+ channels in voltage clamped lobster axons:

$$La^{3+} > Zn^{2+} \approx Co^{2+} > Cd^{2+} \approx Ba^{2+} > Ca^{2+} > Mg^{2+}$$

Interestingly, this order of potency is similar to the efficacy of cations used in the present study, in causing twitch and tetanic depression which was: $La^{3+} >> Co^{2+} > Zn^{2+} \geq Cd^{2+} > Ca^{2+} \approx Mg^{2+}$. This order is an approximation since some of the depressing effects of Zn^{2+} and Cd^{2+} may have initially been masked by twitch potentiation. In Section 4.3.6, the strength of the depolarising pulse eliciting the action potential was increased in an attempt to overcome the effects of a possible shift in the voltage gating of Na^+ channels towards more positive potentials. Increasing the stimulus strength resulted in a partial recovery of the depressed action potential amplitude, but had no effect on the increased duration. This result suggests that the depression of action potential amplitude is at least partially caused by a Cd^{2+}-induced shift in the voltage dependence of channel activation. The increased action potential duration on the other hand, may not be due to an effect of Cd^{2+} on the voltage dependence of channel activation since increasing the stimulus amplitude did not reverse this effect.

Apart from effects on Na^+ channel gating, Na^+ channel block by Cd^{2+} might also contribute to action potential depression. Cd^{2+} is reported to block skeletal muscle Na^+ channels although not as effectively as the cardiac isoform of the Na^+ channel (Benitah, 1989; Backx, *et al.*, 1992; Satin *et al.*, 1992). Single channel recordings from Na^+ channels expressed in Xenopus oocytes show that the cardiac channel is blocked by 100 μM Cd^{2+} (Backx *et al.*, 1992) a concentration which does not effect the skeletal channel (Gellens *et al.*, 1992). However, the skeletal channel was blocked if the $[Cd^{2+}]$ was increased to 2 mM (Backx *et al.*, 1992), providing yet another reason why in the present study, twitch and tetanic depression was not observed at $[Cd^{2+}] < 1$ mM. The difference in Cd^{2+} sensitivity between the skeletal and cardiac Na^+ channel isoforms has been localised to a single amino acid site in the pore lining region of the channel, the "P loop" (Perez-Garcia *et al.*, 1996, Chiamvimonvat *et al.*, 1996). In cardiac channels, this site contains a cysteine residue which is known to have a high affinity for Cd^{2+} and Zn^{2+} (Martell and Smith, 1989). In the skeletal channel, the same site contains a

tyrosine residue which binds Cd^{2+} with a lower affinity than cysteine. Mutation of the tyrosine residue in the skeletal channel to a cysteine produces a Na^+ channel with a cardiac like sensitivity to Cd^{2+} (Perez-Garcia *et al.*, 1996). The block of skeletal Na^+ channels by Cd^{2+} (2 mM) is reported to be voltage dependant with a greater reduction in Na^+ current occurring at more negative potentials (Backx *et al.*, 1992). This observation could also explain why the depression of action potentials by Cd^{2+} in the present study was partially reversed by increasing the strength of the stimulating depolarisation.

Cd^{2+} washout resulted in a partial recovery of action potential amplitude. The blockade of Na^+ channels by Cd^{2+} on the other hand is reported to be fully reversible (Chiamvimonvat *et al.*, 1996), which suggests that the depression of action potentials in the presence of this cation might not be solely the result of Na^+ channel block. However, the Na^+ channels in the study by Chiamvimonvat and coworkers (1996), were expressed in Xenopus oocytes and thus do not necessarily reflect the properties of Na^+ channels present in the surface membrane of soleus fibres. The increase of action potential duration by Cd^{2+} was not reversed upon Cd^{2+} washout. This result correlates well with the observation that twitches remain highly potentiated after Cd^{2+} removal and reinforces the conclusion that twitch potentiation by Cd^{2+} is the result of increased action potential duration. Interestingly, Bretag and colleagues (1984) report that the block of surface membrane Cl^- channels by Cd^{2+} in rat diaphragm muscle is also not reversed by Cd^{2+} removal, further supporting the suggestion that the increased action potential duration in Cd^{2+} is due to a block of Cl^- channels.

In summary the effect of Cd^{2+} to increase action potential duration is likely to be the result of Cl^- and/or K^+ channel block because:

1. the increased action potential duration is not reversed by increasing stimulus strength;
2. a block of Cl^- or K^+ channels would not necessarily depend on membrane field;
3. action potential duration is not decreased by Cd^{2+} washout and the block of K^+ and Cl^- channels is not necessarily reversible

The depressing effect of Cd^{2+} on action potential amplitude on the other hand, is likely to be due to a combination of shifts in Na^+ channel gating arising from surface charge

screening/binding and Na^+ channel block.

Part C: The effect of Cd^{2+} on K contractures

3 mM Cd^{2+} caused a reduction in submaximal K contracture tension in rat soleus fibres. This indicated a Cd^{2+}-induced shift in the voltage dependence of contractile activation by 8.3 mV towards more positive membrane potentials since maximal tension was not affected.. This result is consistent with those obtained in the original study by Dulhunty and Gage (1989) and with the reported depressing effects of other cation species on K contractures including Zn^{2+}, La^{3+}, Ni^{2+}, Mn^{2+} and Co^{2+} (Oota *et al.*, 1972; Parry *et al.*, 1974; Dorrscheidt-Kafer and Luttgau, 1974; Caputo, 1981; Lorkovic and Rudel, 1984; Dulhunty and Gage, 1989). This voltage shift is probably mediated by an indirect action of Cd^{2+} on the voltage sensor by screening/binding of membrane surface charges. A shift in the voltage dependence of contractile activation by Cd^{2+} might also contribute substantially to the depression of twitches and tetani by this cation and would explain why the depressing effects of Cd^{2+} on twitches, tetani and K contractures were largely reversible. A problem with this explanation is that one would expect a larger depressing effect on twitches which are submaximal contractions compared to maximal tetani. It is possible that the depressing effect of Cd^{2+} on twitches by a positive shift in the contractile activation curve was initially counteracted by the potentiating effects of increased action potential duration.

Cd^{2+} also caused a decrease in the slope of the force activation curve which cannot be explained by a simple voltage shift. Instead it is proposed that the cation also interacts directly with the voltage sensor. An interaction of this kind was proposed in Chapter 3 and has been proposed by Oba *et al.*, (1992) as the mechanism for Ag^+ induced contractures in frog skeletal fibres. Ag^+ supposedly binds to sulfhydryl groups on the DHPR which are thought to be important in modulating the voltage sensor (Oba *et al.*, 1992; Caputo *et al.*, 1993; Gonzalez *et al.*, 1993). Cd^{2+} is known in general to bind with high affinity to protein sulfhydryl groups and an interaction of this kind is proposed to be the mechanism by which heavy metals modulate the RyR Ca^{2+} release channel (Brunder *et al.*, 1988; Abramson *et al.*, 1983; Salama *et al.*, 1992). Thus a direct effect of Cd^{2+} on the voltage sensor is likely.

In conclusion, the following mechanisms and sites of Cd^{2+} action are proposed to explain the observed results:

1. twitch potentiation is attributed to increased action potential duration which could be caused by:
   (a) Cd^{2+} block of K^+ and Cl^- channels involved in action potential repolarisation;
   (b) slowing of Na^+ channel activation/inactivation by Cd^{2+} binding to a site on the channel which affects channel kinetics;
2. depression of twitch and tetanic tension attributed to:
   (a) a decrease in action potential amplitude due to hyperpolarisation of the membrane field which shifts activation of Na^+ channel activation towards more positive potentials or by a block of Na^+ channels;
   (b) a shift in the voltage dependence of contractile activation towards more positive potentials due to hyperpolarisation of the membrane field by binding/screening of surface charge;
3. depression of submaximal K contracture tension indicating:
   (a) a shift in the voltage dependence of contractile activation due to hyperpolarisation of the membrane field;
   (b) a decrease in the slope of the contractile activation curve due to a direct interaction between Cd^{2+} and the voltage sensor.

Chapter 5

The Cd^{2+}-Induced Contracture

5.1 Introduction

The Cd^{2+}-induced contracture (CIC), is a slow, non declining increase in tension which develops in bundles of rat soleus fibres shortly after their exposure to 3 mM Cd^{2+} (Dulhunty and Gage, 1989).

Since the CIC appears in the presence of Cd^{2+}, instead of during Cd^{2+} washout, the mechanism of this contracture is presumed to be different from that of the Cd^{2+}-withdrawal contracture (Chapter 3). Furthermore, the slow development and irreversibility of this contracture suggests that its mechanism differs from those by which Cd^{2+} affects twitch, tetanic and K contracture tension (Chapter 4). The possibility that Cd^{2+} acts externally is investigated here whereas the possibility that Cd^{2+} might enter a muscle fibre and produce tension by directly activating the contractile proteins is considered in Chapter 6.

There are reports in the literature of contraction occurring in the presence of metal cations. However, none of these reports include Cd^{2+}. Oba and colleagues (1992) have reported that Ag^{+} ions cause contraction in frog skeletal muscle fibres, presumably by activating the voltage sensor for E-C coupling.

Metal cations are also reported to induce membrane currents in a wide variety of cell types. The currents are proposed to be the result of an interaction with metal ions and sites on the cell surface (Smith *et al.*, 1989; Kiss and Osipenko, 1994). It is possible that the CIC is the result of such currents.

In this Chapter, the mechanism of the CIC is addressed and the possibilities that it might depend on voltage sensor activation or the induction of membrane currents are considered.

5.2 Aims

1. To further characterise the Cd^{2+}-induced contracture in rat soleus muscle;
2. To determine the mechanism underlying this contracture.

5.3 Results

Part A: Further characterisation of the Cd^{2+}-induced contracture

5.3.1 *The Cd^{2+}-induced contracture*

A typical CIC evoked by the method outlined in Chapter 2 (General Methods) is shown in Fig. 5.1. The contracture which appeared shortly after the addition of 3 mM Cd^{2+} to the bath, progressively increased in amplitude with time, reaching on average (n = 12), 18.4 ± 2.5 % of control tetanic tension after 20 minutes.

5.3.2 *Dependence of the Cd^{2+}-induced contracture amplitude on time in Cd^{2+}*

The dependence of CIC amplitude on incubation time in 3 mM Cd^{2+} is shown in Fig. 5.2. The contracture appeared on average within 4.0 ± 0.4 minutes of Cd^{2+} addition (n = 12). Generally, the CIC amplitude increased steadily with time, up to ~ 20 minutes after which time the rate of increase appeared to slow. However, a great deal of variation in the rates of contracture development was observed between preparations. For example, the average CIC amplitude after 30 minutes reached 21.0 ± 2.6 % of control tetanic tension (n = 12) whereas in some individual preparations (2 of 12), the CIC amplitude after 30 minutes approached that of the control tetanus. Variation in the rate of CIC development was also observed within the same preparation such that CIC tension often appeared to oscillate.

5.3.3 *Dependence of Cd^{2+}-induced contracture amplitude on $[Cd^{2+}]$*

The effect of increasing $[Cd^{2+}]$ on CIC amplitude was examined. CIC tension was measured after a 10 minute exposure of different preparations to $[Cd^{2+}]$, ranging from 1 to 10 mM (Fig. 5.3). A CIC was barely visible after preparations had been incubated in 1 mM Cd^{2+} for 10 minutes: tension reached on average 0.5 ± 0.7 % of control tetanic tension (n = 4). The amplitude of the CIC increased rapidly with higher $[Cd^{2+}]$. CIC tension measured after a 10 minute exposure to 10 mM Cd^{2+} for example, had increased to 30.0 ± 3.8 % of control tetanic tension (n = 6). The results imply that greater tension may have been recorded if higher $[Cd^{2+}]$ had been used. The results so

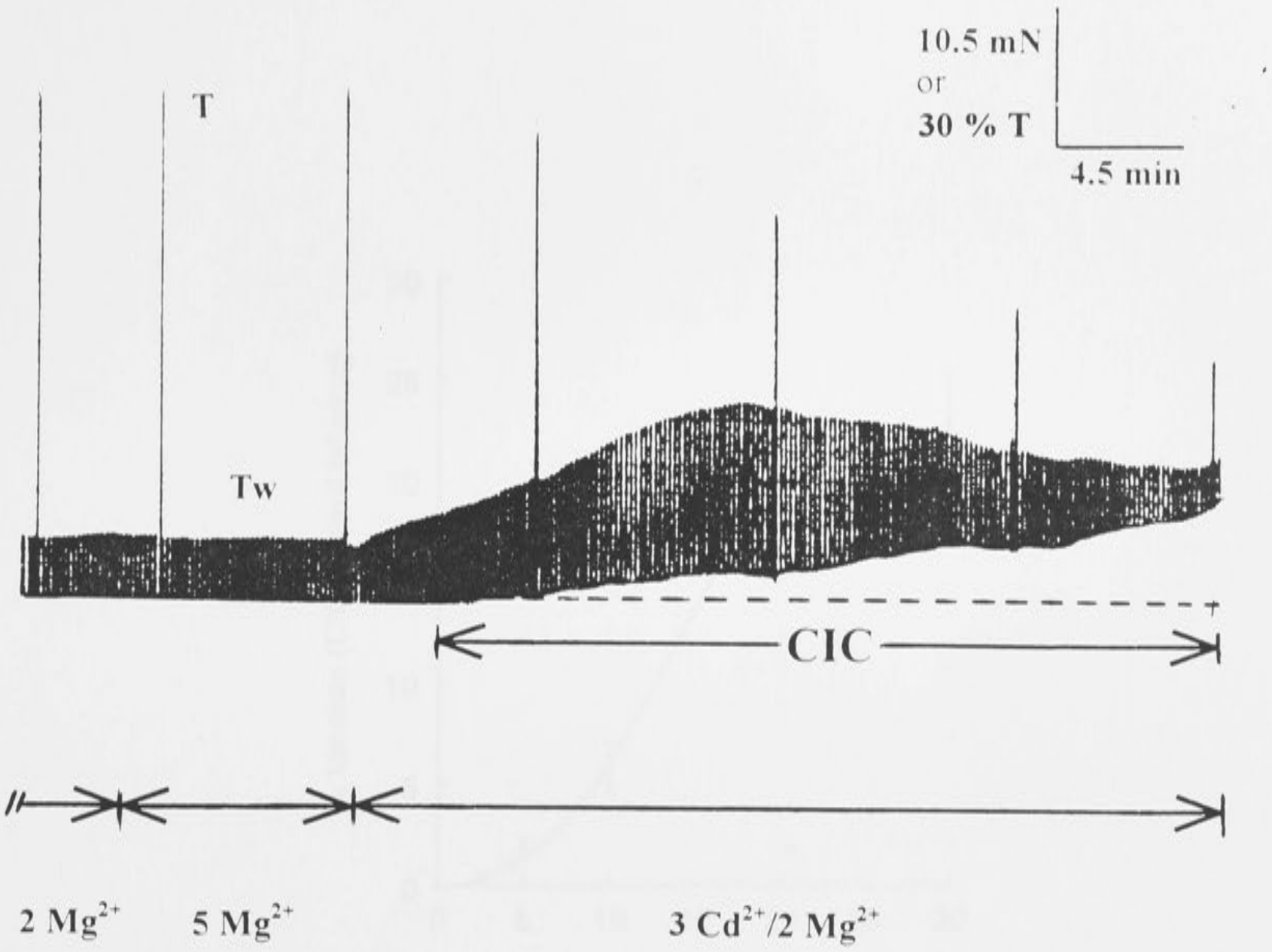


Figure 5.1 *An example of the Cd^{2+}-induced contracture*

A record showing a CIC in rat soleus fibres. The induced contracture is the slow, non-declining increase in tension seen shortly after the addition of 3 mM Cd^{2+} to the bath. Initially, fibres were bathed in Krebs (solution 1A) containing (mM): 150 NaCl, 2 $MgCl_2$, 2 KCl, 2.5 $CaCl_2$, 11 glucose, 2 TES, pH 7.4 ± 0.1. The [Mg^{2+}] of the bathing solution was then increased to 5 mM (solution 1B) for 10 minutes so that there would be no change in divalent [cation] when 3 mM Cd^{2+} was added. Cd^{2+} was then introduced by exchanging the 5 mM Mg^{2+} solution with another containing 3 mM Cd^{2+}/2 mM Mg^{2+} (solution 1C). Changes to the bathing solution are indicated below the record (all divalent cation concentrations are in mM). The dotted line indicates the level of the baseline prior to Cd^{2+} addition. Also shown in the same record are electrically induced twitches (**Tw**), and tetanic contractions (**T**). CIC tension in this and subsequent figures is indicated on the vertical scale bar as force (mN) or as a percentage of the control tetanic tension (%T). Time is indicated on the horizontal scale bar in minutes.

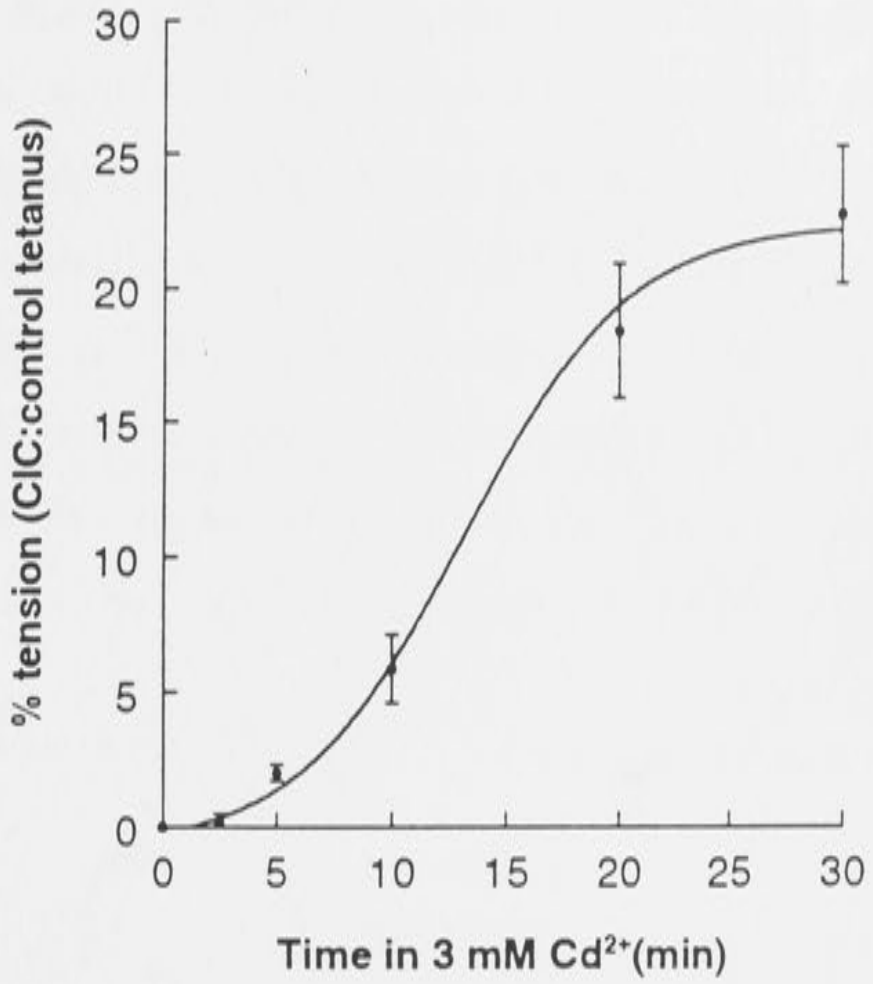


Figure 5.2 *Cd^{2+}-induced contracture amplitude as a function of time*

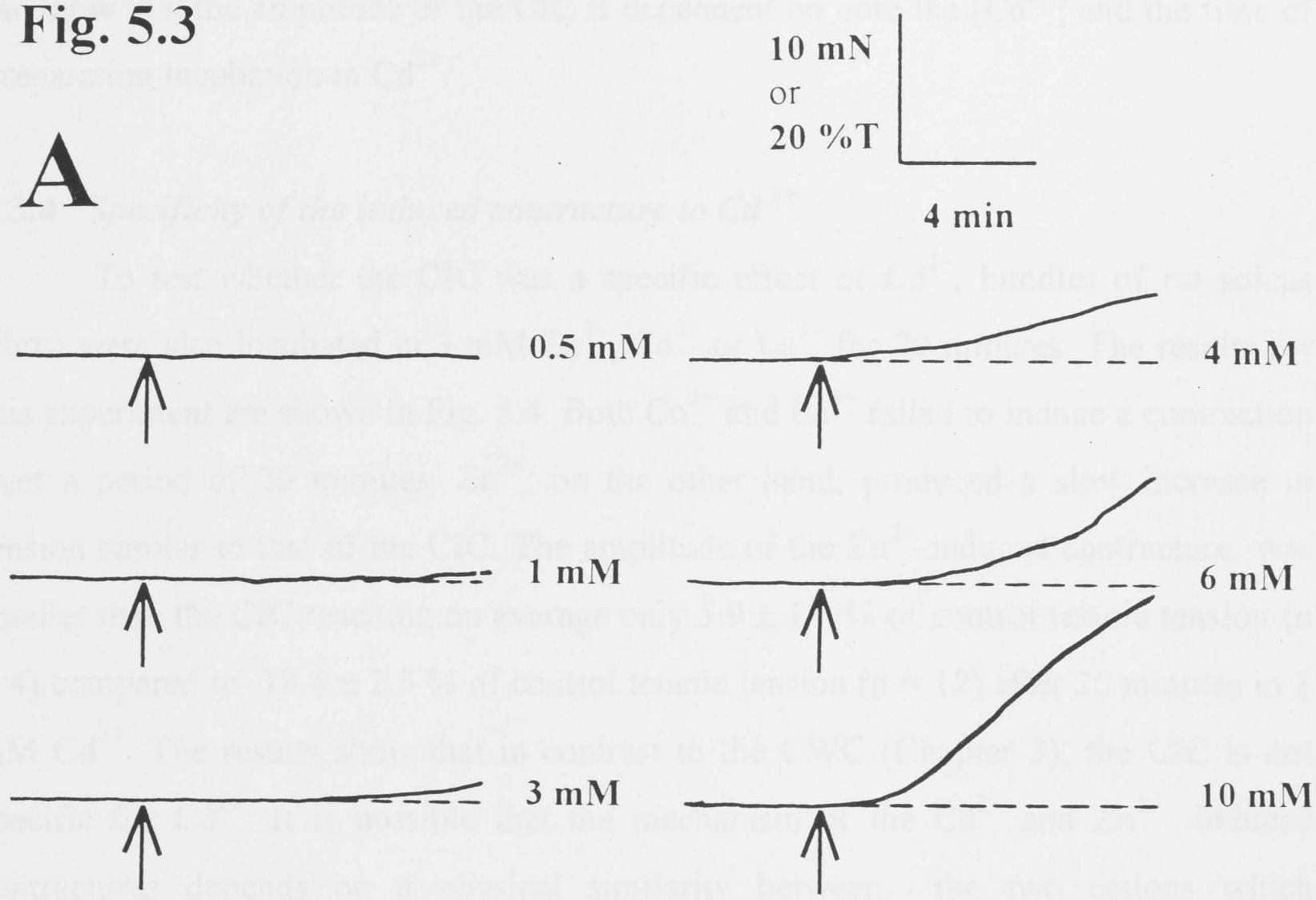
In this graph, the relationship between CIC amplitude (normalised to control tetanic tension) and the time of exposure to 3 mM Cd^{2+} is shown (in minutes). The data points show the average CIC amplitude (obtained from ≥ 4 preparations). Vertical bars indicate ± 1 SEM. A sigmoid function has been fitted to the data but has no physical significance. However, the sigmoidal curve may indicate that the data represents a saturation phenomenon.

Figure 5.3 *Cd^{2+}-induced contracture amplitude as a function of $[Cd^{2+}]$*

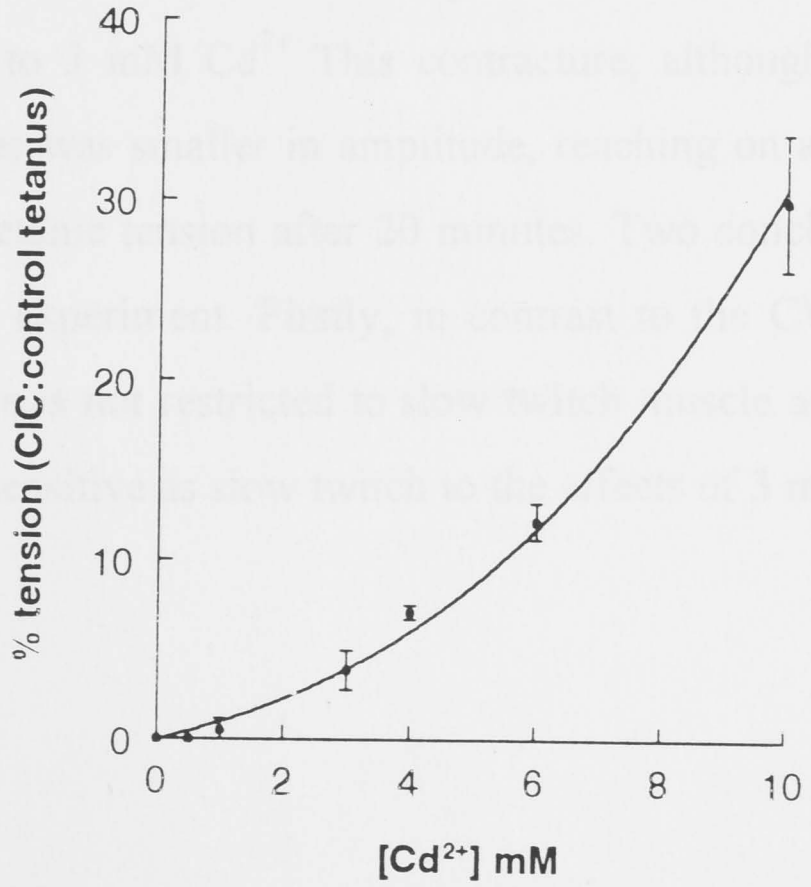
(A) Records showing CIC's produced in different preparations each exposed to a different $[Cd^{2+}]$ for 10 minutes. The arrows indicate the point at which Cd^{2+} was added to the bath and the dotted line represents the baseline. Twitches and tetani have been omitted from this and subsequent figures to allow the CIC to be seen more clearly. The $[Cd^{2+}]$ of the bathing solution is indicated to the right of each record (in mM). **(B)** The average normalised CIC amplitude (obtained from ≥ 4 preparations) produced after a 10 minute incubation at varying $[Cd^{2+}]$ is shown plotted as a function of $[Cd^{2+}]$. Vertical bars show $\pm$ 1 SEM. An exponential function has been fitted to the data but has no physical significance.

Fig. 5.3

A


10 mN
or
20 %T
4 min
0.5 mM
4 mM
1 mM
6 mM
3 mM
10 mM

B


% tension (CIC:control tetanus)
40
30
20
10
0
0
2
4
6
8
10
[Cd2+] mM

far show that the amplitude of the CIC is dependent on both the [Cd^{2+}] and the time of preparation incubation in Cd^{2+}.

5.3.4 *Specificity of the induced contracture to Cd^{2+}*

To test whether the CIC was a specific effect of Cd^{2+}, bundles of rat soleus fibres were also incubated in 3 mM Zn^{2+}, Co^{2+} or La^{3+} for 20 minutes. The results for this experiment are shown in Fig. 5.4. Both Co^{2+} and La^{3+} failed to induce a contraction over a period of 20 minutes. Zn^{2+}, on the other hand, produced a slow increase in tension similar to that of the CIC. The amplitude of the Zn^{2+}-induced contracture, was smaller than the CIC reaching on average only 5.9 ± 1.0 % of control tetanic tension (n = 4) compared to 18.4 ± 2.5 % of control tetanic tension (n = 12) after 20 minutes in 3 mM Cd^{2+}. The results show that in contrast to the CWC (Chapter 3), the CIC is not specific for Cd^{2+}. It is possible that the mechanism of the Cd^{2+} and Zn^{2+} -induced contractures depends on a physical similarity between the two cations which distinguishes them from Co^{2+} and La^{3+}.

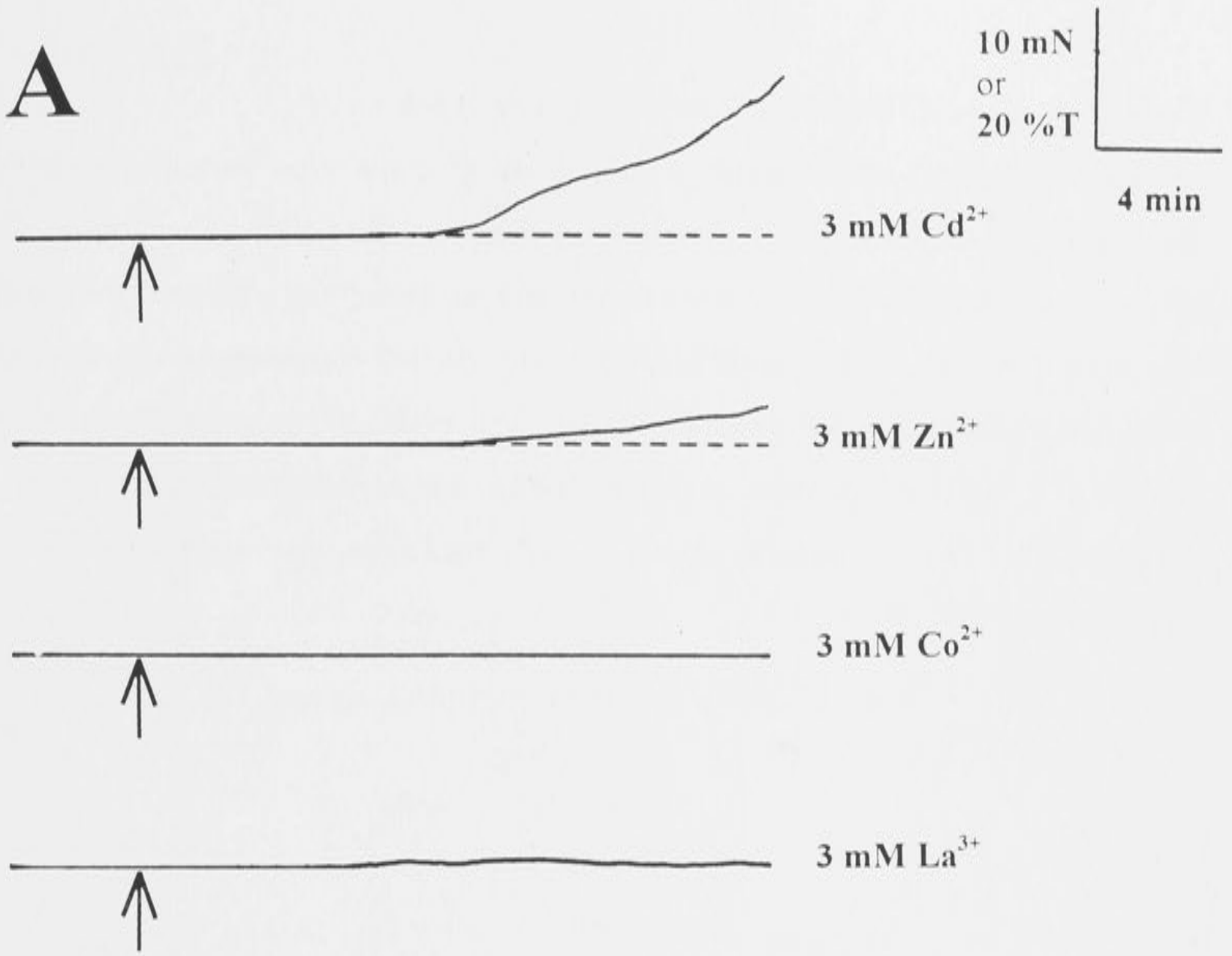
5.3.5 *Does Cd^{2+} induce contraction in fast twitch muscle?*

The effect of 3 mM Cd^{2+} on tension in small bundles (10-15 fibres) of rat EDL muscle was investigated to determine whether the CIC was restricted to soleus fibres. The results for this experiment are shown in Fig. 5.5. A tension increase was observed in EDL fibres exposed to 3 mM Cd^{2+} This contracture, although similar to the CIC described in soleus fibres was smaller in amplitude, reaching on average (n = 3), only 5.5 ± 3.1 % of control tetanic tension after 20 minutes. Two conclusions can be drawn from the results of this experiment. Firstly, in contrast to the CWC (Chapter 3), the Cd^{2+}-induced contracture is not restricted to slow twitch muscle and secondly that fast twitch muscle is not as sensitive as slow twitch to the effects of 3 mM Cd^{2+} .

Figure 5.4 *Is the induced contracture specific to Cd^{2+} ?*

(A) Records showing the effect of 3 mM Cd^{2+}, Zn^{2+}, Co^{2+} and La^{3+} on tension in rat soleus fibres. Arrows indicate the time of addition of each cation to the bath and the baseline is indicated by a dotted line. Each record was obtained from a different preparation. **(B)** The average tension produced by each of the cations (normalised to control tetanic tension) after 20 minutes is shown. Vertical bars show ± 1 SEM. The number of preparations in each group is displayed above each error bar. *** indicates a value significantly different from the CIC ($P \leq 0.05$, student T-test).

Fig. 5.4


A
10 mN
or
20 %T
4 min
3 mM Cd^{2+}
3 mM Zn^{2+}
3 mM Co^{2+}
3 mM La^{3+}

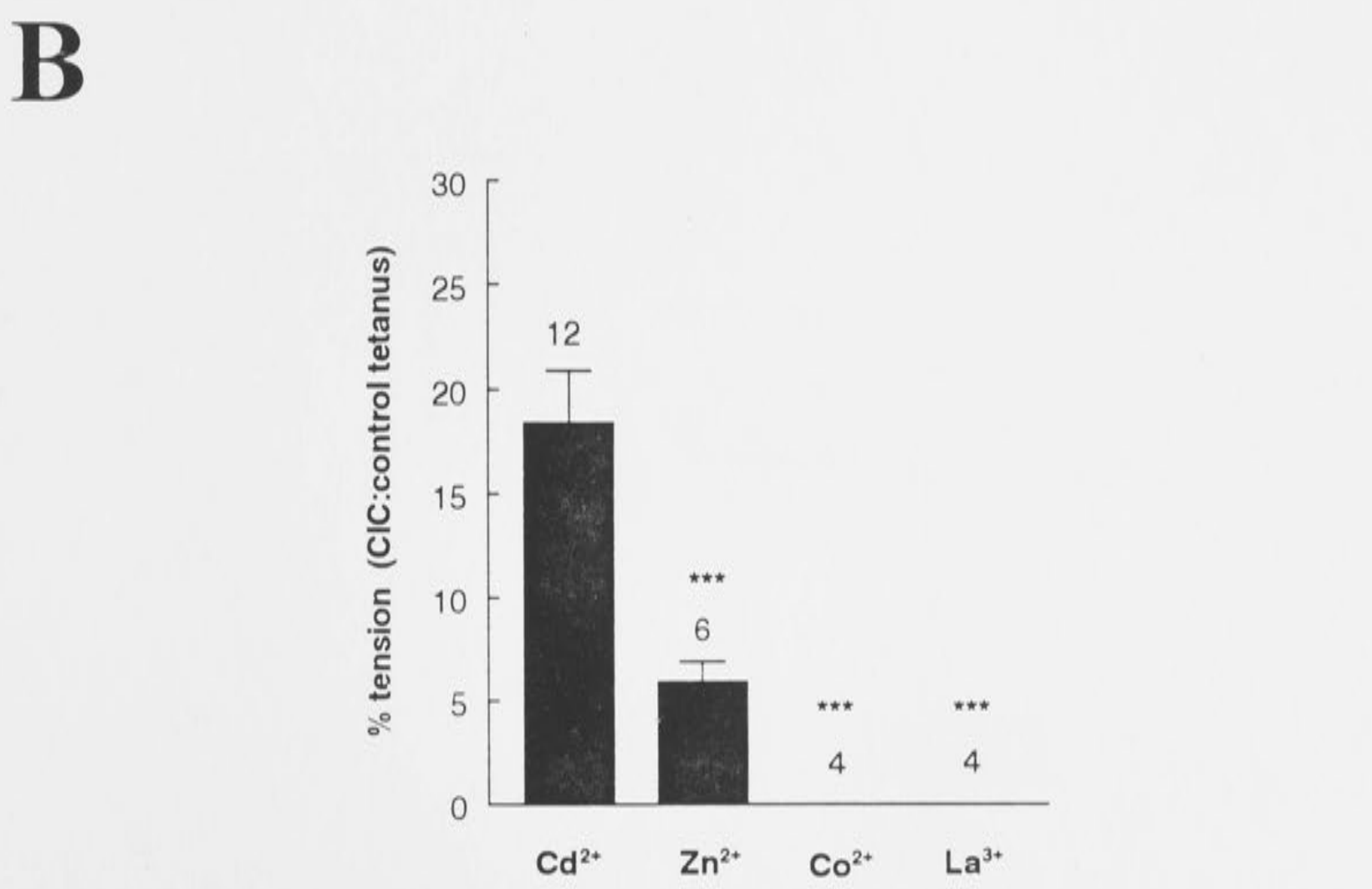
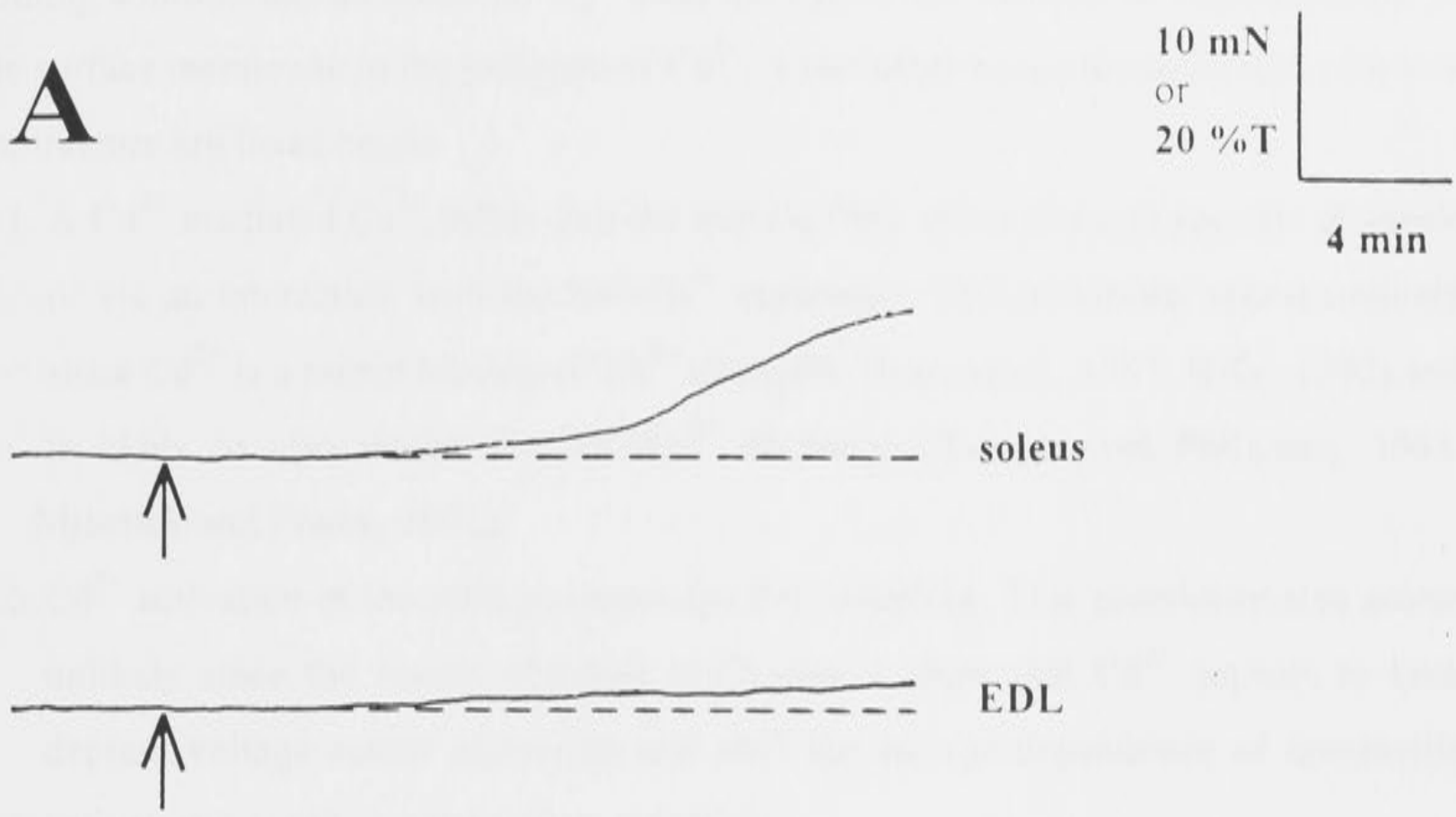

B
% tension (CIC:control tetanus)
30
25
20
15
10
5
0
12
***
6
***
4
***
4
Cd^{2+}
Zn^{2+}
Co^{2+}
La^{3+}

Figure 5.5 *The Cd^{2+}-induced contracture in fast twitch muscle*

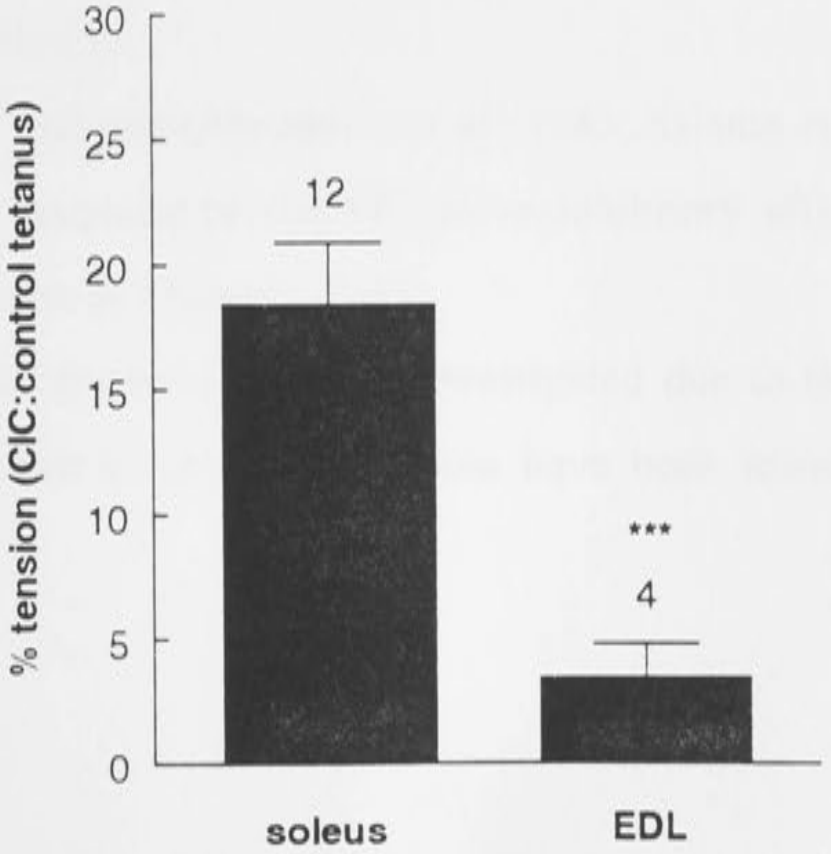
(A) Records showing development of the CIC in rat soleus (slow twitch) and rat EDL (fast twitch) fibres during a 20 minute incubation in 3 mM Cd^{2+}. The point of Cd^{2+} addition to the bath is indicated by the arrows and the dotted line represents the baseline tension. **(B)** Average CIC tension (normalised to control tetanic tension) produced in either soleus or EDL fibres after 20 minutes in 3 mM Cd^{2+} is shown. Vertical bars indicate ± 1 SEM. The number of preparations in each experiment is indicated above the error bars. *** indicates a significant difference from the response obtained in soleus fibres ($P \leq 0.05$).

Fig. 5.5

A


10 mN
or
20 %T
4 min
soleus
EDL

B


% tension (CIC:control tetanus)
30
25
20
15
10
5
0
12
***
4
soleus
EDL

Part B: The mechanism of the Cd^{2+}-induced contracture

It was shown in Chapter 3 (Fig. 3.6), that the addition of 3 mM Cd^{2+} to the bathing solution has no effect on V_m. Thus the CIC is not induced by depolarisation of the surface membrane in the presence of Cd^{2+}. Some other possible mechanisms for this contracture are listed below.

1. A Cd^{2+} mediated Ca^{2+} influx into the muscle fibre either through specific channels or via an interaction with the Na^{+}/Ca^{2+} exchanger. This possibility seems unlikely since Cd^{2+} is a potent blocker of Ca^{2+} channels (Beaty *et al.*, 1987; Hille, 1992) and is likely to also inhibit the Na^{+}/Ca^{2+} exchanger (Trosper and Philipson, 1983; Milanick and Frame, 1991).
2. Cd^{2+} activation of the voltage sensor for E-C coupling. This possibility also seems unlikely since the results obtained in Chapter 4 show that Cd^{2+} appears to both depress voltage sensor activation and shift the voltage dependence of contractile activation towards more positive potentials.
3. Mobilisation of intracellular Ca^{2+} via a second messenger system, eg IP_3, which could be activated by Cd^{2+} interacting with receptors in the surface membrane of the fibre.
4. Small amounts of Cd^{2+} leak into the myoplasm, causing an increase in tension by:
   (a) interacting directly with contractile proteins (Stephenson and Thieleczek, 1986; see Chapter 6);
   (b) causing SR Ca^{2+} release (Abramson *et al.*, 1983; Salama *et al.*, 1992);
   (c) inhibiting Ca^{2+} reuptake by the SR via an inhibitory effect on the SR Ca^{2+} ATPase (Herrmann & Shamoo, 1983).

Whilst not all of these mechanisms could be investigated due to time constraints and equipment limitations, some of these possibilities have been tested in the following experiments.

5.3.6 *Is the Cd^{2+} induced contracture the result of a Cd^{2+} mediated Ca^{2+} influx?*

5.3.6A *The effect of removing external Ca^{2+} on the Cd^{2+} -induced contracture*

To test the possibility that the CIC is the result of a Ca^{2+} influx, the effect of removing external Ca^{2+} on CIC amplitude was examined. The results are shown in Fig. 5.6. Ca^{2+} removal abolished the CIC in all of 10 preparations. The effect of Ca^{2+} removal on the CIC was specific for Ca^{2+} since the removal of external Mg^{2+} did not have the same effect. Instead, removal of Mg^{2+} increased the CIC amplitude to 170 ± 0.8 % of control CIC tension (n = 4). The results show whilst Mg^{2+} appears to modulate the CIC, external Ca^{2+} is essential for the production of a CIC and suggests that the mechanism of this contracture possibly involves a Cd^{2+} -mediated influx of Ca^{2+} into the fibre.

5.3.6B *Are L-type Ca^{2+} channels involved?*

Although the influx of Ca^{2+} through L-type channels in the presence of Cd^{2+} seemed unlikely since Cd^{2+} is known to block L-type Ca^{2+} channels (Stefani and Chiarandini, 1982; Hille, 1992; Spedding and Paoletti, 1992), the effects of 1 μM nifedipine on the CIC were nonetheless examined. The results are shown in Fig. 5.7. Nifedipine (1 μM) had no significant effect on the amplitude of the CIC measured after 20 minutes in 3 mM Cd^{2+} which was 131.0 ± 38.1 % of control CIC amplitude, (n = 6). The amplitude of the CIC in the presence of 1μM nifedipine tended to be larger than the control although, this increase was not statistically significant. This result therefore suggests that the contracture is not produced by a Ca^{2+} influx through L-type Ca^{2+} channels.

5.3.6C *Via a nifedipine insensitive mechanism?*

Since nifedipine did not reduce the amplitude of the CIC, it was possible that Cd^{2+} may activate other types of Ca^{2+} channels in the surface membrane. To test this possibility, Cd^{2+} was added following a 10 minute preincubation of the preparation in 2 mM Co^{2+}, a nonspecific Ca^{2+} channel blocker (Lorkovic and Rudel, 1983; Beaty *et al.*, 1987; Hille, 1992). The results for this experiment are also shown in Fig 5.7. On

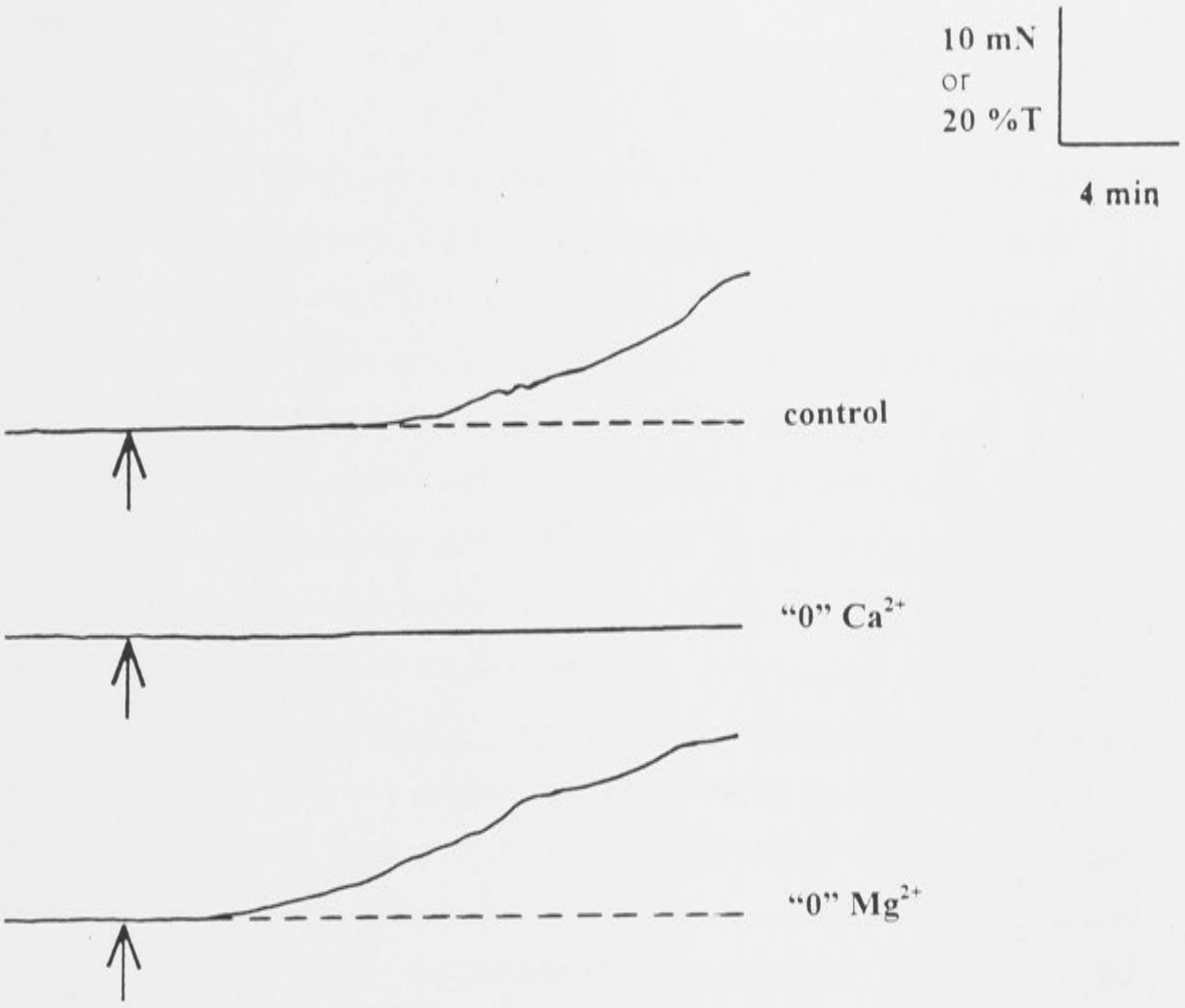


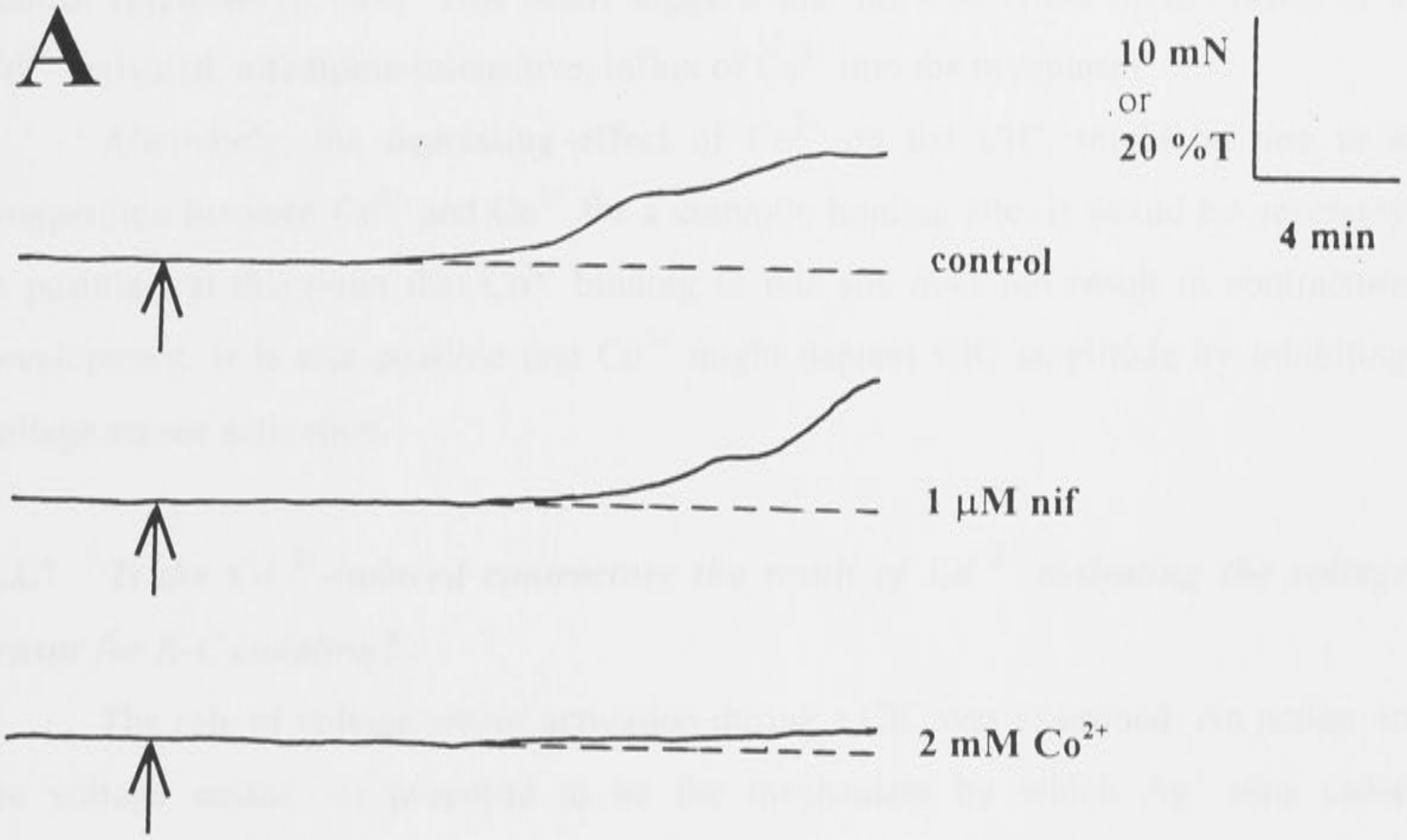
Figure 5.6 *The Cd^{2+}-induced contracture depends on external Ca^{2+} but not Mg^{2+}*
Records showing CIC development in rat soleus fibres exposed to 3 mM Cd^{2+} for 20 minutes in the presence and absence of external Ca^{2+} or Mg^{2+}. The records were obtained from different preparations. Fibres were initially bathed in control Krebs solution (solution 1A, Chapter 2). Fibres were then equilibrated in a "Ca^{2+}-free" (solution 1O) or "Mg^{2+}-free" (solution 1I) solution for 10 minutes before exposure to another "Ca^{2+} or Mg^{2+}-free" solution containing 3 mM Cd^{2+} (solutions 1P or 1J, respectively). The addition of the Cd^{2+}-containing solution is indicated by the arrow below each record. The "Ca^{2+} and Mg^{2+} free" solutions were made by omitting Ca^{2+} or Mg^{2+} from the standard Krebs solution. The "Ca^{2+} free" solution contained extra Mg^{2+} in place of Ca^{2+} to prevent depolarisation of the preparation. However, the "Mg^{2+} free solution" did not contain replacement cations and hence had a lower total [divalent cation] than the control Krebs solution. Identical results were obtained in 10 separate experiments.

Figure 5.7 *Is the Cd^{2+}-induced contracture the result of a Ca^{2+} influx?*

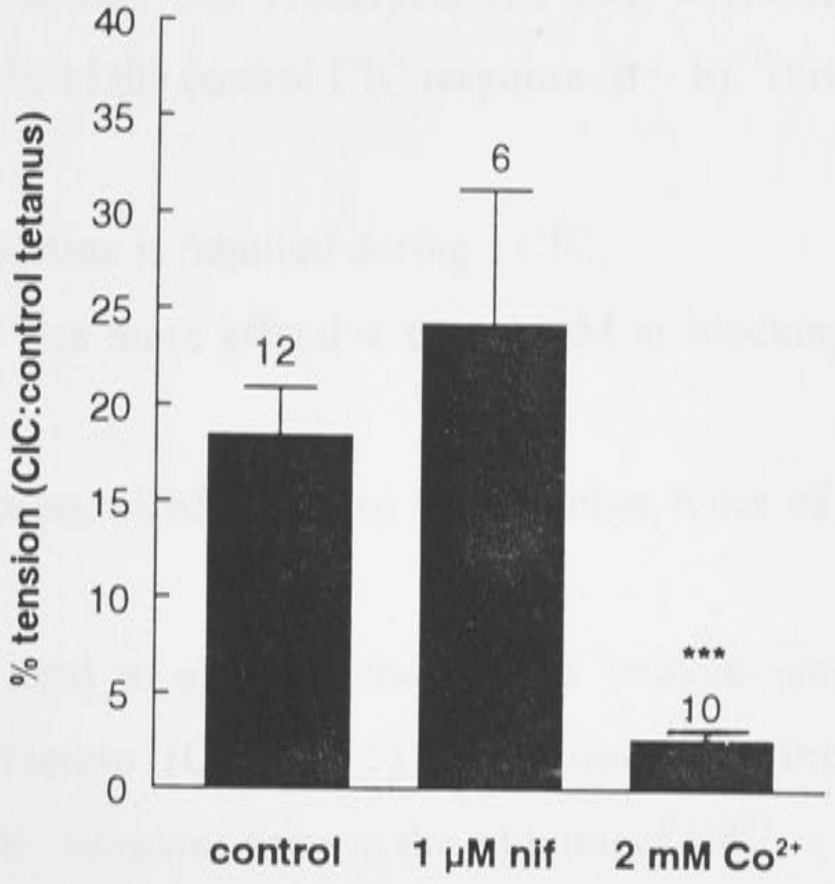
The effect on CIC amplitude of blocking L-type Ca^{2+} channels, using 1 μM nifedipine, and L-type as well as non-L-type Ca^{2+} channels using 2 mM Co^{2+}, is shown in **(A).** The muscle fibres were exposed to solutions containing either 2 mM Co^{2+} (solution 1G, Table 2.1) or 1 μM nifedipine (added directly to solution 1B) for 10 minutes after which the solution was replaced by another also containing 3 mM Cd^{2+} in addition to nifedipine or Co^{2+} (solution 1C+ 1 μM nifedipine or solution 1H, respectively). Addition of the Cd^{2+}-containing solution is indicted below each record by an arrow. Each record was obtained from a different preparation. The dotted line represents the baseline tension. **(B)** The average normalised CIC amplitude measured after 20 minutes in 3 mM Cd^{2+} is shown for the control (no nifedipine or Co^{2+}) and in the presence of 1μM nifedipine or 2 mM Co^{2+}. Vertical bars show ± 1 SEM. The number of preparations used in each experiment is indicated above the error bars. *** indicates a value significantly different from the CIC ($P \leq 0.05$).

Fig. 5.7

A


10 mN
or
20 %T
4 min
control
1 μM nif
2 mM Co2+

B


% tension (CIC:control tetanus)
40
35
30
25
20
15
10
5
0
12
6
***
10
control
1 μM nif
2 mM Co2+

average, Co^{2+} (2 mM) substantially depressed CIC tension to 14.4 ± 3.2 % of the control response, (n = 10). This result suggests that the CIC could be the result of a Cd^{2+}-activated, nifedipine-insensitive, influx of Ca^{2+} into the myoplasm.

Alternately, the depressing effect of Co^{2+} on the CIC, might be due to a competition between Cd^{2+} and Co^{2+} for a common binding site. It would be necessary to postulate at this point that Co^{2+} binding to this site does not result in contracture development. It is also possible that Co^{2+} might depress CIC amplitude by inhibiting voltage sensor activation.

5.3.7 *Is the Cd^{2+}-induced contracture the result of Cd^{2+} activating the voltage sensor for E-C coupling?*

The role of voltage sensor activation during a CIC was examined. An action on the voltage sensor is proposed to be the mechanism by which Ag^{+} ions cause contraction in frog skeletal muscle fibres (Oba, *et al.*, 1992).

5.3.7A *The effect of 50 μM nifedipine on the amplitude of the Cd^{2+}-induced contracture*

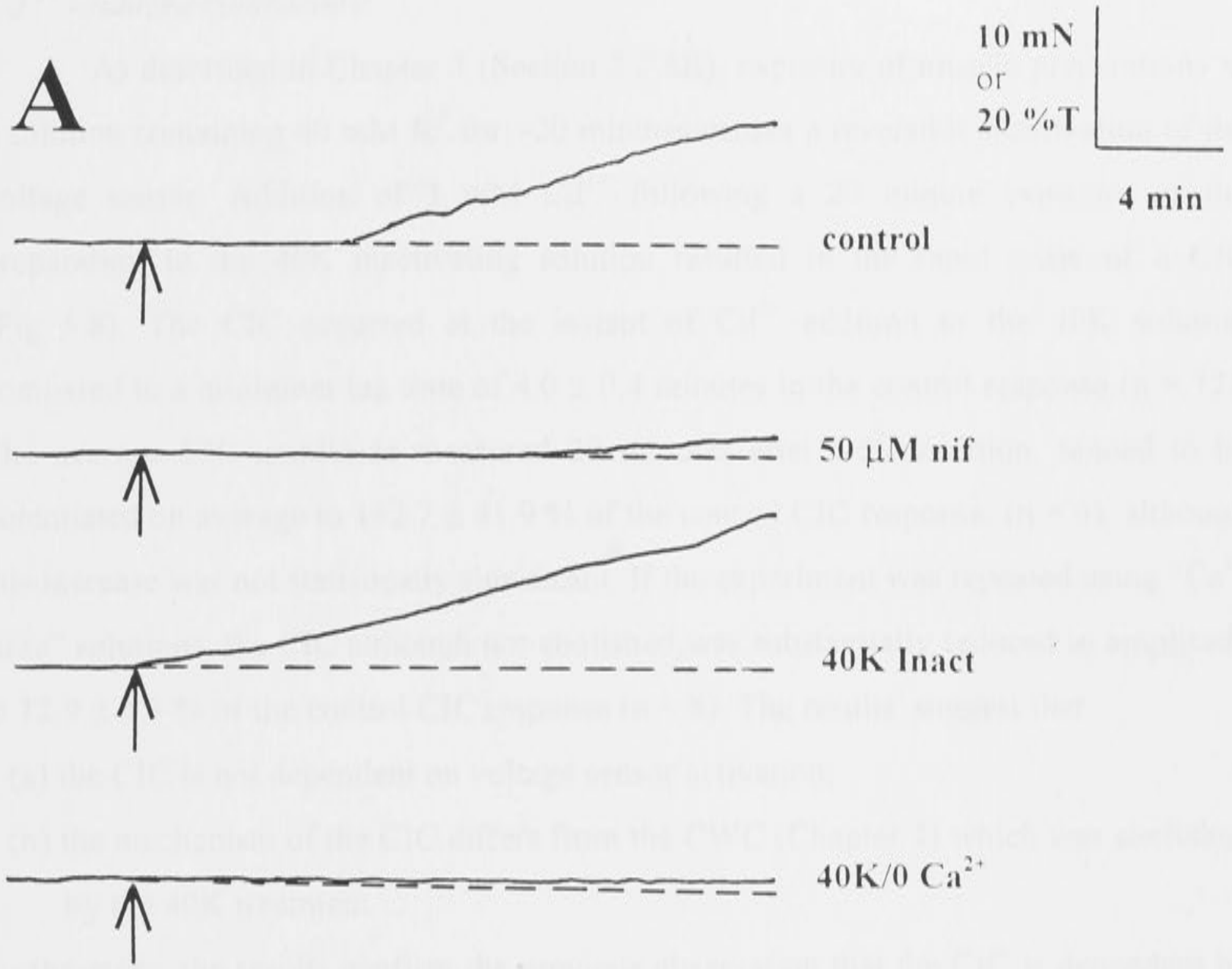
Nifedipine (50 μM) was used to inhibit the voltage sensor (Rios & Brum, 1987; Dulhunty and Gage, 1988; Neuhaus, 1990; Rios & Pizarro, 1991). The results for this experiment are shown in Fig. 5.8. Nifedipine (50 μM) depressed CIC amplitude on average to 17.8 ± 14.6 % of the control CIC response (n = 8). This result could indicate that:

1. voltage sensor activation is required during a CIC;
2. 50 μM nifedipine was more effective than 1 μM at blocking Ca^{2+} entry through L-type channels;
3. at high concentrations, nifedipine also blocks other types of channels involved in the contracture.

A more specific test used to examine the role of voltage sensor activation in the Cd^{2+}-withdrawal contracture (Chapter 3) is to inactivate the voltage sensor by depolarisation in high K^{+} solutions prior to the addition of Cd^{2+}.

Figure 5.8 *Is the Cd^{2+}-induced contracture the result of voltage sensor activation?*
(A) Records showing CIC's following voltage sensor paralysis in 50 μM nifedipine or voltage sensor inactivation in 40 mM K^+ (40K) and 40K/0 Ca^{2+}. Muscle fibres were exposed to nifedipine, 40 K, or 40K/0 Ca^{2+} for at least 10 minutes prior to the addition of 3 mM Cd^{2+} (indicated by the arrows), and each of these treatments were continued during Cd^{2+} exposure. The baseline tension level (prior to Cd^{2+} addition) is indicated by a dotted line. **(B)** The average normalised CIC tension obtained after 20 minutes in 3 mM Cd^{2+} for the control and various treatments is shown. Vertical bars show ± 1 SEM. The number of preparations used in each experiment is indicated above each error bar. *** indicates a value significantly different from the control, ($P \leq 0.05$).

Fig. 5.8


A
10 mN
or
20 %T
4 min
control
50 μM nif
40K Inact
40K/0 Ca2+
B
% tension (ClC:control tetanus)
40
35
30
25
20
15
10
5
0
12
***
8
6
***
8
cont
50 μM nif
40K
40K/0Ca

5.3.7B *The effect of voltage sensor inactivation in 40K on the amplitude of the Cd^{2+}-induced contracture*

As described in Chapter 3 (Section 3.3.8B), exposure of muscle preparations to a solution containing 40 mM K^+ for ~20 minutes causes a reversible inactivation of the voltage sensor. Addition of 3 mM Cd^{2+} following a 20 minute exposure of the preparation to the 40K inactivating solution resulted in the rapid onset of a CIC (Fig. 5.8). The CIC occurred at the instant of Cd^{2+} addition to the 40K solution compared to a minimum lag time of 4.0 ± 0.4 minutes in the control response (n = 12). The average CIC amplitude measured 20 minutes after Cd^{2+} addition, tended to be potentiated on average to 152.7 ± 41.9 % of the control CIC response, (n = 6), although this increase was not statistically significant.* If the experiment was repeated using "Ca^{2+}-free" solutions, the CIC although not abolished, was substantially reduced in amplitude to 12.9 ± 3.3 % of the control CIC response (n = 8). The results suggest that:

(a) the CIC is not dependent on voltage sensor activation;

(b) the mechanism of the CIC differs from the CWC (Chapter 3) which was abolished by the 40K treatment.

Furthermore, the results confirm the previous observation that the CIC is dependent on the presence of external Ca^{2+}.

5.3.7C *The effect of no stimulation on the Cd^{2+}-induced contracture*

To test whether the CIC was stimulation dependent, the effect of not electrically stimulating the muscle on the amplitude of the CIC was investigated (Fig. 5.9). Not stimulating the preparation electrically during exposure to 3 mM Cd^{2+} had no effect on the amplitude of the CIC, which was on average 110.3 ± 38.6 % of the control response, (n = 4).

5.3.8 ***The effect of other cations on the Cd^{2+}-induced contracture***

The effect of elevating the concentration of either Ca^{2+} or Mg^{2+} on the amplitude of the CIC was examined. The raised concentrations of these cations may alter the CIC if:

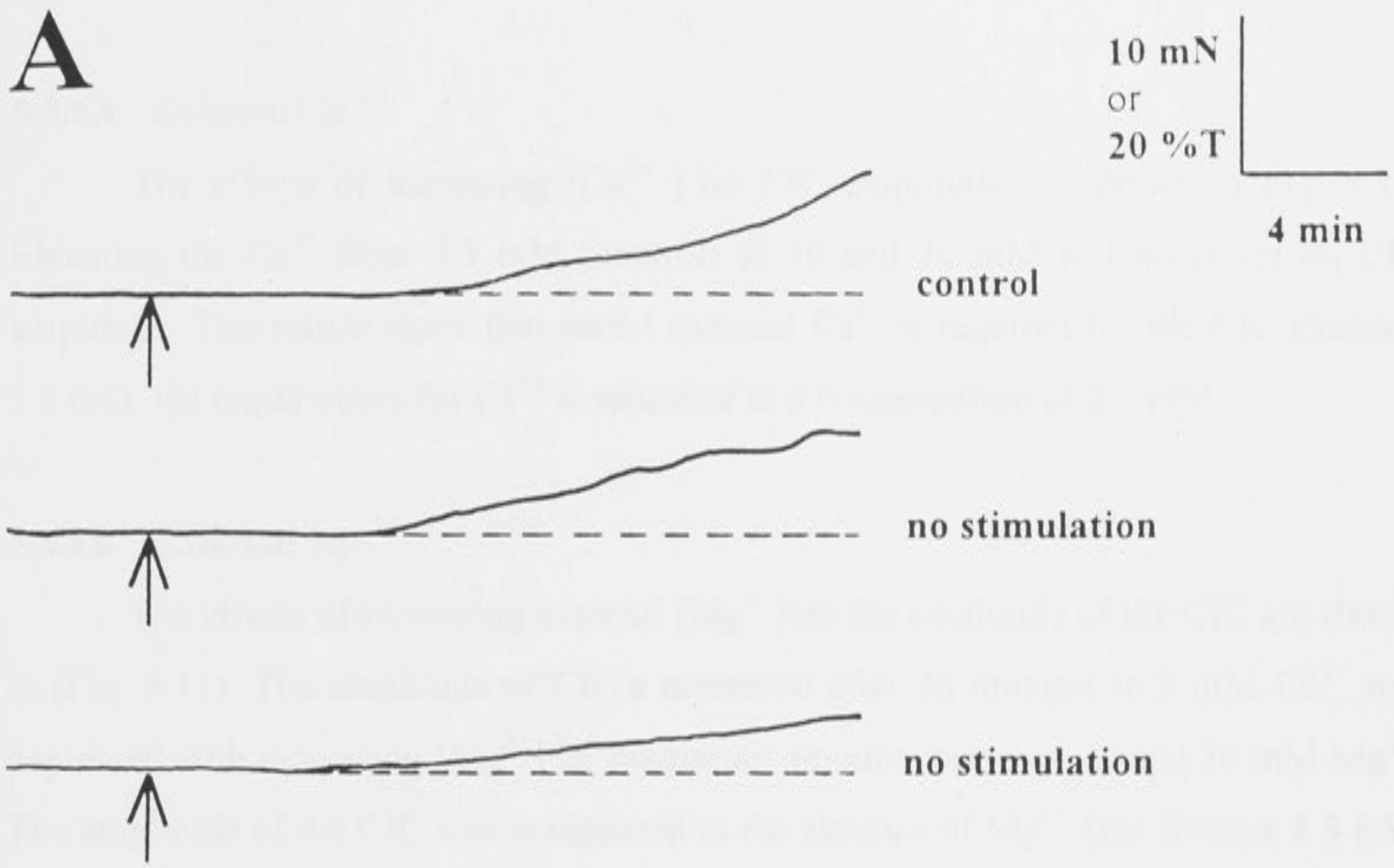
1. Ca^{2+} and Mg^{2+} compete for the site occupied by Cd^{2+};

* NB:- The size of the CIC following 40K treatment is still substantial considering that the free [Ca] in this solution is only 0·2-0·4mM.

Figure 5.9 *The effect of no stimulation upon the amplitude of the Cd^{2+}-induced contracture*

(A) Records showing CIC development during a 20 minute exposure of rat soleus fibres to 3 mM Cd^{2+} with (control) and without stimulation. 2 examples of CIC in non-stimulated preparations are shown so that the variability in contracture amplitude can be seen. In the non-stimulated preparations, electrically evoked twitches and tetanic contractions were ceased immediately prior to the addition of 3 mM Cd^{2+} to the bath (indicated by the arrows). The dotted line represents the baseline tension immediately preceding Cd^{2+} addition. **(B)** Average normalised CIC tension measured after a 20 minute exposure to 3 mM Cd^{2+} in stimulated and unstimulated preparations is shown. Vertical bars show ± 1 SEM. The number of preparations used in each experiment is indicated above the error bars.

Fig. 5.9


A
10 mN
or
20 %T
4 min
control
no stimulation
no stimulation
B
% tension (CIC:control tetanus)
30
25
20
15
10
5
0
12
4
control
no stim

2. the CIC is the result of an effect of Cd^{2+} on membrane surface charge.

5.3.8A *External Ca^{2+}*

The effects of increasing [Ca^{2+}] on CIC amplitude are shown in Fig. 5.10. Elevating the Ca^{2+} from 2.5 mM (control) to 10 and 30 mM had no effect on CIC amplitude. The results show that whilst external Ca^{2+} is required for the CIC (Section 5.3.6A), the requirement for Ca^{2+} is saturated at a concentration of 2.5 mM.

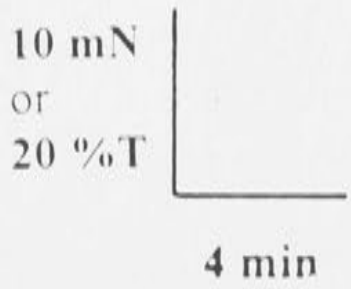
5.3.8B *External Mg^{2+}*

The effects of increasing external [Mg^{2+}] on the amplitude of the CIC are shown in (Fig. 5.11). The amplitude of CIC's measured after 20 minutes in 3 mM Cd^{2+} was depressed with increasing [Mg^{2+}] by maximum amounts between 10 and 30 mM Mg^{2+}. The amplitude of the CIC was potentiated in the absence of Mg^{2+} (see Section 5.3.6A). The results show that whilst Mg^{2+} is not required for CIC development, the amplitude of the CIC is modulated by this cation. The results also show that the CIC is not the result of an effect of Cd^{2+} on surface charge since high Ca^{2+} and Mg^{2+} did not have the same effect on CIC amplitude.

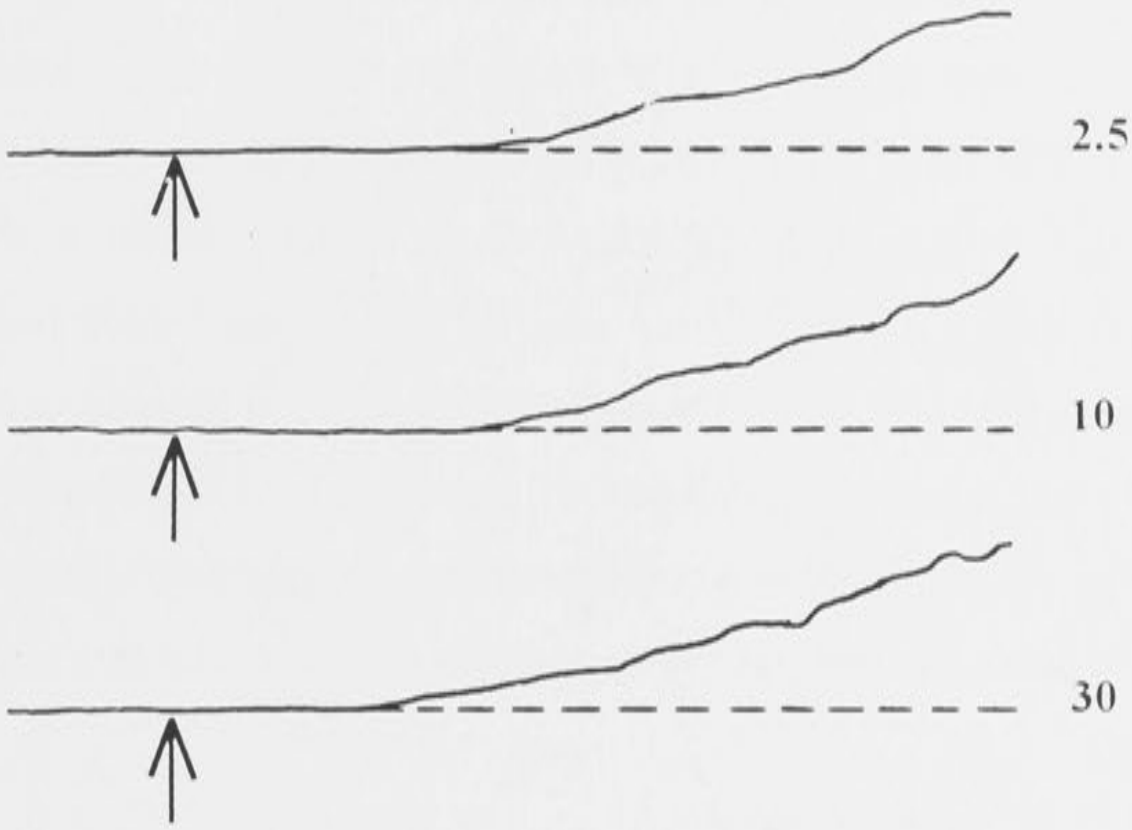
Figure 5.10 *The Cd^{2+}-induced contracture is not affected by elevated [Ca^{2+}]*

(A) Records showing CIC's produced during a 20 minute exposure of rat soleus fibres to 3 mM Cd^{2+} at varying external [Ca^{2+}]. Each record represents a different preparation. The normal "control" CIC response was obtained in 2.5 mM Ca^{2+}. Preparations were equilibrated in Krebs solution containing 2.5, 10, or 30 mM Ca^{2+} (solutions 1A, 1Q or 1S respectively, Table 2.1). After 10 minutes in the elevated Ca^{2+} solution, preparations were exposed to another solution containing 3 mM Cd^{2+} in addition to the elevated Ca^{2+} (solutions 1C, 1R or 1T respectively). The addition of Cd^{2+} to the bath is indicated by the arrows, and the dotted line represents the baseline tension. The [Ca^{2+}] of the bathing solutions (mM) is indicated to the right of each record. **(B)** The average normalised CIC tension (obtained from $n \geq 4$ preparations) at each [Ca^{2+}] is shown. Vertical bars indicate $\pm$ 1 SEM. A sigmoid function has been fitted to the data but has no physical significance.

Fig. 5.10


10 mN
or
20 %T
4 min

A


2.5
10
30
[Ca2+] mM

B

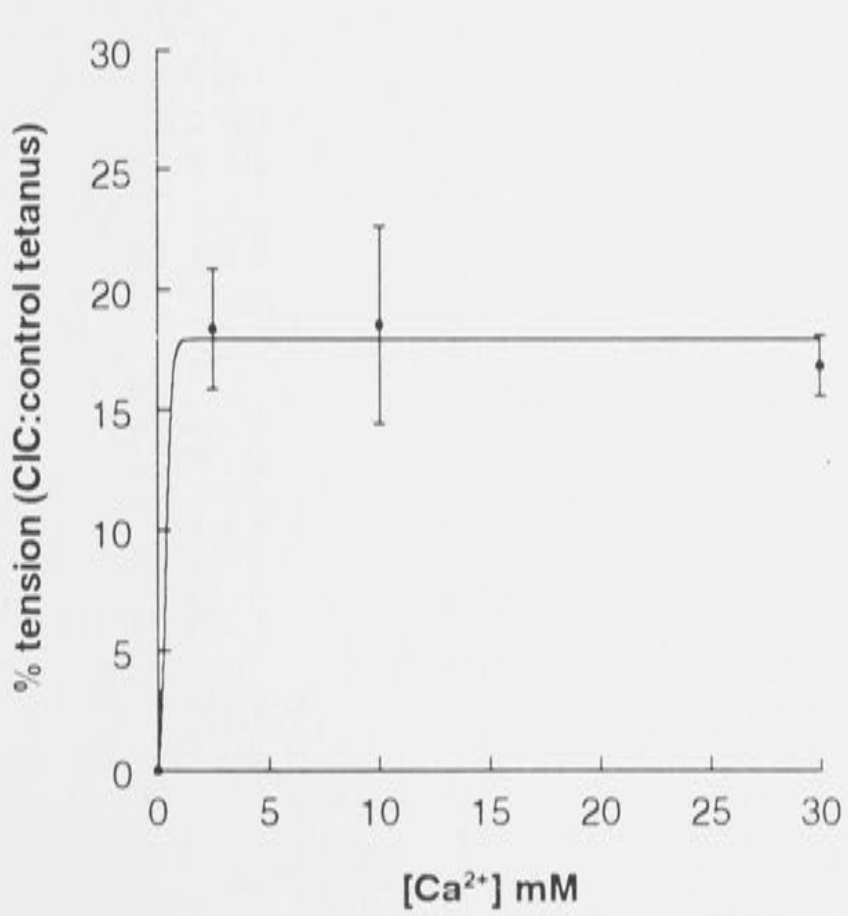
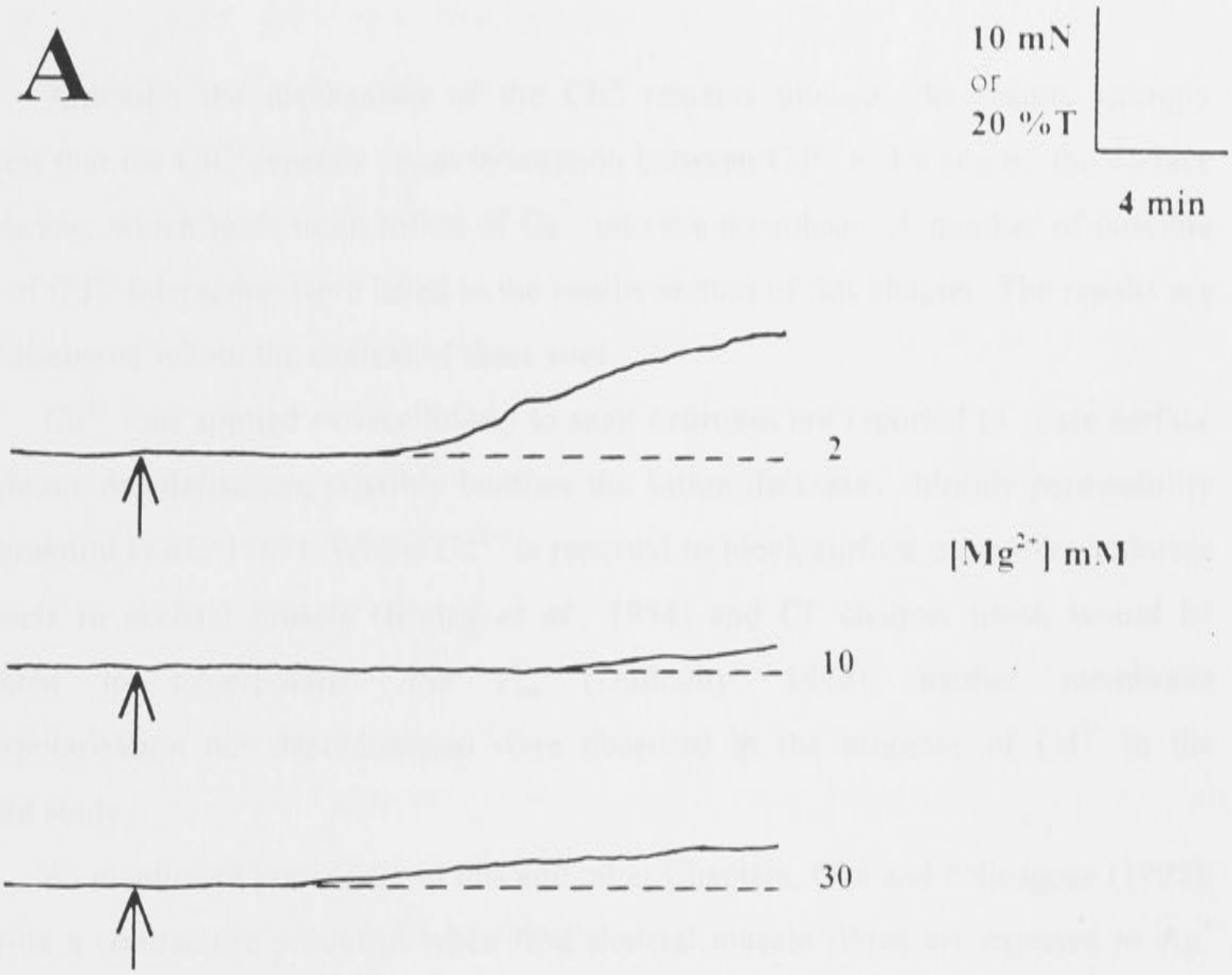

% tension (CIC:control tetanus)
0
5
10
15
20
25
30
[Ca2+] mM

Figure 5.11 *The Cd^{2+}-induced contracture is modulated by external Mg^{2+}*

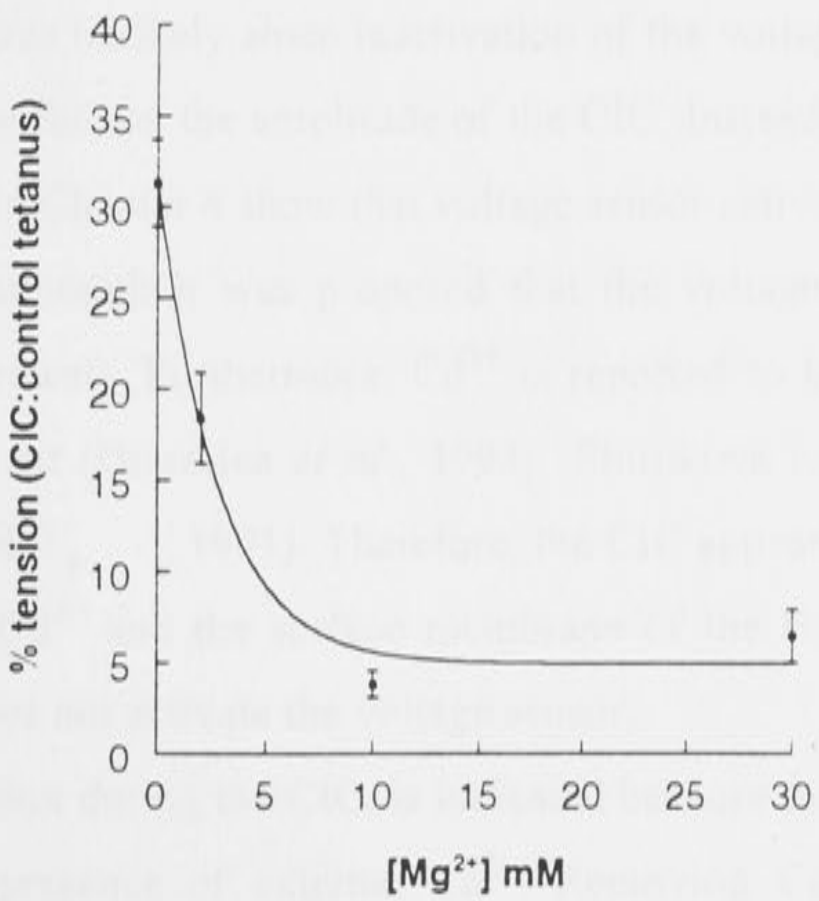
(A) Records showing CIC's produced in rat soleus fibres exposed to 3 mM Cd^{2+} for 20 minutes at varying [Mg^{2+}]. Each record represents a different preparation. The procedure is essentially the same as in Fig. 5.10 except that [Mg^{2+}] was varied and [Ca^{2+}] was kept constant at 2.5 mM. The addition of Cd^{2+} to the bath is indicated by the arrows and the dotted line represents the baseline tension. [Mg^{2+}] of the bathing solutions (mM) is indicated to the right of each record. The normal "control" CIC response was obtained in 2 mM Mg^{2+}. **(B)** Average normalised CIC tension (measured from $n \geq 4$ preparations) after 20 minutes in 3 mM Cd^{2+} at each [Mg^{2+}] are shown. The result obtained in "0" Mg^{2+} (Fig. 5.6) has been included in the graph for comparison. Vertical bars show ± 1 SEM. An exponential function has been fitted to the data but has no physical significance.

Fig. 5.11

A


10 mN
or
20 %T
4 min
2
[Mg2+] mM
10
30

B


% tension (CIC:control tetanus)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
[Mg2+] mM

5.5 Discussion

Although the mechanism of the CIC remains unclear, the results strongly suggest that the CIC depends on an interaction between Cd^{2+} and a site on the surface membrane, which leads to an influx of Ca^{2+} into the myoplasm. A number of possible sites of Cd^{2+} interaction were listed in the results section of this chapter. The results are now discussed within the context of these sites.

Cu^{2+} ions applied extracellularly to snail neurones are reported to cause surface membrane depolarisation, possibly because the cation decreases chloride permeability (Chiarandini *et al.*, 1967). Whilst Cd^{2+} is reported to block surface membrane chloride channels in skeletal muscle (Bretag *et al.*, 1984) and Cl^- channel block would be expected to hyperpolarise the V_m, (Dulhunty, 1978), neither membrane hyperpolarisation nor depolarisation were observed in the presence of Cd^{2+} in the current study.

As mentioned previously in this and other Chapters, Oba and colleagues (1992), describe a contracture produced when frog skeletal muscle fibres are exposed to Ag^+ ions. The contracture is thought to be the result of voltage sensor activation by Ag^+ since the increase in tension was blocked by voltage sensor antagonists. It was proposed that Ag^+ causes voltage sensor activation by interacting with sulfhydryl groups present on the DHPR molecule which are thought to be critical to E-C coupling. Such a mechanism for the CIC seems unlikely since inactivation of the voltage sensor using a 40 mM K^+ solution had no effect on the amplitude of the CIC. Instead, the results from K contracture experiments in Chapter 4 show that voltage sensor activation is depressed in the presence of Cd^{2+} (although it was proposed that the voltage sensor becomes activated upon Cd^{2+}-withdrawal). Furthermore, Cd^{2+} is reported to have no effect on asymmetric charge movement (Gonzalez *et al.*, 1993; Shirokova *et al.*, 1994) apart from altering its kinetics (Hui , 1991). Therefore, the CIC appears to be caused by some interaction between Cd^{2+} and the surface membrane of the fibre that does not cause depolarisation and does not activate the voltage sensor.

A role for a Ca^{2+} influx during the CIC, is indicated because the contracture was highly dependent on the presence of external Ca^{2+}. Removing Ca^{2+} abolished the

contracture. On the other hand, increasing the Ca^{2+} concentration from 2.5 mM to 10 and 30 mM had no effect on contracture amplitude. As argued in Chapter 3 for the CWC, one might expect that increasing the Ca^{2+} concentration would increase the CIC amplitude if the mechanism was based on a Ca^{2+} influx due to a larger Ca^{2+} driving force, provided the current did not saturate at high [Ca^{2+}],which is highly likely. 1 μM nifedipine did not change the amplitude of the CIC suggesting that Ca^{2+} entry through L-type channels was not involved. This was expected since Cd^{2+} is a potent blocker of these channels. Again as suggested in Chapter 3, this might be due to the reduced binding of dihydropyridines which occurs in the presence of Cd^{2+} (Peterson and Catterall, 1995). However, contractures were depressed by nifedipine if the dosage was increased to 50 μM. It is thought that this depression is probably the result of a non-specific effect since nifedipine at this concentration (50 μM) is also known to block Ca^{2+} activated K^{+} channels (Kaji, 1990), and thus possibly other ion channel species. Concomitant incubation of preparations in 3 mM Cd^{2+} with 2 mM Co^{2+}, a non-specific Ca^{2+} channel blocker (Lorkovic and Rudel, 1983; Beaty *et al.*, 1987; Hille, 1992), caused a significant depression of the amplitude of the CIC. The depression of the CIC by Co^{2+} occurred only if Co^{2+} was added before Cd^{2+}. This result could be interpreted by Co^{2+} blocking a Cd^{2+} mediated Ca^{2+} influx or by a competition between Co^{2+} and Cd^{2+} for a common binding site. Cations competing for a common binding site might also explain the dose dependent decrease of CIC tension seen when the external [Mg^{2+}] was increased. It is necessary to postulate that (a) the binding of Co^{2+} or Mg^{2+} to this site does not result in contraction and (b) that the site is not occupied by Ca^{2+} since elevating [Ca^{2+}] did not depress the CIC. Alternatively, it is possible that Co^{2+} and Mg^{2+} but not Ca^{2+}, bind to a nearby site which physically prevents Cd^{2+} from accessing its binding site. Furthermore, the depressing effects of Co^{2+} and Mg^{2+} on CIC amplitude cannot be the result of charge screening since high (10 and 30 mM) Ca^{2+} would be expected to have similar depressing effects, and this was not observed.

The CIC was not restricted to Cd^{2+}. Exposure of rat soleus fibres to 3 mM Zn^{2+} also resulted in a contracture although the amplitude of the Zn^{2+}-induced contracture was smaller than the contracture seen in Cd^{2+}. Application of 3 mM Co^{2+} and La^{3+} to fibres on the other hand failed to elicit a contractile response. These results immediately

suggest that the induced contracture is not the result of a general electrostatic interaction between cations and negative surface charges. If this was a charge screening/binding effect, then all cations would be expected to produce a similar response, especially at millimolar concentrations. The observation that only Cd^{2+} and Zn^{2+} cause contraction could be due to the fact that either these cations either bind to the same site as other cations but with higher affinity; or they bind to different a site which is not accessible to other cations (see also discussion, Chapter 4).

Another possible mechanism for the CIC is based upon reports that Cd^{2+} causes a rise in cytosolic Ca^{2+} in human skin fibroblasts mediated by an increase in IP_3 production (Smith *et al.*, 1989). The increased production of IP_3 is thought to occur as the result of an interaction between Cd^{2+} and specific receptors present on the cell surface. However, this type of mechanism is probably not the basis of the CIC as the effect on skin fibroblasts was not affected by the removal of external Ca^{2+}, which in the present study was found to abolish the CIC.

An increase in non-specific membrane conductance by Cd^{2+}-induced membrane lipid peroxidation provides yet another possible mechanism for the CIC. Kiss and Osipenko (1994), argue that lipid peroxidation does not contribute significantly to metal cation induced membrane currents. This conclusion is based on a study by Weinreich and Wonderlin, (1987) who found that copper induced inward currents in molluscan neurones were not affected by the presence of superoxide dismutase, an enzyme which protects against lipid peroxidation. However, lipid peroxidation may occur in mammalian skeletal muscle. It is unlikely that Ca^{2+} entry through the lipid peroxidised membrane is the basis of the CIC since Ca^{2+} entry would continue after Cd^{2+} removal and tension would continue to increase. Instead, CIC tension did not decline upon Cd^{2+} washout, but stopped increasing and remained at a constant level. Conversely, it is possible that the CIC is the result of a Ca^{2+}-dependent influx of Cd^{2+} into the fibre through the damaged membrane, which might also explain the observation that preparations did not fully recover from prolonged (> 20 min) exposure to 3 mM Cd^{2+}. However, one would expect that a membrane damaged through lipid peroxidation would not be so selective and that Cd^{2+} would pass through the membrane whether Ca^{2+} was present or not and yet the CIC was highly dependent on the presence of Ca^{2+}.

If a small amount of Cd^{2+} entered the muscle fibre, it could cause direct activation of the contractile proteins (Chapter 6). This seems unlikely because no ion channels have been reported to conduct Cd^{2+}, and the contracture is abolished in zero external Ca^{2+}. However, the possibility that there is a Ca^{2+}-dependent Cd^{2+} influx cannot be fully excluded. Furthermore, metal cations which have been shown to induce surface membrane currents in various cells when applied extracellularly, fail to do so when injected into the same cells (Kiss and Osipenko, 1994), suggesting that these cations do not normally enter the cells.

It could be argued that the CIC could also arise from a Cd^{2+}-mediated Ca^{2+} influx through the Na^{+}-Ca^{2+} exchanger. This is not thought to be likely since Cd^{2+} is reported to bind with high affinity and block Ca^{2+} exchange in isolated canine sarcolemmal vesicles (Trosper & Philipson, 1983) and ferret red blood cells (Milanick and Frame, 1991). However, no information about the effect of Cd^{2+} on Na^{+}-Ca^{2+} exchange in skeletal muscle is available.

In summary, the CIC is proposed to be the result of an external effect of Cd^{2+} on the muscle fibre which produces an influx of external Ca^{2+} resulting in contraction. The mode of Ca^{2+} entry is not understood and could possibly occur via the following pathways:

1. a Cd^{2+}-activated non-specific cation channel;
2. a Ca^{2+}-dependent entry of Cd^{2+} into the fibre, possibly following lipid peroxidation by Cd^{2+};
3. an effect of Cd^{2+} on the Na^{+}-Ca^{2+} exchanger.

Chapter 6

Activation of the Contractile Apparatus by Cd^{2+}

6.1 Introduction

Ca^{2+} is the major cation concerned with contraction in skeletal muscle. Not only is this cation essential for the normal functioning of the voltage sensor and prolonged activation of the RyR Ca^{2+} release channel, but it also directly activates the contractile machinery allowing the generation of tension.

Development of the skinned fibre technique (Natori, 1954), has greatly contributed to the understanding of the role of internal Ca^{2+} in contraction. In skinned fibres, the sarcolemma is either chemically or mechanically removed leaving the SR and myofibrils exposed to the external environment. During the skinning process, the T-tubules which are torn from the sarcolemma, reseal and repolarise allowing contraction via T-tubule depolarisation. Contraction in skinned fibres can also be achieved by (Lamb and Stephenson, 1991 , 1992):

1. directly activating SR Ca^{2+} release (eg. using caffeine or by lowering $[Mg^{2+}]$);
2. directly activating the contractile proteins using elevated Ca^{2+} solutions.

The technique is useful because it allows the internal and external events involved in E-C coupling to be manipulated and overcomes problems associated with the drug permeability across the surface membrane.

Skinned fibre studies have revealed that cations other than Ca^{2+} can also directly activate the skeletal muscle contractile apparatus. Stephenson and Theileczek (1986) report that Ba^{2+}, Sr^{2+}, Ni^{2+} and Cd^{2+} all produce myofibrillar tension in skinned frog iliofibularis fibres, although none of the cations are as effective as Ca^{2+} in this role.

So far in this report, it has been proposed that the action of Cd^{2+} is primarily external. This seems especially likely in the case of the Cd^{2+}-withdrawal contracture (Chapter 3) and the effects of Cd^{2+} on twitch and tetanus (Chapter 4) since both occur rapidly on Cd^{2+} washout or addition, respectively. However, the Cd^{2+}-induced contracture (Chapter 5) develops more slowly and is not readily reversible upon removal of the cation. In Chapter 5 it was suggested that the CIC is the result of a Cd^{2+}-mediated influx of Ca^{2+} into the fibre. In this chapter, the possibility that the CIC could be the result of a direct activation of the contractile apparatus by Cd^{2+} following Cd^{2+} leakage into the fibre is examined.

6.2 Aims

1. To examine the relative sensitivity of the contractile apparatus of skinned rat soleus fibres to activation by Cd^{2+} and Ca^{2+};
2. To determine whether a direct activation of the contractile apparatus could play a role in the Cd^{2+}-induced contracture described in Chapter 5.

6.3 Results

6.3.1 *Activation of the contractile apparatus by Ca^{2+}*

Isometric tension produced by directly activating the contractile apparatus of a skinned rat soleus fibre with elevated Ca^{2+} solutions is shown in Fig. 6.1. The lowest [Ca^{2+}] which produced a recordable increase in tension was 0.1 μM (pCa 7). The amplitude of the contractile response increased rapidly with higher [Ca^{2+}] and reached a plateau at a free [Ca^{2+}] of approximately 10 μM (pCa 5). Maximal tension was therefore defined as the tension produced by exposing fibres to a solution containing 100 μM free Ca^{2+} (pCa 4). The average contractile responses (normalised against maximal tension) obtained from a total of 8 fibres is shown plotted against pCa in Fig. 6.3 (solid purple line). A sigmoidal curve has been fitted to the data and the following values were obtained from the equation to the curve as described in Section 2.3.5 (Chapter 2):

*T_{Max} (Max normalised tension in Ca^{2+}) = 1

*$p_{50\%}$ (pCa causing 50 % maximal tension) = 5.97 (~1.07 μM)

*k_x (slope factor) = -2.61.

6.3.2 *Activation of the contractile apparatus by Cd^{2+}*

The isometric tension produced by exposing a skinned rat soleus fibre to elevated Cd^{2+} solutions (containing minimal free Ca^{2+}) is shown in Fig. 6.2. In contrast to the Ca^{2+} induced responses, no contracture was produced when fibres were exposed to 0.1 μM Cd^{2+}. Tension did develop in fibres exposed to solutions estimated to

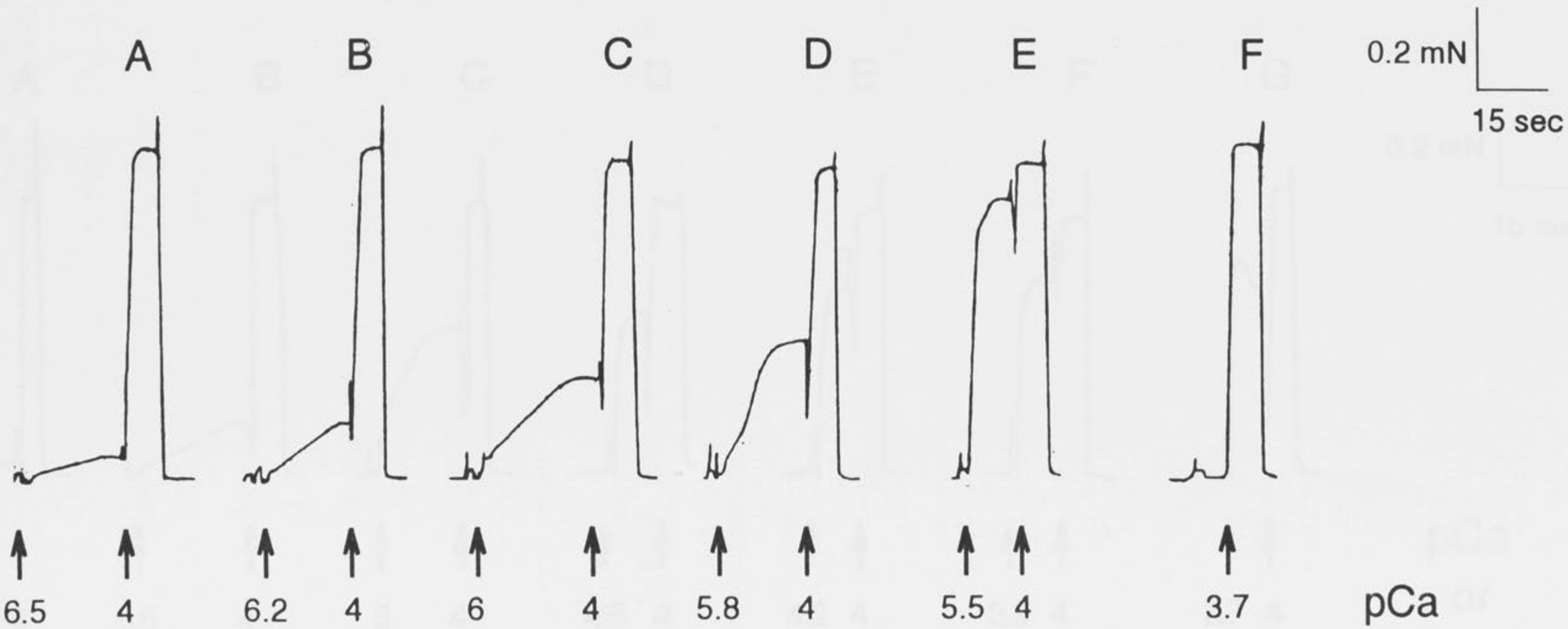


Figure 6.1 *Activation of the contractile apparatus by* Ca^{2+}

Records (A-F) show isometric tension produced by exposing a skinned rat soleus fibre to solutions of varying Ca^{2+} concentration. The composition of all bathing solutions and contracture protocol are described in detail in Chapter 2. The elevated Ca^{2+} solutions were made by adding appropriate amounts of $CaCl_2$ to solution "S". Each test contracture was followed immediately by a maximal contracture in a solution containing 100 μM Ca^{2+} (pCa 4). The pCa of the bathing solutions used to produce each of the contractures are indicated below each record. In this and the following figure, the tension spikes occurring at the beginning and end of each contracture are artefacts produced by the solution change. Isometric tension is shown as force (mN) on the vertical scale bar and time (sec) is indicated on the horizontal scale bar.

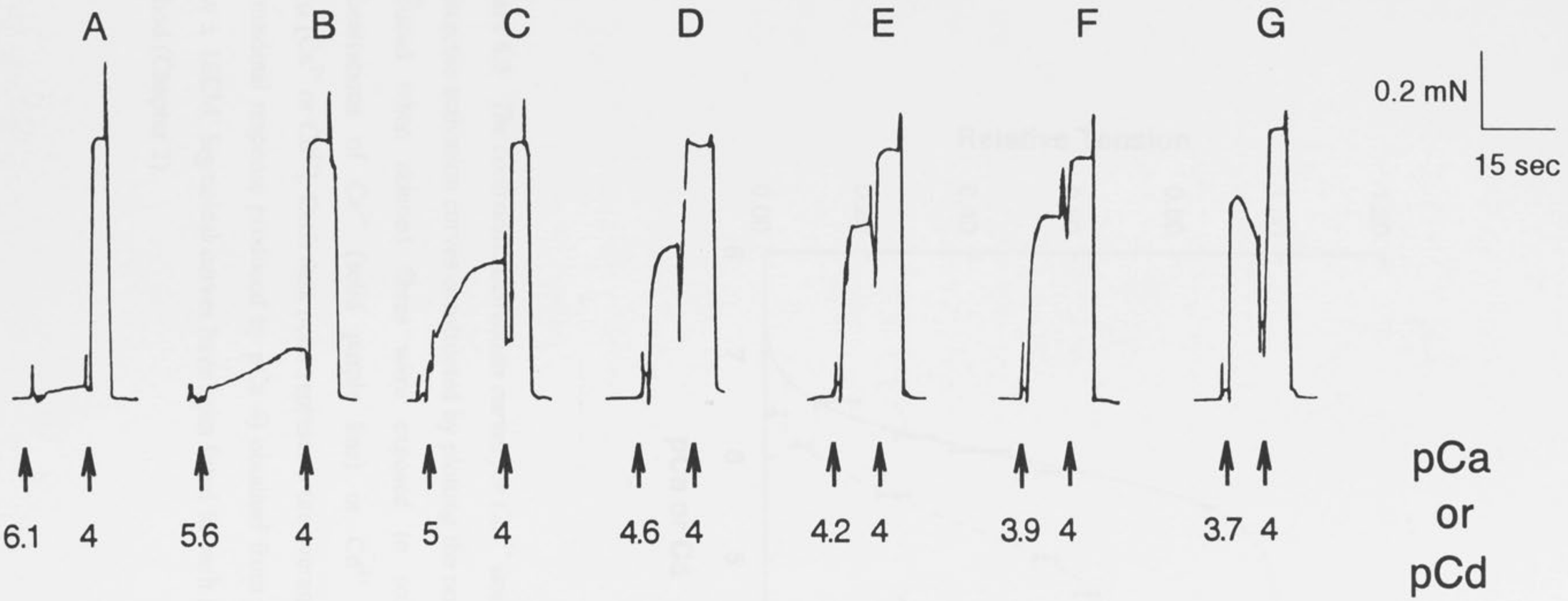


Figure 6.2 *Activation of the contractile apparatus by Cd^{2+}*

Records (A-G) show isometric tension development in a skinned rat soleus fibre exposed to solutions of varying Cd^{2+} concentration. The Cd^{2+} solutions were prepared by adding appropriate amounts of $CdCl_2$ to solution "S" (Chapter 2). Each Cd^{2+} contracture was followed by a maximally activating contracture in a solution containing 100 μM Ca^{2+} (pCa 4). The pCd or pCa of the bathing solutions used to produce each contracture is shown below each record. Isometric tension is shown as force (mN) on the vertical scale bar and time (sec) is indicated on the horizontal scale bar.

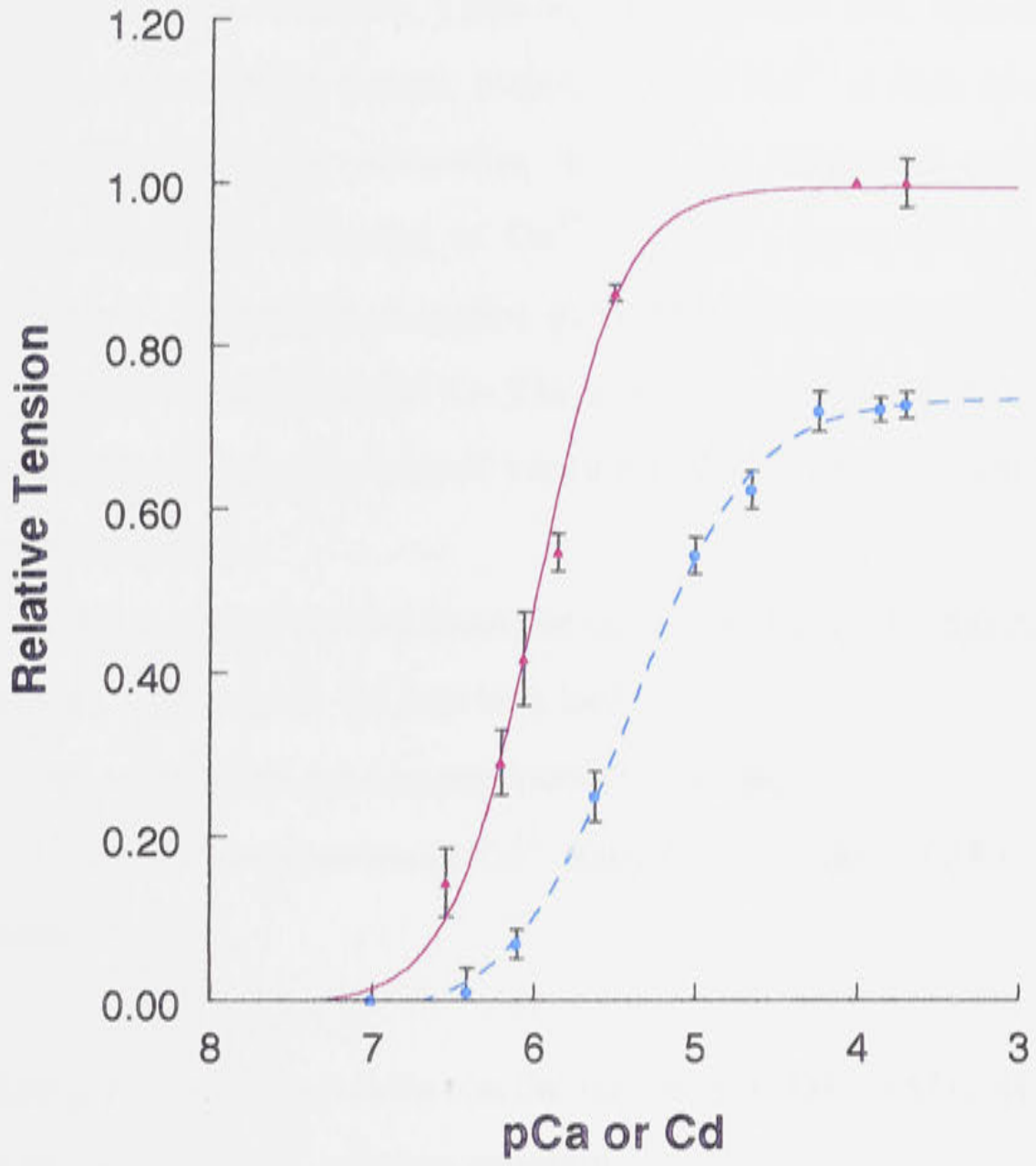


Figure 6.3 *The contractile activation curves for Ca^{2+} and Cd^{2+}*

Contractile activation curves constructed by plotting the normalised tension (Chapter 2), produced when skinned fibres were exposed to solutions containing different concentrations of Ca^{2+} (solid purple line) or Cd^{2+} (dotted blue line) against $-\log_{10}$ [Ca^{2+} or Cd^{2+}]. Each data point represents the average tension (normalised against the maximal response produced by pCa 4) obtained from 8 preparations. Vertical bars show ± 1SEM. Sigmoidal curves have been fitted to each set of data by a least squares method (Chapter 2).

contain 0.4 μM free Cd^{2+} (pCd 6.4). The amplitude of the tension response increased with free [Cd^{2+}] greater than 0.4 μM, and reached a plateau at approximately 100 μM free Cd^{2+} (pCd 4). If the free [Cd^{2+}] was increased to 200 μM, tension spontaneously decayed shortly after reaching a peak, suggesting that Cd^{2+} at high concentrations also has some inhibitory effect on contraction. So that the contractile activating effects of Cd^{2+} could be compared with that of Ca^{2+}, all Cd^{2+} contractions were normalised against the maximal contractile response produced by exposing fibres to a solution containing 100 μM free Ca^{2+} (pCa 4). The average normalised tension produced by exposing 8 skinned fibres to solutions of varying [Cd^{2+}] is shown plotted against pCd in Fig. 6.3 (dotted blue line).

The following values were obtained from the equation of a sigmoidal curve fitted to the data as described previously in the previous Section:

*T_{Max} (maximum normalised tension produced by Cd^{2+}) = 0.76

*$p_{50\%}$ (pCd causing 50 % of maximum Cd^{2+} tension response) = 5.38 (~ 4.17 μM)

*k_x (slope factor) = -3.7

A number of differences between the response of the contractile apparatus of rat soleus fibres to Ca^{2+} and Cd^{2+} are thus apparent:

1. fibres are more sensitive to activation by Ca^{2+};
2. the maximum tension produced by Cd^{2+} is smaller than the response in Ca^{2+}, and the slope of the force-pCd^{2+} curve is less steep compared to that obtained for Ca^{2+}, suggesting possible inhibitory effects of Cd^{2+} on the contractile apparatus.

6.4 Discussion

Activation of the contractile apparatus of skinned rat soleus fibres by Cd^{2+} is described in this Chapter. Exposure of skinned fibres to solutions containing 0.4 μM Cd^{2+} (pCa 6.1) or higher concentrations induced tension which reached a maximum at a $[Cd^{2+}]$ of approximately 100 μM. The maximum tension generated in Cd^{2+} corresponded to about 75 percent of the maximal tension response generated in 100 μM Ca^{2+}. The contractile apparatus had a fourfold higher sensitivity to activation by Ca^{2+} than by Cd^{2+} and the slope of the curve relating tension to $[Ca^{2+}]$ was steeper than the curve obtained for Cd^{2+}. The results in general show that whilst Cd^{2+} is capable of directly activating myofibrillar tension, it does not do so as effectively as Ca^{2+}. The results from this study support results obtained by Stephenson and Thieleczek (1986) using skinned frog iliofibularis fibres, although some important differences between the two studies were found. In Stephenson's study, the maximum force generated by Cd^{2+} and Ca^{2+} was the same in frog fibres and the contractile apparatus exhibited a 10-fold higher sensitivity to activation by Ca^{2+}. A possible reason for the differences in results could be that the contractile apparatus of mammalian fibres is more sensitive to activation by Cd^{2+} than amphibian fibres;

Tension produced by high concentrations of Cd^{2+} (eg 200 μM) spontaneously decayed. This was proposed by Stephenson and Thieleczek (1986) to be the result of an increase in the formation of Cd-ATP which has an inhibitory effect on the tension response. It is possible that the failure of Cd^{2+} to generate a maximal force similar to Ca^{2+} in this study was the result of the inhibitory effects of Cd^{2+} at high concentrations having a stronger influence in mammalian fibres than in amphibian fibres.

Apart from directly activating the contractile apparatus, a leak of Cd^{2+} into the myoplasm could also cause tension via Cd^{2+} -induced SR Ca^{2+} release. Cd^{2+} and other heavy metals (2-5 μM), including Hg^{2+}, Cu^{2+}, Ag^{+} and Ni^{2+}, are reported to induce Ca^{2+} release from isolated SR vesicles (Abramson *et al.*, 1983) and from the SR of skinned rabbit psoas fibres (Salama *et al.*, 1992). This effect is thought to be due to an interaction between heavy metals and critical sulphydryl groups on the RyR Ca^{2+} release channel since Ca^{2+} release is blocked by ruthenium red and magnesium and is

dependant on [Ca^{2+}]. Furthermore, heavy metals have been shown to displace the binding of [^{3}H] ryanodine (Salama *et al.*, 1992). Cd^{2+} is also known to inhibit the SR Ca-ATPase (Ca^{2+} pump). Hechtenberg and Beyersman (1991) report that ATP hydrolysis in rabbit skeletal muscle is inhibited by 50% in 0.95 μM Cd^{2+}. Such an effect would prevent the re-uptake of Ca^{2+} into the SR and augment the elevation of myoplasmic Ca^{2+}. Although the CIC could be the result of an effect of Cd^{2+} on the RyR, SR Ca^{2+} release was not thought to contribute to the skinned fibre tension in this study since:

1. the SR was destroyed by exposure to the detergent Triton X-100;
2. 10 mM caffeine was present in all solutions which would have caused any sequestered Ca^{2+} to be released.

The results presented in this Chapter, show that micromolar Cd^{2+} causes tension development in skinned rat soleus fibres via a direct interaction with the fibres contractile machinery. Since Cd^{2+} is reported to also cause SR Ca^{2+} release at micromolar concentrations (Abramson *et al.*, 1983; Salama *et al.*, 1992), it seems possible that the leakage of Cd^{2+} across the surface membrane of intact fibres could indeed be responsible for, or contribute to, the Cd^{2+}-induced contracture. Because the external [Cd^{2+}] used to initiate the CIC was in the mM range, only a small fraction of external Cd^{2+} would be required to enter the myoplasm to induce contraction. The absolute dependence of the CIC on external Ca^{2+} (Chapter 5) could be explained by assuming that the entry of Cd^{2+} into the fibre is mediated by external Ca^{2+}. The key question therefore is whether it is likely that Cd^{2+} could enter the myoplasm. Fukuda and Kawa (1977) report that Cd^{2+}, Zn^{2+} , Mn^{2+} and Be^{2+} all carry currents through the surface membrane of larval insect skeletal muscle cells. Interestingly, Co^{2+}, Ni^{2+} and Mg^{2+} did not traverse the membrane but instead competitively inhibited the permeation of the other cations. In another study, Lansman and colleagues (1986) speculate that potent Ca^{2+} channel blockers such as Co^{2+}, Cd^{2+} and La^{3+} might also permeate the Ca^{2+} channels and gain entry to cells under appropriate conditions. This evidence was based observations that membrane hyperpolarisation rapidly speeds up the unblocking reaction of these ions on cardiac L-type Ca^{2+} channels. However, patch clamp studies have so far failed to provide direct evidence of Cd^{2+} entry into cardiac cells (Hess *et al.*,

1986) and there is no evidence to show the entry of Cd^{2+} into mammalian skeletal muscle.

In conclusion, it is possible that the CIC is the result of an internal effect of Cd^{2+} on both the RyR and contractile apparatus. The major argument against this type of mechanism for the CIC is the lack of evidence supporting Cd^{2+} movement across the sarcolemma into the myoplasm of mammalian skeletal muscle.

Chapter 7

General Conclusions

Conclusions

From the results presented in this report it is evident that the effects of Cd^{2+} on contraction in rat soleus fibres are complex. The problem with studying this cation is that it has more than one site of action which makes the identification of specific effects extremely difficult. Whilst the main action of this cation is proposed to be external, the contribution of possible internal effects have also been considered. Three main sites of Cd^{2+} action (summarised in Fig. 7.1) are proposed:

1. the surface membrane;
2. T-tubule (voltage sensor);
3. internal (RyR and contractile apparatus).

Each of these sites are discussed below in context with the proposed mechanisms for the CWC, CIC and effects of the cation on twitches, tetani and K contractures.

Interaction of Cd^{2+} with the surface membrane

In general, it is proposed that Cd^{2+} binds to negative charges present on the surface of the muscle fibre. The possible sources of these charges are varied, arising both from sialic acid residues in the membrane lipids, and various negatively charged amino acid sidechains such as cysteine present in membrane proteins. The binding of Cd^{2+} to these sites could have the following consequences:

1. hyperpolarise the surface membrane thus shifting the gating of voltage activated ion channels towards more positive potentials
2. block of surface membrane ion channels
3. shift the voltage dependence of contractile activation towards more positive potentials

Both 1 and 2 would effect action potential propagation, and this is proposed to be the mechanism by which Cd^{2+} affects twitch and tetanic tension (Chapter 4). Although the effects of Cd^{2+} on specific surface membrane ion channels were not investigated, the results in this study have been tentatively explained by looking at other reports on the effects of this cation on surface membrane channels in other cell types. Cd^{2+} caused the depression of submaximal K contractures. Both the effects of Cd^{2+} on action potentials

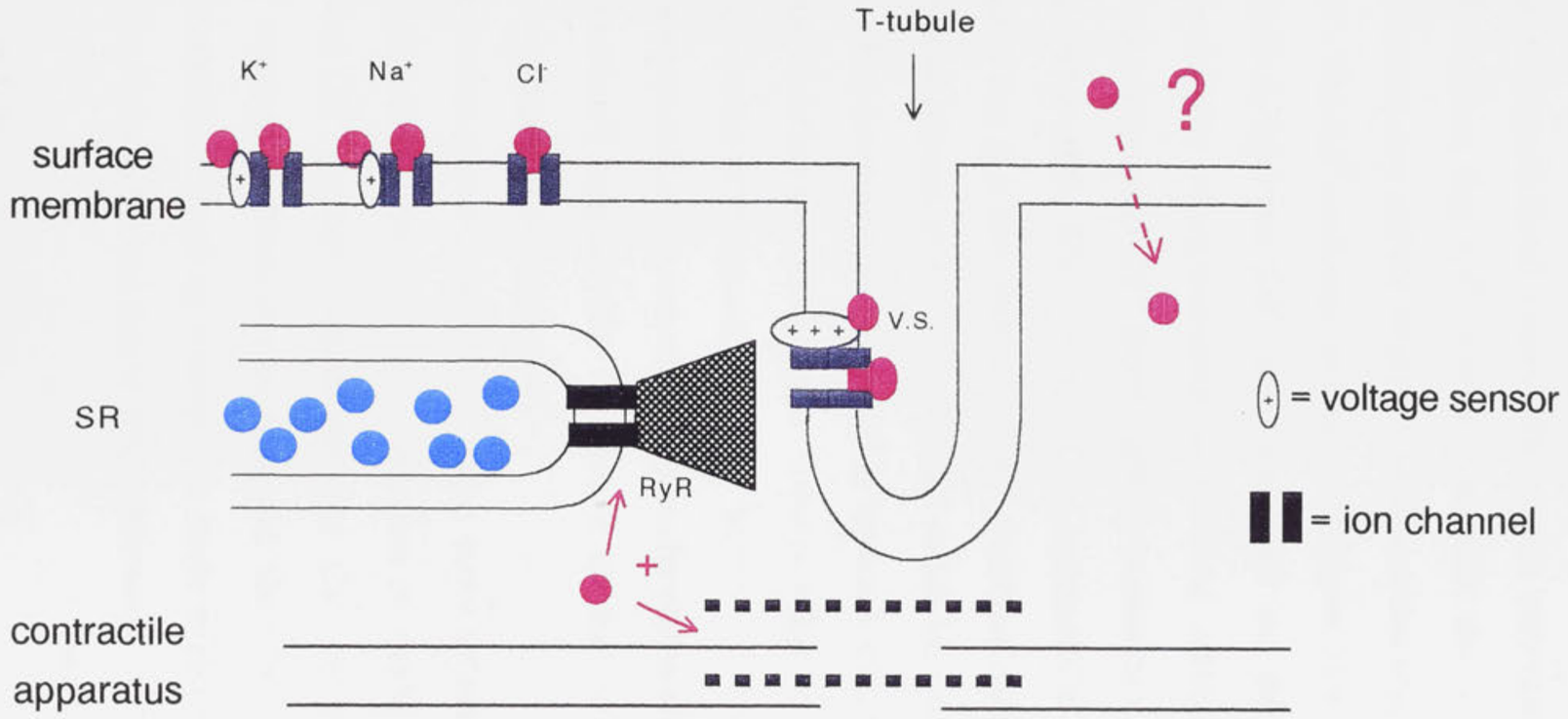


Figure 7.1 *Proposed sites of Cd^{2+} action on rat soleus fibres*

A diagram summarising the sites at which Cd^{2+} is proposed to interact with rat soleus fibres to cause the CWC (Chapter 3), CIC (Chapter 5), and effect of Cd^{2+} on twitch, tetanus, and K contracture tension (Chapter 4). The filled red circles represent Cd^{2+} ions and the filled blue circles represent Ca^{2+} ions. From the diagram, Cd^{2+} is shown to block ion channels in the surface membrane as well as interfering with the channel voltage sensors. Most important is the interaction of Cd^{2+} with the voltage sensor for E-C coupling located in the T-tubule. Possible internal effects of Cd^{2+} on the contractile apparatus and RyR Ca^{2+} release channel are also indicated.

and K contractures are consistent with similar reports in the literature for the effects of other cations. It is likely therefore that the effect of Cd^{2+} on these parameters is the result of a generalised cation effect at the membrane surface. If this is true, then one might expect that Cd^{2+} would also shift the voltage dependence of steady state inactivation in a similar direction. However, this was not examined.

The Cd^{2+}-induced contracture (Chapter 5) is also proposed to be the result of an interaction between Cd^{2+} and some site on the surface membrane although the mechanism by which this occurs is less clear. Although this contracture is more specific it is not restricted to Cd^{2+} since a similar contracture occurs in the presence of Zn^{2+}. The dependence of the CIC on external Ca^{2+} but not voltage sensor activation, suggests that it is the result of a Cd^{2+} -mediated influx of Ca^{2+} into the fibre. There are numerous reports in the literature of metal ion induced currents in a variety of cell types. The currents may be inward or outward and carried by various ions depending on the metal cation and the cell type. Some possible routes through which Ca^{2+} might enter the cell whilst in the presence of Cd^{2+} are:

1. a novel metal ion induced non-specific cation channel
2. increased non-specific membrane conductance arising from disruption of the membrane structure by Cd^{2+}.

As a further experiment, Ca^{2+} currents could be measured in the surface membrane of skeletal muscle fibres during the presence of 3 mM Cd^{2+} to determine whether a Cd^{2+} induced Ca^{2+} current could be detected. Ca^{2+} currents could also be measured during Cd^{2+} washout to check that as proposed, the CWC was not the result of a Ca^{2+} influx mechanism. Although an attempt was made to conduct some of the above experiments, the idea was abandoned due to time constraints.

Cd^{2+} and the voltage sensor

The depression of submaximal K contracture tension and the shift in voltage dependence of tension to more positive membrane potentials can be explained by the binding of Cd^{2+} to negative membrane surface charges. On the other hand, the change in the slope of voltage activation curve cannot be explained by this mechanism and instead suggests a direct and specific effect of Cd^{2+} on the voltage sensor. The Cd^{2+}-

withdrawal contracture (Chapter 3) was proposed to be due to an activating effect of Cd^{2+} withdrawal on the voltage sensor. Whilst the exact mechanism of the CWC remains unknown, the effect could be explained by changes in surface charge associated with Cd^{2+} binding and unbinding to the voltage sensor, possibly to sulfhydryl groups. An interesting further experiment therefore would be to investigate the effects of compounds which protect free sulfhydryl groups or reverse the effects of sulfhydryl oxidation on the amplitude of the CWC.

Possible internal effects

As discussed in Chapter 6, it is possible that the CIC (Chapter 5), could be the result of Cd^{2+} entering the myoplasm and causing tension by activating SR Ca^{2+} release (via an effect on the RyR) or by directly activating the contractile apparatus.

Whilst an internal effect of Cd^{2+} could explain or contribute to the CIC, the dependency of CIC amplitude on external Ca^{2+} and lack of evidence supporting the movement of Cd^{2+} into skeletal muscle cells, make the likelihood of this type of mechanism somewhat doubtful. To clarify whether Cd^{2+} does enter the myoplasm of rat soleus fibres some possible further experiments could involve: radioactively labelling Cd^{2+} to trace the location of Cd^{2+} ions after Cd^{2+} washout using autoradiographic techniques; or using cation sensitive fluorescent dyes injected into the fibre which fluoresce when bound to Cd^{2+} (although it is doubtful that dyes with such specificity exist).

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Appendices

Appendix 1

Approximate free cation concentrations present in the SO_4^{2-} containing solutions from Table 2.2 (Chapter 2).

solution		approx free [cation] mM		
code	**type**	**Ca^{2+}**	**Mg^{2+}**	**Cd^{2+}**
2A	**control**	1.27	0.19	
B	**3Cd**			~0.40
C	**30K**	0.69	0.10	
D	**30K/3Cd**			~0.20
E	**40K**	0.69	0.10	
F	**40K/3Cd**			~0.20
G	**60K**	0.70	0.11	
H	**60K/3Cd**			~0.20
I	**80K**	0.71	0.11	
J	**80K/3Cd**			~0.20
K	**120K**	0.72	0.11	
L	**120K/3Cd**			~0.21
M	**200K**	0.76	0.12	
N	**200K/3Cd**			~0.22

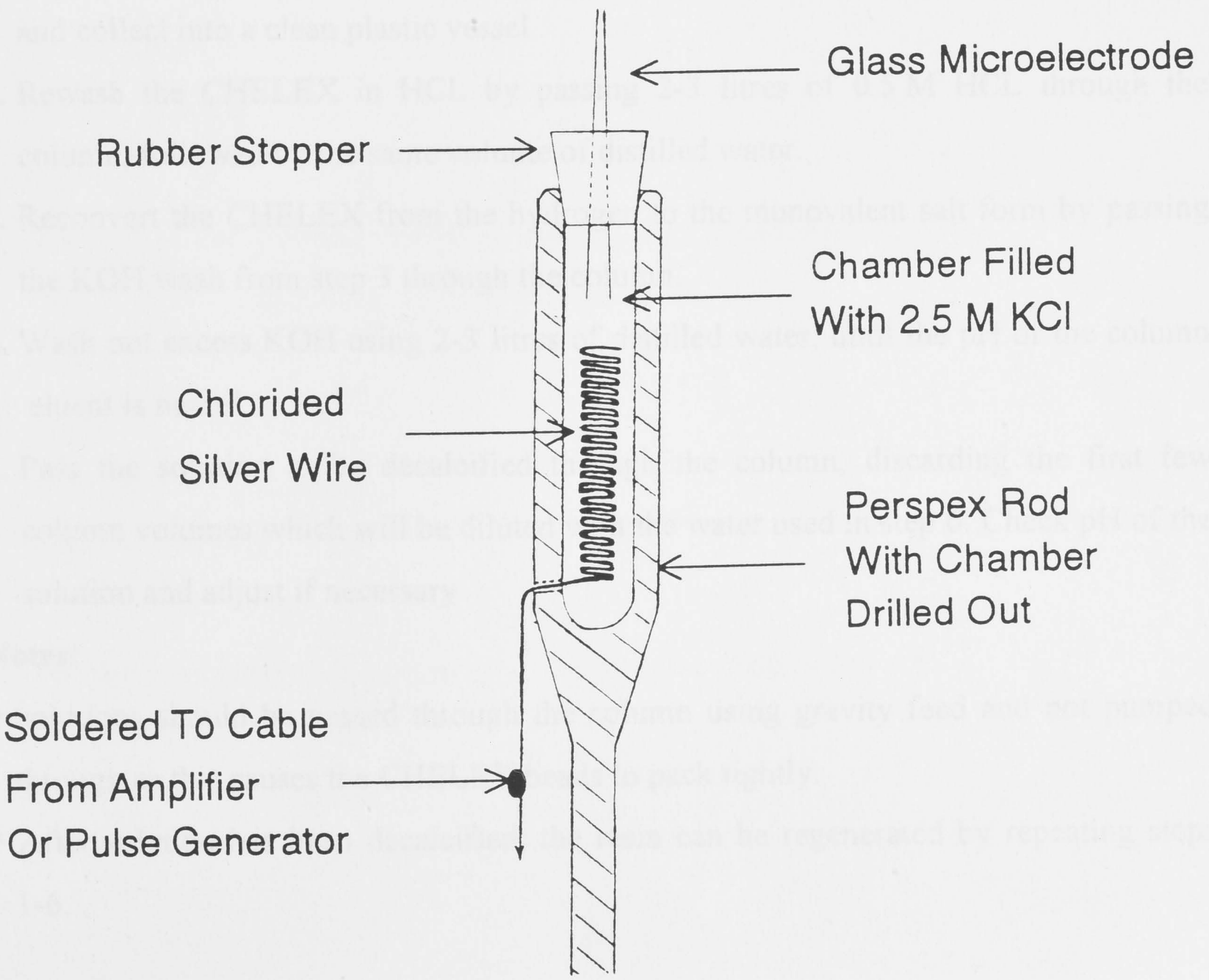
*Free cation concentrations were calculated using "BUFFA" (see Appendix 4). The following stability constants were used: $CaSO_4$, 204 M^{-1}; $MgSO_4$, 170 M^{-1}; K_2SO_4, 7 M^{-1}; Na_2SO_4, 5 M^{-1}; $CdSO_4$, 288 M^{-1} (from Dulhunty and Gage, 1989).

NOTE:

1. The free Cd^{2+} concentrations are very approximate because the programme "BUFFA" is limited to calculations with a maximum of 4 metal ions, and there were 5 metal ions in the Cd^{2+} containing solutions (Na^{+}, Ca^{2+}, Cd^{2+}, K^{+}, and Mg^{2+}). To overcome this problem, Mg^{2+} was omitted from the calculations and as a result, the actual free concentration of Cd^{2+} would be expected to be slightly higher, since some of the SO_4^{2-} would have been bound to Mg^{2+}.
2. The calculated free Ca^{2+} concentrations of the Cd^{2+} containing solutions were not shown in the above table because they were only slightly different from the values listed for the Cd^{2+} -free solutions.

Appendix 2

Structural details of microelectrodes used for recording of membrane and action potentials (Section 2.2.6, Chapter 2).



Appendix 3

Method of Ca^{2+} removal from skinned fibre solutions using CHELEX 100 resin (BIO-RAD).

CHELEX 100 is an ion exchange resin which has a high preference for heavy metals over monovalent cations. The resin was used to decalcify solution S (Table 2.3, Chapter 2) by the method outlined below.

1. Place CHELEX beads into a clean plastic vessel and wash beads several times in 0.5 M HCI followed by several washes in distilled water by decantation..

2. Half fill a plastic column eg. Pharmacia type (dimensions ~ 2.0 × 30 cm) with washed CHELEX beads. The column should only be half filled because the CHELEX resin doubles it's volume when converted from the hydrogen form to a monovalent salt form. It is important that the column contains a gel bead support mesh small enough to retain the CHELEX.

3. Pass several litres of 0.5 M KOH (or any other OH^- salt depending on the principal monovalent cation in the solution to be decalcified) through the CHELEX column and collect into a clean plastic vessel.

4. Rewash the CHELEX in HCL by passing 2-3 litres of 0.5 M HCL through the column, followed by the same volume of distilled water.

5. Reconvert the CHELEX from the hydrogen to the monovalent salt form by passing the KOH wash from step 3 through the column.

6. Wash out excess KOH using 2-3 litres of distilled water, until the pH of the column eluent is near 7.

7. Pass the solution to be decalcified through the column, discarding the first few column volumes which will be diluted with the water used in step 6. Check pH of the solution and adjust if necessary.

Notes:

* solutions should be passed through the column using gravity feed and not pumped through as this causes the CHELEX beads to pack tightly.

* After solution has been decalcified, the resin can be regenerated by repeating steps 1-6.

Appendix 4

Method for calculating the free concentration of cations in the SO_4^{2-}-containing and skinned fibre solutions (Chapter 2) using the software program: "BUFFA" Version. January 1985 (R.G Ryall, Dept. of Haematology, Flinders Medical Centre, Bedford Park. S.A. 5042).

Listed below are the sequence of steps encountered throughout the program and examples of entries used for the calculation of free [Cd^{2+}] resulting from the addition of varying amounts of $CdCl_2$ to solution S (Table 2.3, Chapter 2). The relative affinity constants for the complexing agents and cations used in the example below are listed in Table 2.4 (Chapter 2) and were obtained from Stephenson and Thieleczek (1986).

1. Number of metals in reaction mixture (max 4) = ?

enter 3: (Ca^{2+}, Cd^{2+} and Mg^{2+})

2. Metal 1 = ? Cd^{2+} (enter the cation of main interest first)

Metal 2 = ? Ca^{2+}

Metal 3 = ? Mg^{2+}

3. Number of complexing agents in the reaction mixture (max 5) = ?

enter 3 ie ATP, succinate, creatine phosphate (CP)

4. Agent # 1 = ? ATP

Agent # 2 = ? succinate

Agent # 3 = ? CP

5. Number of complexed and protonated species (Max = 30) = ?

ie. ATP binds 1 Ca^{2+}

1 Mg^{2+}

1 Cd^{2+}

succinate binds 1 Ca^{2+}
1 Mg^{2+}
1 Cd^{2+}

CP binds 1 Mg^{2+}
1 Ca^{2+}
1 Cd^{2+} Total = 9

6. Enter 9

7. Complexed species # 1= ?

eg (for ATP and Ca^{2+})

H^+ = **?** enter 0

Cd^{2+} = **?** enter 0

Ca^{2+} = **?** enter 1

Mg^{2+} = **?** enter 0

succ = **?** enter 0

ATP = **?** enter 1

CP = **?** enter 0

Log constant = ? 3.79

8. pH of the reacting solution = ? enter 7.4

9. concentration of succinate (M) = ? enter 50 mM (ie 0.05 M)

10. concentration of ATP (M) = ? enter 8 mM (ie 0.008 M)

11. concentration of CP (M) = ? enter 10 mM (ie 0.01 M)

12. Lowest concentration of Cd^{2+} (added) = ? say enter 0

13. highest concentration of Cd^{2+} (added) = ? (sets range) say 100 μM (0.0001 M)

14. incremental change required = ? say 20 μM (0.00002 M)

15. concentration of Ca^{2+} (added) = ? (contaminant) say 0.1 μM (ie 0.0000001 M)

16. concentration of Mg^{2+} (added) = ? enter 8.6 mM (ie 0.0086 M)

The following list of calculated cation concentrations will appear on the screen. Shown are the free concentrations of Cd^{2+}, Ca^{2+} and Mg^{2+} resulting from adding different amounts of Cd^{2+} to the solution.

Metal	Total concn added (M)	Free concn (M)
Cd^{2+}	2.000E -05	3.763E -07
Ca^{2+}	1.000E -07	1.642E -08
Mg^{2+}	8.600E -03	9.247E -04
Cd^{2+}	4.000E -05	7.569E -07
Ca^{2+}	1.000E -07	1.649E -08
Mg^{2+}	8.600E -03	9.296E -04
Cd^{2+}	6.000E -05	1.142E -06
Ca^{2+}	1.000E -07	1.656E -08
Mg^{2+}	8.600E -03	9.346E -04
Cd^{2+}	8.000E-05	1.531E -06
Ca^{2+}	1.000E-07	1.663E -08
Mg^{2+}	8.600E -03	9.397E -04

Appendix 5

List of chemicals used

Chemical	Source	Notes
Cl^- salts		
NaCl	BDH Chemicals	
KCl	AJAX Chemicals	
HCl	SIGMA Chemical Company	
$CaCl_2$	AJAX	
$MgCl_2$	SIGMA	Ultrapure
$CdCl_2$	SIGMA	Ultrapure
$CoCl_2$	AJAX	
SO_4^{2-} salts		
Na_2SO_4	BDH	
K_2SO_4	AJAX	
$MgSO_4$	AJAX	
$CaSO_4$	AJAX	
OH^- salts		
NaOH	AJAX	
KOH	AJAX	
Buffers		
EGTA	SIGMA	
HEPES	SIGMA	
TES	SIGMA	
Succinic acid	ALDRICH Chemical Company	Titrated with KOH
Drugs + other		
Nifedipine	SIGMA	Dissolved in ethanol
Caffeine	AJAX	
ATP	SIGMA	Disodium salt

List of chemicals cont...

Chemical	Source	Notes
Phosphocreatine	SIGMA	Disodium salt
Triton X-100	Boehringer Mannheim	
Sucrose	AJAX	
d-Glucose	AJAX	
Paraffin oil	Faulding	