SKELETAL MUSCLE AND MALIGNANT HYPERPYREXIA

by

PAUL STEPHEN FOSTER

STATEMENT

The investigations described in this thesis are my own original work.

Paul Stephen Foster
May, 1987
DEDICATION

This thesis is dedicated to Kim.
ACKNOWLEDGEMENTS

I would like to thank Dr M.A. Denborough for his guidance and constant encouragement throughout the course of this study and his assistance in the preparation of this thesis.

I am indebted to Drs B. Creese and M.D. White for their invaluable assistance and constructive comments during the preparation of this thesis. Gratitude is also expressed to Mr K. Hopkinson for his technical assistance.

A special thanks is extended to my wife Kim for her unerring support during my university studies, for the maintenance of my nutritional state and the excellent work in typing this manuscript.

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Finally, I would like to thank my family for their support during my tertiary endeavours and Mr G. Cooper and colleagues for duplicating the thesis.
Susceptibility to the anaesthetic-induced syndrome Malignant Hyperpyrexia (MH) is diagnosed by in vitro contractile responses of muscle fibres. In both human and porcine MH, skeletal muscle fibres contract in the presence of 3% halothane, 2mM caffeine and 80mM KCl, while normal or control muscle contracts only at higher caffeine concentrations.

Like the skeletal muscle relaxant dantrolene, the calcium channel antagonist diltiazem inhibited and reversed the abnormal contractile responses of isolated MH susceptible human and porcine skeletal muscle fibres. Dantrolene and diltiazem also inhibited contractile responses to caffeine in control muscle. However, in detubulated MH susceptible and control porcine muscle diltiazem failed to affect contractile responses to caffeine while dantrolene inhibited responses. Furthermore, dantrolene could still induce relaxation in the presence of maximally effective concentrations of diltiazem and the converse was also true.

In the presence of diltiazem MH susceptible pigs failed to express the clinical features of MH, after being challenged with the precipitating agents succinylcholine and halothane. The same pigs all developed MH in response to succinylcholine and halothane in the
absence of diltiazem. Administration of diltiazem during a MH crisis dramatically prevented any further expression of the syndrome. Diltiazem may be an effective therapeutic agent in the treatment of MH if administered early in the onset of a crisis or before the initiation of anaesthesia. These results also suggest that Ca\(^{2+}\) influx through transverse tubules may be important in the aetiology of the MH syndrome.

The calcium channel antagonists verapamil and nifedipine failed to be effective in inhibiting all the abnormal drug-induced responses of isolated MH susceptible human and porcine muscle. Nifedipine and verapamil potentiated halothane-induced contractures in MH susceptible pig muscle. Nifedipine also potentiated responses to caffeine and halothane in isolated control human muscle preparations. Verapamil also had no effect on Ca\(^{2+}\) release by isolated terminal cisternae membrane fractions. In view of these observations it is unlikely that these calcium channel antagonists would inhibit a MH response \textit{in vivo}.

A detailed biochemical characterisation of the sarcoplasmic reticulum membrane from MH susceptible and control pig muscle was conducted with the aim of defining the site of the abnormality predisposing to MH. The SR membrane from MH susceptible and control porcine skeletal muscle was separated into two vesicular fractions enriched
in the membrane elements of the terminal cisternae and longitudinal tubules. Characteristics of membrane fractions derived from MH susceptible muscle did not differ from those of control preparations. Morphological studies and protein polyacrylamide gel electrophoresis showed the same membrane elements in corresponding fractions. Ca$^{2+}$ fluxes associated with the terminal cisternae membrane did not vary nor did Ca$^{2+}$ uptake by the longitudinal tubules. In this study no distinction could be made between the membrane elements or Ca$^{2+}$ fluxes associated with these functionally distinct regions of MH susceptible and control sarcoplasmic reticulum membranes.

Diltiazem did not affect Ca$^{2+}$ fluxes associated with isolated porcine SR membrane fractions from the terminal cisternae or longitudinal tubules. Dantrolene inhibited Ca$^{2+}$ release from the terminal cisternae membrane but had no affect on the sequestration of Ca$^{2+}$ by the longitudinal tubules. Both drugs failed to effect Ca$^{2+}$-dependent ATPase activities of the terminal cisternae and longitudinal tubules. These results suggested that diltiazem and dantrolene have a separate site of action. Diltiazem probably acts by modifying contractile responses at the level of the transverse tubular membrane while dantrolene acts directly on the terminal cisternae membrane to inhibit Ca$^{2+}$ release. Results with diltiazem also implicate abnormal transverse tubular Ca$^{2+}$ fluxes in the
pathogenesis of MH. Caffeine did not modify \( \text{Ca}^{2+} \) uptake or \( \text{Ca}^{2+} \)-dependent ATPase activities of longitudinal sarcoplasmic reticulum membranes, but increased \( \text{Ca}^{2+} \) efflux and exchange across the terminal cisternae membrane. This suggested that caffeine acts selectively at the junctional region of the sarcoplasmic reticulum to release \( \text{Ca}^{2+} \).

Changes in the phosphate metabolite profile of MH susceptible skeletal muscle occurs more readily under conditions of anoxia when compared to control muscle. Increased oxygen consumption may occur in anoxic MH muscle which leads to accelerated glycolysis, a rapid fall in intracellular high energy phosphates and acidosis. Accelerated muscle metabolism is also observed in the presence of 2mM caffeine and 3% halothane in MH muscle. Changes in the concentrations of metabolites and intracellular pH can be mapped under anoxic conditions using Topical \(^{31}\text{P}-\text{NMR}\). \(^{31}\text{P}-\text{NMR}\) spectroscopy may therefore have the potential to aid in the non-invasive diagnosis of MH.
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<td>AAS</td>
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<tr>
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<td>CCA</td>
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<td>PSE</td>
<td>pale soft exudative</td>
</tr>
<tr>
<td>PSS</td>
<td>porcine stress syndrome</td>
</tr>
<tr>
<td>31P-NMR</td>
<td>31&lt;sup&gt;P&lt;/sup&gt;Phosphorus nuclear magnetic resonance</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulphate</td>
</tr>
<tr>
<td>s.e.m</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SIDS</td>
<td>sudden infant death syndrome</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>T-tubules</td>
<td>transverse tubules</td>
</tr>
<tr>
<td>T-system</td>
<td>sarcoplasmic reticulum transverse tubules</td>
</tr>
<tr>
<td>VOCC</td>
<td>voltage-operated calcium channels</td>
</tr>
</tbody>
</table>
1.1 MALIGNANT HYPERPYREXIA

Malignant Hyperpyrexia (MH) was first described by Denborough and Lovell as familial hypersensitivity to general anaesthesia in 1960. Ten relatives of a patient who was to undergo general anaesthesia for the reduction of a compound fracture had died during or shortly after ether anaesthesia. During surgery the propositus also exhibited a drastic reaction to general anaesthesia, developing unexplained fever, tachycardia, a falling blood pressure, sweating and cyanosis of the skin. The anaesthetic was ceased, the patient was packed in ice and he survived. Later, investigations on the genetic relationship elucidated the mode of inheritance of MH susceptibility as autosomal dominant, (Denborough, Forster, Lovell, Maplestone and Villiers, 1962).

MH is now widely recognised as an inherited muscular abnormality which presents clinically as a syndrome of life threatening complications during general anaesthesia. An explosive and sustained rise in core body temperature and gross muscle rigidity are striking clinical features of an MH episode. Clinical manifestations of MH follow the administration of a skeletal muscle relaxant or
inhalation anaesthetic. Halothane and succinylcholine are most commonly incriminated (Britt and Kalow, 1970a; Gordon, Britt and Kalow, 1973). The onset of an MH episode can occur immediately after induction, several hours after initiation or shortly after cessation of general anaesthesia (Britt and Kalow, 1970b; Denborough, 1980; Fletcher, Blennow, Olsson, Ranklev and Tornebrant, 1982). Masseter muscle rigidity and subsequent difficulty in intubating a patient who has received a skeletal muscle relaxant is an early warning sign of an MH crisis (Denborough, 1980; Flewellen and Nelson, 1984; Ellis and Halsall, 1984). During anaesthesia unexplained ventricular arrhythmia, tachycardia, sweating, patchy cyanosis of the skin, a falling blood pressure and increased respiratory rate and depth are indicative of a pending MH crisis (Britt, 1977, 1979; Nissen and Yonkers, 1982). As the episode develops, a rapid and sustained rise in body temperature, elevated serum potassium concentration and high blood carbon dioxide levels are observed. Severe metabolic and respiratory acidosis also occur. Skeletal muscle rigidity develops in 70-80% of reactions (Britt, 1969; Stephen, 1977; Britt, 1979; Denborough, 1980). Large increases in the serum levels of enzymes such as creatine phosphokinase (CPK), glutamate oxaloacetate transaminase, lactate dehydrogenase and hydroxybutyryl dehydrogenase are observed, indicating sarcolemmal damage (Britt, 1979; Gronert, 1980). Myoglobinuria may cause acute renal failure and late neurological changes
such as fixed dilated pupils, convulsions and coma may also occur.

Successful management of MH requires early diagnosis, immediate cessation of anaesthesia, prompt intravenous administration of the drug dantrolene sodium, peripheral cooling with ice, hyperventilation with oxygen and correction of metabolic acidosis by intravenous administration of sodium bicarbonate (Britt and Gordon 1969; Brițt and Kalow, 1970a; Denborough, 1975; Kolb, Horne and Martz, 1982).

While the incidence of MH is rare, ranging from 1:14,000 to 1:75,000 general anaesthetics (Ellis, Keany, Harriman, Sumner, Kyei-mensah, Tyrrell, Hargreaves, Parikh and Mulrooney, 1972; Williams, 1976; McPherson and Taylor, 1982) a mortality rate of 60-70% indicates that awareness of MH is important for safe anaesthetic practice (Britt and Kalow, 1970b).

MH may also present after severe physical stress as heat stroke (Denborough, 1982) or as rhabdomyolysis after serious infections (Denborough, Collins and Hopkinson, 1984). An association between MH and sudden infant death syndrome (SIDS) has also been described (Denborough, Galloway and Hopkinson, 1982). In this investigation, 5 out of 15 parents whose children had died from SIDS were diagnosed as MH susceptible. The
association between SIDS and MH has been confirmed (Peterson and Davis, 1986).

Although various musculoskeletal abnormalities had been reported in patients with MH (Saidman, Havard and Eger, 1964; Hogg and Renwick, 1966; Relton, Creighton, Johnston, Pelton and Conn, 1966) it was not until 1968 that the skeletal musculature was first incriminated as the site of the abnormality predisposing to MH (editorial British Medical Journal, 1968). It was proposed that there was a relationship between the administration of succinylcholine, hypermetabolism in skeletal muscle tissue and the onset of an MH crisis. The observation of generalized muscular rigidity in an MH susceptible individual undergoing anaesthesia, while a limb which had a tourniquet on it remained flaccid, further suggested skeletal muscle as the sight of the anaesthetic-induced abnormality (Drury and Gilbertson, 1970). The finding of markedly elevated serum CPK and other muscle enzyme levels in a susceptible individual after an MH episode indicated that the anaesthetic was indeed acting on skeletal muscle, inducing severe muscle damage (Denborough, Forster, Hudson, Carter and Zapf, 1970a). Subsequent investigations of families susceptible to MH showed abnormally high resting serum CPK levels and it was proposed that MH was associated with a subclinical myopathic process (Isaacs and Barlow, 1970; Denborough, Ebeling, King and Zapf, 1970b).
Three myopathies which predispose to MH have now been described (King, Denborough and Zapf, 1972; Denborough, 1978). The Evans myopathy, which is the most common myopathy predisposing to MH, is inherited as a Mendelian dominant characteristic and is usually subclinical although some muscle wasting in the thigh region may occur (Denborough et al., 1970b; King et al., 1972). The second myopathy occurs in young boys and is probably inherited as a recessive characteristic (King and Denborough, 1973). The boys are usually small in stature and have unusual facial appearances with a small chin, low set ears and antimongoloid obliquity of the palpebral fissures. Other physical abnormalities may include undescended testes, lumbar lordosis, thoracic kyphosis and pectus carinatum. Central core disease is also associated with MH (Denborough, Dennett and Anderson, 1973). This myopathy is only evident after histological examination of muscle tissue and is characterised by 'core' lesions in type I fibres (Denborough, 1979). MH episodes have also been reported in individuals with Duchennes and congenital muscular dystrophies (Rowland, 1980; Fletcher et al., 1982; Brownell, Paasuke, Elash, Fowlow, Seagram, Diewold and Friesen, 1983; Kelfer, Singer and Reynolds, 1983). However, Moulds and Denborough (1974a) failed to find any pharmacological abnormality in muscle from patients with myotonia congenitia, dystrophia myotonica and hypokalaemic periodic paralysis. Furthermore, general anaesthesia in Duchennes
muscular dystrophy is normally uneventful (Richards, 1972).

Further evidence that MH is an underlying disease of skeletal muscle came from experiments using isolated skeletal muscle fibres from susceptible individuals. Isolated susceptible skeletal muscle fibres display hypercontractility to caffeine (Kalow, Britt, Terreau and Haist, 1970), halothane (Ellis, Harriman, Keaney, Kyei-mensah and Tyrrell, 1971; Moulds and Denborough, 1974b) and potassium chloride (KCl) (Moulds and Denborough, 1974b). The distinction between control and MH muscle can be made by challenging the muscle preparation with 2mM caffeine, 3% halothane, or 80mM potassium chloride (Denborough, 1980). Also, MH susceptible muscle strips pretreated with 4mM caffeine produce a larger contracture than control strips in response to succinylcholine (6.29mM) (Halsall and Ellis, 1979).

While the exact aetiiological mechanism which predisposes to the occurrence of MH is unknown, it is now widely accepted that anaesthetic-induced MH results from an increased level of free calcium ions (Ca$^{2+}$) in the myoplasm (Kalow et al., 1970; Moulds and Denborough, 1972; Nelson, Jones, Venable and Kerr, 1972; Moulds and Denborough, 1974c; Denborough, 1980). The strength of muscular contraction is related to the concentration of Ca$^{2+}$ in the myoplasm (Weber and Murray, 1973). This
suggests that the observed hypercontractility of MH skeletal muscle \textit{in vitro}, and the generalised muscular rigidity usually associated with MH \textit{in vivo}, is due to a dysfunction in the mechanism of Ca$^{2+}$ regulation by the muscle cell which leads to a raised myoplasmic Ca$^{2+}$ concentration.

A rise in the myoplasmic Ca$^{2+}$ will also have several heat producing effects (Blinks, Rudel and Taylor, 1978; Britt, 1979). Phosphorylase will become activated resulting in the catabolism of glycogen to lactic acid, carbon dioxide and the liberation of heat (Ozawa, Hosi and Ebashi, 1967; Heilmeyer, Meyer, Haschke and Fischer, 1970). Further increases in the myoplasmic Ca$^{2+}$ concentration would induce muscle contraction. Actomyosin ATPase would be activated, hydrolysing ATP to ADP, inorganic phosphate and heat. Prolonged elevation of Ca$^{2+}$ levels required to sustain muscular rigidity during an MH episode may result in the sequestration of Ca$^{2+}$ by the mitochondria. Consequently oxidative phosphorylation would be uncoupled from electron transport, thereby decreasing ATP production and further increasing oxygen consumption and lactate, carbon dioxide and heat output (Britt, 1979). Once the muscle stores of creatine phosphate are depleted in an attempt to maintain ATP levels, ATP depletion will be rapid. ATP is necessary for the separation of actin and myosin and subsequent muscle relaxation. ATP also serves as a substrate for
sarcoplasmic reticulum (SR) ATPases. A decrease in the ability of these enzymes to function effectively would further exacerbate the MH crisis. Once ions and molecules can flow freely into and out of the muscle cell serum pH and electrolyte composition will change. This will have adverse effects on the functioning of other body tissues, which are expressed as the clinical manifestations of the MH syndrome (Gronert, 1980; Denborough, 1980; Aldrete, 1981; Britt, 1983).

Raised serum CPK levels, identification of mild or subclinical myopathies, abnormal skeletal muscle function in vitro, and muscular rigidity and fever in vivo indicate that the primary abnormality in MH is an underlying disease of skeletal muscle (Britt, 1979; Denborough, 1980; Gronert, 1980). However, the site of the inherited muscle anomaly which predisposes to the abnormally high myoplasmic Ca^{2+} concentration is yet to be defined, although a number of possibilities have been investigated (these are discussed in Section 2.3 in more detail). These include a dysfunction in the regulation of intracellular Ca^{2+} by the SR (Nelson, 1978; Gronert, Hefferon and Taylor, 1979; Blank, Gruener, Suffecool and Thompson, 1981; Nelson, 1983a; Ohnishi, Taylor and Gronert, 1983), an abnormality in the mechanism of excitation-contraction coupling (EC coupling) (Nelson and Denborough, 1977; Denborough, 1980; Okumura, Crocker and Denborough, 1980), defective Ca^{2+} storage by the
mitochondria (Cheah and Cheah, 1978, 1979), an abnormality of Ca\textsuperscript{2+} binding proteins (Lorkin and Lehman, 1983 a,b) and increased adenylate cyclase activity leading to raised cyclic AMP levels (Willner, Cerri and Wood, 1981). Biochemical and pharmacological investigations on MH muscle tissue indicate that the major lesion is most likely to involve processes that primarily regulate myoplasmic Ca\textsuperscript{2+} levels which are post synaptic (Nelson, Flewelling and Arnett, 1983) and prior to the activation of the contractile proteins (Wood, Mozo and Willner, 1979). Late events of EC coupling, such as the mechanism of signal transmission at the triadic junction or Ca\textsuperscript{2+} release from the SR have been incriminated (Nelson et al., 1983).

Susceptibility to MH is not only confined to humans but also occurs in cats (De Jong, Heavner and Amory, 1974), dogs (Short and Paddleford, 1973; Leary, Anderson, Manning, Blache and Zweber, 1976), horses (Williams, 1976) and pigs (Hall, Wolf, Bradley and Jolly, 1966). Much of the understanding of MH stems from the predominant use of the pig as the animal model for human MH.

The reports of muscle rigor associated with fulminant hyperthermia in pigs exposed to succinylcholine (Hall et al., 1966) or halothane (Harrison, Biebuyck, Terblanche, Dent, Hickman and Saunders, 1968; Berman, Harrison, Bull and Kench, 1970) first led to the association of porcine
MH with human MH. The MH syndrome has been described in the Dutch Pietrain (Sybesma and Eikelenboom, 1969), Poland China (Steward and Thomas, 1971) and Dutch Landrace (Nelson, 1973) breeds of swine. The expression of the MH syndrome in swine may be more florid and extreme than in man, but the clinical manifestations of the syndrome on exposure to a precipitating agent are similar in both species (Berman et al., 1970; Berman and Kench, 1973; Gronert and Theye, 1976 a, b; Gronert, Milde and Theye, 1976; Sybesma and Eikelenboom, 1978; Lucke and Hall, 1983). These include hyperpyrexia, muscular rigidity, metabolic acidosis, elevated plasma potassium, CPK and inorganic phosphate levels. Tachycardia, dysrhythmia, increased blood PaCo₂ and a falling blood pressure are also consistently observed. Also, isolated muscle contracture responses to a variety of chemical stimuli from pigs which are susceptible to MH (Okumura, Crocker and Denborough, 1979) are similar to those observed in muscle from humans who are susceptible to MH (Moulds and Denborough, 1974c). The use of MH susceptible swine as a model for human MH is now firmly established (Denborough, 1980; Gronert, 1980).

The porcine MH syndrome occurs in swine which develop the porcine stress syndrome (PSS). PSS susceptible swine die suddenly when subjected to stressful situations (Topel, Bricknell, Preston, Christian and Matsushima, 1968; Sybesma and Eikelenboom, 1969). Characteristic
features of a stressed animal are identical to the clinical and biochemical changes observed in the porcine MH syndrome (Sybesma and Eikelenboom, 1978). Pigs susceptible to PSS or MH have a high liability to develop a pale soft exudative (PSE) condition of the musculature postmortem (Nelson, 1973). The PSE meat condition is associated with the rapid anaerobic breakdown of glycogen to lactic acid (Briskey, 1964). This causes intracellular pH to fall, denaturing the muscle proteins and reducing the water holding capacity of the carcass.

1.2 SUMMARY

Malignant hyperpyrexia, a life threatening complication of general anaesthesia is characterised by skeletal muscle rigidity and elevated body temperature. The identification of mild or subclinical myopathies, elevated serum CPK levels and abnormal skeletal muscle function in vitro, suggest that susceptible individuals have an underlying disease of skeletal muscle. Clinical features of MH and the observed hypercontractility of isolated MH susceptible muscle fibres indicate that the basic abnormality in MH is a defect in the process which regulates myoplasmic Ca\(^{2+}\) concentration. The precise site of the lesion which predisposes to the abnormally high Ca\(^{2+}\) level is unknown. However, the processes which primarily control myoplasmic free Ca\(^{2+}\) levels, such as the events of EC coupling or Ca\(^{2+}\) transport by the SR, have
been implicated. Much of the information on MH stems from the use of MH susceptible swine as a model for human MH.

1.3 **AIMS OF THESIS**

Many Ca$^{2+}$-dependent cellular processes can be inhibited by calcium channel antagonists (CCA). The basic abnormality in MH susceptible muscle led to an elevation in the level of free Ca$^{2+}$ in the myoplasm. The main aim of the investigations described in this thesis was to examine the effects of CCA on Ca$^{2+}$-dependent functions which are primarily involved in the regulation of myoplasmic Ca$^{2+}$ in skeletal muscle. Thus investigations focused on 3 main aspects:

1) The effects of CCA on the hypercontractility of isolated MH susceptible porcine and human skeletal muscle fibres.

2) A detailed biochemical characterisation of the SR membrane, isolated from MH susceptible and control porcine skeletal muscle. This included a comparative study on the properties of Ca$^{2+}$ uptake and release and Ca$^{2+}$-dependent ATPase activity from functionally distinct regions of control and affected SR preparations. The effects of CCA on these Ca$^{2+}$-dependent processes were also examined.
3) An investigation into the effect of diltiazem on the porcine MH syndrome in vivo.

These studies were conducted with a view to establishing the effectiveness of CCA in regulating the abnormal Ca\(^{2+}\) fluxes in MH susceptible muscle and to further defining the site of the muscle abnormality. Access to human MH susceptible muscle was limited and therefore investigations were predominantly focussed on the porcine MH model.

Another aim was to investigate the phosphate metabolite profile of isolated MH susceptible and control porcine muscle under various conditions using \(^{31}\)Phosphorus nuclear magnetic resonance spectroscopy with a view to defining parameters for the development of a non-invasive test to identify susceptibility in vivo.
CHAPTER 2

THE MUSCLE CELL ABNORMALITY IN MALIGNANT HYPERPYREXIA

2.1 INTRODUCTION

The mechanism whereby depolarization of the surface membrane results in an increase in myoplasmic Ca\(^{2+}\) is called EC coupling (Ebashi, 1979). An abnormality in the mechanism of EC coupling has been proposed to result in the raised myoplasmic Ca\(^{2+}\) concentration in MH susceptible skeletal muscle (Denborough, 1980). Relatively little is known about the events of EC coupling that lead to the release of stored Ca\(^{2+}\) from the SR. This chapter briefly describes the structure and function of systems involved in EC coupling and the evidence incriminating this process as the primary dysfunction in MH skeletal muscle.

2.2 EXCITATION CONTRACTION COUPLING

2.2.1 The Role of Calcium in Contractile Activation

An action potential on the surface membrane of a muscle fibre is followed by a transient rise in myoplasmic Ca\(^{2+}\) and soon thereafter by a rapid increase in force. The importance of Ca\(^{2+}\) for the initiation of muscular contraction has been known for some time. Heilbrunn and Wiercinski (1947) demonstrated that of those ions
normally present in muscle, calcium was the only one which when injected at very low concentrations could cause rapid shortening of muscle fibres. The role of Ca$^{2+}$ as the sole physiological activator of the contractile proteins has since been firmly established (Ebashi and Endo, 1968).

Muscle contraction is initiated by the nerve impulse that depolarizes the surface membrane of the muscle cell. The depolarization wave is conducted longitudinally along the sarcolemma and radially into the interior of the muscle fibres along the membranes of the transverse tubules (T-tubules) (Huxley and Taylor, 1958; Costantin, 1975). T-tubules terminate at specialized junctions, where the action potential initiates EC coupling events that lead to a massive release of Ca$^{2+}$ from the SR, raising the myoplasmic Ca$^{2+}$ concentration above the threshold for contractile activation (Sandow, 1965; Costantin, 1975). The binding of Ca$^{2+}$ to the C-subunit of the troponin-tropomyosin-actin complex (thin filament) facilitates the interaction of actin and myosin (thick filament), the hydrolysis of ATP and the generation of tension (Ebashi, Endo and Ohtsuki, 1969; Potter and Gergely, 1974). During relaxation the sarcolemma and T-tubules are repolarized and Ca$^{2+}$ is actively sequestered by the Ca$^{2+}$-dependent transport ATPase of the SR, lowering the myoplasmic free Ca$^{2+}$ concentration to 10$^{-7}$ M or below thus allowing the inhibition of the contractile proteins.
by the I-subunit of the troponin-tropomyosin system (Ebashi et al., 1969; Ebashi, 1976; Asheley, 1978; Ebashi, 1979; Martonosi, 1982). A schematic drawing of part of a mammalian skeletal muscle fibre is shown in Figure 2.1.

2.2.2 The Role of the Transverse Tubule in Excitation Contraction Coupling.

EC coupling involves the activity of the internal membrane systems, the T-tubular and SR networks, and is composed of two successive steps: signal transmission from the T-tubular system to the terminal cisternae of the SR, and Ca$^{2+}$ release from the terminal cisternae (Ebashi, 1979).

In vertebrate skeletal muscle, two components of SR are connected with one T-tubule forming a triad or triadic junction (Franzini-Armstrong, 1980) (Fig.2.2). The junctional surfaces of a T-tubule and the lateral sacs or terminal cisternae of the SR, face each other at a distance of 110 - 130 Å. The SR and T-tubule surfaces are connected by electron dense feet at intervals of about 300 Å (Franzini-Armstrong, 1975; Heusser, Reese, Somlyo and Somlyo, 1978; Franzini-Armstrong, 1980). Membranous pillars with electron lucent interiors also span the junctional gap directly connecting the myoplasmic components of the T-tubule and SR membranes (Somlyo, 1979). The role of the feet and pillars in EC coupling is
FIGURE 2.1 Schematic Drawing of Part of a Mammalian Skeletal Muscle Fibre.

The relationship of the sarcoplasmic reticulum transverse tubules (T-system), terminal cisternae and mitochondria to myofibrils are shown (From Eisenberg, Kuda and Peter, 1974).
FIGURE 2.2 Longitudinal Section of Toadfish Swim-Bladder Muscle

Four triads are shown. T, T-tubule; C, terminal cisternae. Arrows point to the feet of the triadic junction. Junctional sarcoplasmic reticulum are the portions of the lateral sacs that are located between arrowheads. Magnifications x 110,000. (From Franzini-Armstrong, 1980).
unclear. However, the T-tubule membrane is essential for the inward transmission of excitation from the sarcolemma to the triadic junction. This has been dramatically demonstrated in muscle fibres detubulated by osmotic shock treatment with glycerol. Detubulated fibres fail to contract in response to electrical stimulation although an action potential is still produced (Eisenberg and Gage, 1967). Also, potassium-induced depolarization and subsequent muscle contraction is abolished after osmotic shock treatment (Eisenberg and Eisenberg, 1968; Howell, 1969; Gage and Eisenberg, 1969a; Eisenberg, Howell and Vaughan, 1971). Caffeine, a drug that is thought to act directly on the SR, can still induce contractures in detubulated fibres, suggesting that SR function and the contractile system are unaffected by the treatment (Axelsson and Thesleff, 1968; Weber, 1968).

The skinned fibre preparation is obtained by immersing a fibre in oil and mechanically peeling off the sarcolemma (Natori, 1965). Skinned fibres contract in response to electrical stimulation, or when the potassium concentration is reduced in the extrareticular space (Costantin and Podolsky, 1967; Stephenson and Podolsky, 1977). After removal of the sarcolemma, T-tubules may reseal maintaining a functional connection with the SR (Costantin and Podolsky, 1967). A response to electrical stimulation after the sarcolemma has been removed implies that the T-tubular elements are repolarized, probably
after resealing by re-establishing a potassium gradient across the internal membrane, making the lumina of the T-tubule more positive than the myofibrillar space and generating a resting potential (Costantin and Podolsky, 1967; Caputo, 1983). The skinned fibre preparation, although not physiological, provides further evidence of T-tubular participation in EC coupling.

2.2.3 The Mechanism of Signal Transmission

Hodgkin and Horowicz (1960) demonstrated that potassium is the major carrier of ionic current across the T-tubule membrane and induces muscular contraction. The density of feet and pillars in the triadic junction increases after potassium-induced depolarization or low frequency stimulation of single muscle fibres, but remains unchanged when Ca\textsuperscript{2+} is directly released from the SR by caffeine, suggesting that the role and formation of these structures is a cause and not an effect of Ca\textsuperscript{2+} release (Eisenberg and Gilai, 1979; Eisenberg and Eisenberg, 1982; Caputo, 1983).

Several hypotheses have evolved in which the feet and pillars of the triadic junction are central in the structural basis of EC coupling mechanisms. According to the Schneider-Chandler model (1973), movement of nonlinear intramembrane charge, which occurs at the level of the T-tubule and is voltage dependent, links T-tubule membrane
depolarization with the opening of Ca\textsuperscript{2+}-channels in the junctional region of the SR, releasing stored Ca\textsuperscript{2+} (Adrian and Almers, 1976; Schneider and Chandler, 1973; Schneider, 1981). Feet located in the triadic junction may link charge movement with Ca\textsuperscript{2+} release. Ebashi (1976) suggested that each charge movement exerts its effect on a corresponding foot. Thus, the magnitude of charge movement and the number of electron dense feet localized in the triadic junction appear to be related (Franzini-Armstrong, 1980). The charge movement may be in the form of a current that is gated (opened or closed), corresponding directly to the opening and closing of Ca\textsuperscript{2+} channels located in the feet of the terminal cisternae of the SR (Martonosi and Beeler, 1983). After glycerol-induced detubulation, a large fraction of intramembrane charge movement is removed, indicating again the essential role of the T-tubule for EC coupling (Chandler, Rakowski and Schneider, 1976). Mathias, Levis and Eisenberg (1980) proposed that the pillars of the triadic junction act as conductive channels. These channels may open transiently during excitation, facilitating the flow of ionic current into the SR through the interiors of the pillars linking the T-tubules and SR membranes. This would produce a voltage-dependent increase in the permeability of the SR to Ca\textsuperscript{2+}, initiating Ca\textsuperscript{2+} release. It was proposed therefore that Ca\textsuperscript{2+} release was controlled by voltage in both membrane systems. Studies using optical birefringence changes and extrinsic fluorescence signals
generated by permeable dyes such as Nile blue A during contractile activity have associated latency relaxation (LR) (observed as a small decrease in tension which precedes a twitch following a stimulus) with potential changes in the SR that lead to Ca\(^{2+}\) release (Bezanilla and Horowicz, 1975; Baylor and Oetliker, 1977; Oetliker and Schumperci, 1979). Recently, this model for the mechanism of EC coupling was withdrawn from consideration as the model imposed unrealistic restrictions on the membrane capacitance and conductance of the SR (Martonosi, 1984).

Alternative theories on the mechanism of EC coupling in skeletal muscle provide no definite role for the pillars and feet of the SR. Heilbrunn (1943) proposed that upon excitation, Ca\(^{2+}\) bound to external regions of the muscle fibre entered the muscle cell to induce contraction. Although Hill (1952) disputed the role of extracellular Ca\(^{2+}\) in muscular contraction, indicating that diffusion of a substance from the extracellular to the intracellular regions of muscle was too slow to account for the rate of contractile activation, further evidence of the involvement of extracellular Ca\(^{2+}\) in muscle excitation was forthcoming. In 1952 Sandow proposed that electrostatic attractions between the myofibrils and Ca\(^{2+}\) increased the rate of Ca\(^{2+}\) mobilization to the interior of the muscle fibre. Bianchi and Shanes (1959) then demonstrated an increase in Ca\(^{2+}\) influx during a single
twitch and potassium-induced contracture in frog sartorius muscles. They also observed that nitrate ions which increase twitch tension, also increased Ca\(^{2+}\) influx per twitch. Frank (1958, 1960, 1962), using frog toe muscles, observed that potassium-induced contractures were abolished in Ca\(^{2+}\) free solutions, while caffeine-induced contractures were still obtained. Furthermore, potassium contractures eliminated in Ca\(^{2+}\) free solutions could be restored by re-exposing the muscle to Ca\(^{2+}\) (Frank, 1962). However, the amount of extracellular Ca\(^{2+}\) entering a fibre during depolarization was calculated to be insufficient to activate all contractile sites. It was considered therefore, that extracellular Ca\(^{2+}\) entering the myoplasm during muscle depolarization must 'trigger' the release of Ca\(^{2+}\) from the SR to facilitate contraction (Bianchi, 1968, 1969). The finding that Ca\(^{2+}\) could induce rapid Ca\(^{2+}\) release from the SR in skinned fibres further strengthened the trigger Ca\(^{2+}\) hypothesis (Ford and Podolsky, 1972; Endo, Tanaka and Ogawa, 1970; Stephen, 1982). Frank (1980) also proposed that LR provided further evidence for the trigger Ca\(^{2+}\) mechanism, the observed latent period between excitation and activation corresponding to the time required for a substance to diffuse from the T-tubule membrane across the triadic junction to the SR membrane.

A model for the trigger Ca\(^{2+}\) mechanism was first proposed by Bianchi (1968, 1969) and later developed by
Frank (1980, 1982). A working model for the trigger calcium mechanism of EC coupling is shown in Figure 2.3. At rest the free Ca\(^{2+}\) concentration in the myoplasm (Camy) is equal to, and in equilibrium, with the Ca\(^{2+}\) concentration in the triadic junction (Catj) which is below the threshold for contractile activation. The majority of Ca\(^{2+}\) is stored in the terminal cisternae of the SR and is known as activator Ca\(^{2+}\) (CaSR). Trigger Ca\(^{2+}\) (Camb) is bound to the intracellular surface, at the junctional region of the T-tubule membrane. Ca\(^{2+}\) is also bound to the luminal surface of the T-tubule (Cats) and is in equilibrium with the extracellular Ca\(^{2+}\) (Cat). When the T-tubule membrane is depolarized trigger Ca\(^{2+}\) is released into the triadic junction and diffuses across the junction inducing the release of activator Ca\(^{2+}\) from the SR. This raises the myoplasmic Ca\(^{2+}\) concentration facilitating muscular contraction. Myoplasmic Ca\(^{2+}\) is sequestered by the SR during relaxation. Ca\(^{2+}\) located in the lumen of the T-tubule facilitates the binding of trigger Ca\(^{2+}\) to the membrane, maintaining sufficient levels of Ca\(^{2+}\) for coupling.

In the absence of Ca\(^{2+}\) in the bathing medium muscle fibres have been reported to maintain twitch responses for long periods of time (Spiecker, Melzer and Luttgau, 1979) indicating that extracellular Ca\(^{2+}\) does not have a primary role in EC coupling. However, extracellular Ca\(^{2+}\) may only be needed to maintain the T-tubule membrane.
FIGURE 2.3 A Model for the Trigger Calcium Mechanism for EC Coupling

Resting, coupling and recovery phases are shown. The T-tubule is on the right and the terminal cisternum on the left. Abbreviations: t, T-tubular lumen; ts, luminal surface of the T-tubule membrane; mb, T-tubule membrane; tj, triad junction; my, myoplasm; SR, sarcoplasmic reticulum; E, efflux; I, influx; Ca, calcium. See text for further details. (From Frank, 1979).
**RESTING**

\[ I_{Ca} = E_{Ca} \]

\[ [Ca]_{SR} \sim 10^{-2} \text{ M} \]

\[ [Ca]_{L} = 1.08 \text{ (or 1.6)} \times 10^{-3} \text{ M} \]

\[ [Ca]_{my} < 0.3 \text{ (or 0.01)} \times 10^{-6} \text{ M} \]

\[ E_{P,Ca} \sim \sim 104 \text{ mV inside +} \]

*: active pump required

---

**E-C COUPLING**

\[ I_{Ca} \uparrow \]

\[ [Ca]_{my} \downarrow \]

\[ \rightarrow [Ca]_{LJ} \uparrow \]

\[ \rightarrow [Ca]_{SR} \downarrow \]

\[ \rightarrow [Ca]_{my} \uparrow \rightarrow \text{ activation} \]

---

**RECOVERY**

\[ [Ca]_{SR} \uparrow \text{(active pump)} \]

\[ E_{Ca} \uparrow \]

\[ [Ca]_{my} \uparrow \]

\[ [Ca]_{LJ} \downarrow \]

\[ [Ca]_{my} \rightarrow \text{relaxation} \]
store of Ca\(^{2+}\) and is not required to trigger directly Ca\(^{2+}\) release (Frank, 1980). Also, certain multivalent cations and buffers (found in physiological solutions in experiments examining the effects of zero Ca\(^{2+}\) on muscle function) delay the loss of trigger Ca\(^{2+}\) into Ca\(^{2+}\) free solutions allowing a functional connection to be maintained (Frank, 1978a, b).

Although electrophysiological studies have demonstrated the existence of an active inward Ca\(^{2+}\) current in striated muscle, its role in EC coupling remains obscure. This current is very slow but is activated by depolarization, and abolished in Ca\(^{2+}\) free solutions and by calcium antagonists (Sanchez and Stefani, 1978; Almers, Fink and Palade, 1981; Cota and Stefani, 1985). After glycerol treatment the Ca\(^{2+}\) current is diminished, indicating that Ca\(^{2+}\) enters through the T-tubule (Siri, Sanchez and Stefani, 1980). The role of this current in the action potential or in contractile activation is unknown. However, it may be activated during sustained activity and could serve to replenish intracellular Ca\(^{2+}\) stores (Curtis, 1970). Recently, Graf and Schatzmann (1984) have proposed a model for the role of external Ca\(^{2+}\) in the activation process. This model integrates Ca\(^{2+}\) into the mechanism of voltage-dependent charge movement, which may supply the trigger Ca\(^{2+}\) required for activation of Ca\(^{2+}\)-induced Ca\(^{2+}\) release.
Like Ca\(^{2+}\), inositol 1,4,5-triphosphate (InsP\(_3\)) has also been proposed as a second messenger in EC coupling (Vergara, Tsien and Delay, 1985). InsP\(_3\) was shown to be released by direct electrical stimulation of muscle fibres and to induce Ca\(^{2+}\) release from skinned muscle fibres. It was suggested that InsP\(_3\) acts as a chemical intermediary between the depolarization of the T-tubule membrane and Ca\(^{2+}\) release from the SR. Voltage changes across the T-tubular membrane may activate the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phosphodiesterase to release InsP\(_3\) and diacylglycerol. InsP\(_3\) in turn opens Ca\(^{2+}\) channels in the SR inducing Ca\(^{2+}\) release (Vergara et al., 1985). However, it still remains to be determined if InsP\(_3\) has a primary role in skeletal muscle EC coupling.

2.3 THE SITE OF THE MUSCLE CELL ABNORMALITY IN MALIGNANT HYPERPYREXIA

Excitation contraction coupling involves the activity of the sarcolemma-transverse tubule and SR membranous networks. Elevated myoplasmic Ca\(^{2+}\) levels in MH muscle may be due to an abnormality in the mechanism of EC coupling (Nelson and Denborough, 1977; Denborough, 1980). Okumura et al. (1980), found that detubulation of MH muscle by osmotic shock treatment with glycerol inhibited the abnormal contracture responses to halothane, caffeine and potassium chloride. Hypercontractility
of MH muscle fibres was also abolished by pretreatment with deuterium oxide, another inhibitor of EC coupling. These authors suggested that the defect in MH muscle may lie at the level of the sarcolemma-T-tubular membrane system.

Gallant, Godt and Gronert (1979) and Galloway, Bornstein and Denborough (1980) investigated the properties of the sarcolemma from pigs susceptible to MH using electrophysiological techniques. They demonstrated that halothane could depolarize the sarcolemma of MH susceptible muscle while having no effect on the resting membrane potential of normal muscle. This depolarization was reduced or partially reversed by dantrolene. While the degree of depolarization was small (5-15mV), and was considered to be insufficient to produce a contracture in normal muscle (Galloway, 1982), studies using voltage clamp techniques found that the mechanical threshold for contractile activation was significantly lower in MH susceptible muscle in comparison with control tissue. Therefore, a small depolarization on exposure to halothane may be sufficient to cause contractile activation of MH susceptible muscle (Gallant, Gronert and Taylor, 1982). The hypercontractility displayed by MH muscle in response to potassium chloride also suggests a lower mechanical threshold for contractile activation (Gronert, 1980). Recent studies have shown abnormalities in the properties of electromechanical coupling in MH muscle. Nelson et al.,
(1983) observed that the time between nerve stimulation and the onset of mechanical activation (tension) was longer in MH susceptible than in control muscle, both in vitro and in vivo. Although prolonged electromechanical coupling time intervals were demonstrated in MH muscle, the kinetics of action potential formation were similar to those of control muscle. Nelson et al. (1983) suggested that this indicated an abnormality in the later process of EC coupling, such as signal transmission at the triadic junction or release of $Ca^{2+}$ from the SR.

Studies on skinned muscle fibres and isolated SR membranes from MH susceptible muscle have shown a defect in the mechanism of $Ca^{2+}$ release from the SR. Ohnishi et al. (1983), and Nelson (1983a) studying $Ca^{2+}$-induced $Ca^{2+}$ release from isolated SR membranes, observed a lower $Ca^{2+}$ concentration threshold for $Ca^{2+}$-induced $Ca^{2+}$ release from MH susceptible vesicles, in comparison to controls. Nelson (1983a) also observed that caffeine decreased the $Ca^{2+}$ threshold for $Ca^{2+}$-induced $Ca^{2+}$ release, to a greater extent in MH than in control isolated SR preparations. The effect of caffeine was inhibited by ruthenium red, implicating the ruthenium red sensitive $Ca^{2+}$ channel in the abnormal release mechanism. Hypersensitivity of the $Ca^{2+}$-induced $Ca^{2+}$ release mechanism has also been observed in skinned muscle fibres from a patient susceptible to MH. $Ca^{2+}$-induced $Ca^{2+}$ release was activated at lower $Ca^{2+}$ concentrations and achieved a higher rate at the
optimal Ca$^{2+}$ concentration, when compared with control fibres (Endo, Yagi, Ishizuka, Horiati, Koga and Amaha, 1983). An abnormality of Ca$^{2+}$-induced Ca$^{2+}$ release may occur in MH muscle.

Dantrolene sodium (DANTRIUM, Norwich - Eaton Pharmaceuticals) a skeletal muscle relaxant, is the drug of choice in the treatment of MH (Denborough, 1980; Gronet, 1980). In vivo, dantrolene is highly effective in preventing and reversing a MH episode (Kolb et al., 1982). Dantrolene also effectively inhibits and reverses the abnormal contracture responses produced by a variety of chemical stimuli in isolated MH muscle fibres (Ellis, Castellion, Honkomp, Wessels, Carpenter and Halliday, 1973; Okumura et al., 1980). The skeletal muscle relaxant primarily acts to lower myoplasmic Ca$^{2+}$ concentrations by suppressing EC coupling, without interfering with neuromuscular transmission or with the conduction of the action potential (Ellis and Carpenter, 1972, 1974; Putney and Bianchi, 1974; Desmedt and Hainaut, 1979). Dantrolene at low concentrations does not affect calcium uptake or Ca$^{2+}$-dependent ATPase activities of the SR (White, Collins and Denborough, 1983a; Yamamota, Suzuki and Hotta, 1977; Green, Heffron and Mitchell, 1976). It appears that dantrolene primarily acts to inhibit Ca$^{2+}$ release from intracellular stores. In resting barnacle giant muscle fibres injected with aequorin, dantrolene (35uM) decreased the myoplasmic free Ca$^{2+}$ concentration and 45$^{Ca}_{2+}$ efflux.
without affecting the rate of Na\(^+\)-Ca\(^{2+}\) exchange (Desmedt and Hainaut, 1977). Dantrolene was also shown to inhibit the amount of Ca\(^{2+}\) released from the SR and to decrease the force of isometric tension after depolarization of the sarcolemma, without affecting Ca\(^{2+}\) uptake during relaxation. Other experiments using intact muscle fibres have also shown that dantrolene may act to selectively inhibit Ca\(^{2+}\) release from the SR (Desmedt and Hainaut, 1979; Lopez, Helland, Wanek, Rudel and Taylor, 1979).

While the precise site of action is yet to be defined, it seems that dantrolene exerts its effect on some element of the triadic junction. Dantrolene may inhibit trigger Ca\(^{2+}\) release from T-tubules (Putney and Bianchi, 1974; Nelson and Denborough 1977; Takauji, Takahashi, Suzuki and Nagai, 1977; Oba and Hotta, 1978) or bind to Ca\(^{2+}\) channels at the junctional region of the SR, on the T-tubules or on other triad elements (Martonosi, 1984). In any event, the ability of dantrolene to preferentially act on EC coupling and its successful use in the treatment of MH is consistent with the primary muscle lesion in MH being associated with this process.

Studies on denervated mammalian skeletal muscle also indicate that the defect in MH is more likely to be postsynaptic. Chronically denervated muscle shows increased sensitivity to acetylcholine (Gutman and Sandow, 1965; Thesleff, 1974). However, acetylcholine at
similar concentrations fails to contract MH muscle (Okumura et al., 1979). Denervated mouse skeletal muscle also behaves differently from human MH muscle (Moulds 1977, 1978). Unlike MH muscle, denervated muscle fails to contract in response to halothane and has a prolonged "active state" in response to electrical stimulation. Although certain similarities exist between denervated and MH muscle, the skeletal muscle abnormality predisposing to MH does not seem to be related to the degree of innervation.

Investigations on other aspects of skeletal muscle function have failed to identify the primary defect predisposing to MH. These include studies on mitochondria, contractile proteins, calmodulin, calsequestrin, adenylate cyclase and adenylate kinase.

Defective Ca\(^{2+}\) storage by the mitochondria has been reported to occur in MH muscle cells. Cheah and Cheah (1978, 1979, 1981) observed an increase in the rate of Ca\(^{2+}\) efflux from mitochondria isolated from MH susceptible muscle which was considered sufficient to activate myofibrillar ATPase, phosphorylase kinase and to induce accelerated glycolysis. Decreased Ca\(^{2+}\) uptake capacity of mitochondria isolated from MH susceptible pig muscle has also been reported (Britt, Endrenyi and Cadman, 1975). However, the suggestion that abnormal calcium handling by the mitochondria is the
primary lesion predisposing to MH is questionable. These Ca\(^{2+}\) efflux studies were conducted with mitochondria that were isolated from muscle obtained from pigs postmortem after conventional slaughter techniques, conditions of stress which are known to trigger metabolic changes in MH susceptible pigs (Nelson, 1983a). Furthermore, the physiological role of Ca\(^{2+}\) transport by the mitochondria is unknown. Ca\(^{2+}\) transport may be important only in cells with a poorly defined endoplasmic reticulum, or after a large influx of Ca\(^{2+}\) into the cell which saturates other Ca\(^{2+}\) regulatory mechanisms (Farber, 1981; Chizzonite and Zak, 1981; Martonosi, 1984).

A number of studies have investigated the possibility that MH is due to an abnormal Ca\(^{2+}\) binding protein of muscle. Lorkin and Lehmann (1983a) using two dimensional gel electrophoresis failed to find any abnormalities in the major contractile proteins, including the Ca\(^{2+}\) binding light chains of myosin, between MH susceptible and control pigs. Lorkin and Lehman (1983b) also found no differences in the Ca\(^{2+}\) binding properties of troponin-C from control and MH susceptible pigs. White (personal communication) isolated calsequestrin, the major Ca\(^{2+}\) binding protein of the SR, from MH susceptible and control pig muscle, but failed to find any differences in the ability of the protein to bind Ca\(^{2+}\). Collins (1984) demonstrated that calmodulin antagonists increased MH susceptible and control skeletal muscle contractility
in vitro. However, the structure and function of this Ca\(^{2+}\) binding protein from MH susceptible pigs was indistinguishable from that of control porcine calmodulin (Marjanen and Denborough, 1983; Collins, 1984).

Deficiencies in the activity of adenylate kinase in human MH susceptible muscle have also been reported (Schmitt, Schmidt and Ritter, 1974; Schmidt and Heller, 1977). Adenylate kinase (AK) catalyses the reaction

\[
2 \text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}
\]

and is important in maintaining cellular levels of ATP. It was proposed that decreased activity (Schmitt et al., 1974) or inhibition of AK by halothane (Sachsenheimer, Pai, Schulz and Schirmer, 1977) may lead to a breakdown of ATP regeneration by this process during a MH crisis. Halothane has been shown to decrease AK activity, but the degree of inhibition was the same in both MH susceptible and control porcine muscle tissue (Marjanen and Denborough, 1982a). Furthermore, a number of investigators failed to find an abnormality in this enzyme from MH susceptible humans (Cerri, Willner and Rowland, 1981) or pigs (Marjanen and Denborough, 1982b; Marjanen and Denborough, 1983). An AK deficiency as the major abnormality predisposing to MH is unlikely.

An abnormality in adenylate cyclase (AC) activity may also occur in MH susceptible muscle (Willner et al.,
1981). Activation of AC results in an increased level of myoplasmic cyclic AMP, activation of cyclic AMP-dependent protein kinases and phosphorylation of certain SR proteins. Increased AC activity has been found in MH susceptible human muscle (Willner et al., 1981; Ellis, Halsall, Allam and Hay, 1984). However, studies on AC in muscle from MH susceptible pigs found activity to be within the normal range (Ono, Topel and Althern, 1977; Sim, 1985).

Pyrophosphate, a product of AC activity, is elevated in the serum of survivors of MH episodes (Van Wormer, Armstrong and Solomons, 1978). Catecholamines stimulate AC activity. Elevated AC activity during MH may be due to the increased levels of circulating catecholamines observed during an episode (Gronert and Theye, 1976a; Lister, Hall and Lucke, 1974).

An abnormality in the ability of the SR to sequester Ca\(^{2+}\) could account for the raised myoplasmic Ca\(^{2+}\) concentration in MH susceptible muscle. However, investigations on the rates of Ca\(^{2+}\) uptake by isolated SR membrane vesicles isolated from susceptible muscle have produced conflicting results. Ca\(^{2+}\) uptake has been reported to be similar (Kalow et al., 1970), reduced (Isaacs and Hefferon, 1975) or increased (Berman and Kench, 1973) in comparison to control rates. (Ca\(^{2+}\) transport by isolated MH susceptible SR membranes will be discussed further in Chapter 5).
2.4 SUMMARY

EC coupling involves the activity of the sarcolemmal-transverse tubular and SR membranous networks and controls the level of myoplasmic Ca\(^{2+}\) (Ebashi, 1979). T-tubules conduct the excitatory stimulus into the interior of skeletal muscle cells. The depolarization of T-tubules may release trigger Ca\(^{2+}\) from the myoplasmic surface of the T-tubular membrane and/or induce charge movement, activating Ca\(^{2+}\) channels located at the terminal cisternae, initiating Ca\(^{2+}\) release into the myoplasm and contractile activation.

An abnormality in the mechanism of EC coupling has been proposed to result in a raised myoplasmic Ca\(^{2+}\) concentration in MH skeletal muscle. Evidence for an abnormality in this process stems from a number of investigations (Nelson and Denborough, 1977; Okumura et al., 1980; Endo et al., 1983, Ohnishi et al., 1983; Nelson et al., 1983). The effective use of dantrolene in the treatment of MH is also consistent with the primary lesion in MH being associated with the EC coupling mechanism. A defect in the ability of the SR to sequester Ca\(^{2+}\) from the myoplasm has also been proposed (Isaacs and Heffron, 1975). Although other abnormalities may occur in MH muscle they do not appear to be the primary cause of elevated myoplasmic Ca\(^{2+}\) levels.
CHAPTER 3

THE EFFECT OF CALCIUM CHANNEL ANTAGONISTS AND DETUBULATION ON THE CONTRACTILITY OF PORCINE SKELETAL MUSCLE FIBRES

3.1 INTRODUCTION

The previous chapter described the pivotal role of Ca\(^{2+}\) in EC coupling, muscle contraction and MH. The pathogenesis of MH leads to an abnormally high myoplasmic Ca\(^{2+}\) concentration. Calcium channel antagonists (CCA) may modify Ca\(^{2+}\) fluxes associated with EC coupling in skeletal muscle (Section 3.1.2).

Inhibition of Ca\(^{2+}\) fluxes associated with EC coupling may lead to a lowering of myoplasmic Ca\(^{2+}\). The effect of CCA on porcine skeletal muscle contractility is investigated in this Chapter with the aim to further defining the site of the muscle abnormality in MH and ascertaining whether CCA are effective in inhibiting the hypercontractility of MH muscle.

3.1.1 Calcium Channels and Calcium Channel Antagonists

Extracellular Ca\(^{2+}\) passively enters the cytosol through specific surface membrane channels in a variety of excitable cells. These channels are opened by critical
changes in cellular polarization and are known as voltage-operated or voltage-regulated Ca\textsuperscript{2+} channels (Reuter, 1983; Tsien, 1983). Voltage-operated calcium channels (VOCC) play a critical role in the generation of the cardiac action potential, neurotransmitter release and EC coupling (Kostyuk, 1981; Reuter, 1983; Tsien, 1983).

Calcium entry via these channels may be modified by a class of drugs called calcium channel blockers or calcium channel antagonists (Fleckenstein, 1977; Nayler and Horowitz, 1983). CCA are a diverse group of organic compounds which consist of three main classes, the dihydropyridine derivatives which include nifedipine and nitrendipine, the papaverine derivatives such as verapamil and the benzothiazepine derivative, diltiazem. As therapeutic agents, these drugs are primarily used in the treatment of cardiovascular disorders such as myocardial ischaemia, angina, arterial hypertension, and cardiac arrhythmias (Fleckenstein, 1977; Antman, Stone, Muller and Braunwald, 1980; Henry, 1980).

The precise mechanism by which CCA inhibit the Ca\textsuperscript{2+} fluxes of excitable cells is not clearly understood. However, several modes of action have been proposed (Lee and Tsien, 1983; Nayler and Horowitz, 1983). They may limit the amount of inwardly permeating ions by directly inhibiting the inward current through the Ca\textsuperscript{2+} channel.
CCA may achieve this by, 1) impeding the approach of Ca\(^{2+}\) to the channel, 2) by inducing configurational changes in the membrane which may affect the carrying capacity of each channel, and 3) by altering the electrical properties of the channels. A number of \(^{45}\)Ca\(^{2+}\) flux studies indicate that CCA may inhibit the activation of Ca\(^{2+}\) channels rather than blocking permeability (Rosenberger, Ticku and Triggle, 1979; Godfraind and Dieu, 1981). Activation of Ca\(^{2+}\) channels may be suppressed by inducing changes in the kinetics of channel activation and/or recovery (Lee and Tsien, 1983; Nayler and Horowitz, 1983).

Two separate binding sites on VOCC have been described for CCA (Rosenberger and Triggle, 1977; Yamamura, Shoemaker, Boles and Roeske, 1980; Murphy, Gould, Largent and Snyder, 1983). Dihydropyridines bind to a high affinity site associated with the channel and can either block or activate the channel (Lee and Tsien 1983; Schramm, Thomas, Towart and Franckowiak, 1983). Diltiazem and verapamil bind to a second site with lower affinity. Both verapamil and diltiazem block Ca\(^{2+}\) flux through the channel, but they have different effects on the binding of dihydropyridine derivatives. Diltiazem increases, while verapamil inhibits, nitrendipine binding.

CCA may also interfere with the intracellular mobilization of Ca\(^{2+}\), inhibiting Ca\(^{2+}\) fluxes associated
with such organelles as the SR and mitochondria (Nayler and Horowitz, 1983). This has been demonstrated in muscle obtained from dog and rabbit mesenteric arteries where CCA inhibited the release of Ca\(^{2+}\) from intracellular storage sites at high concentrations (Walus, Fondacaro and Jacobson, 1981; Saida and van Breeman, 1983). CCA may also act on the Ca\(^{2+}\)-dependent modulator protein, calmodulin, to exert some of their effects (Bostrom, Ljung, Mardh, Forsen and Thulin, 1981; Johnson, Vaghy, Crouch, Potter and Schwartz, 1982).

3.1.2 Calcium Channels, Calcium Channel Antagonists and Skeletal Muscle

Ca\(^{2+}\) channels are primarily localized in the T-tubules of skeletal muscle. In the sarcolemma-T-tubular membrane system of frog skeletal muscle more than 97% of Ca\(^{2+}\) channels reside in the T-tubular membrane (Almers et al., 1981). Almers and Stirling (1984) have also reported that the density of Ca\(^{2+}\)-channels was greater in the T-tubular membrane than in the sarcolemma.

T-tubule Ca\(^{2+}\) channels have been described as VOCC (Potreau and Raymond, 1980a; Almers et al., 1981; Stefani and Chiarandini, 1982) and, like those of other excitable tissues have specific binding sites for CCA. Fosset, Jaimovich, Delpont and Lazdunski (1983) demonstrated that \(^{3}\)H-nitrendipine binding was almost entirely localized
to the T-tubule membranes isolated from frog and rabbit skeletal muscle. Surface membranes had less than 5% of the binding capacity of T-tubules, while SR membranes were devoid of \(^3\)H-nitrendipine binding sites. The low binding capacities of surface membranes was considered to be due to contamination by T-tubular membranes.

Nitrendipine and verapamil were shown to inhibit the binding of \(^3\)H-nitrendipine to the T-tubule membranes. Verapamil, nifedipine and other dihydropyridine derivatives inhibited the binding with high potencies, while diltiazem and dantrolene were without effect, indicating that these binding sites were distinct from that of \(^3\)H-nitrendipine (Dehpour, Mofakham and Mahmoudiam, 1982; Galizzi, Fosset and Lazdunski, 1984a). Verapamil does not act directly at the dihydropyridine receptor to influence \(^3\)H-nitrendipine binding (Murphy et al., 1983), but has a separate binding site which also binds diltiazem with low affinity (Galizzi, Fosset and Lazdunski, 1984b). Dihydropyridines did not affect \(^3\)H-verapamil binding to rat skeletal muscle membranes, while diltiazem was shown to compete for binding (Reynolds, Gould and Snyder, 1983). Recently, the CCA binding sites or receptors were successfully solubilized from guinea pig and rabbit T-tubule membranes (Glossman and Ferry, 1983; Borsotto Norman, Fosset and Lazdunski, 1984; Curtis and Catterall, 1984).

Active inward Ca\(^{2+}\) currents have been described in frog and rat skeletal muscles. The current which
enters through the VOCC in the T-tubule membrane system (Sanchez and Stefani, 1978; Potreau and Raymond, 1980a; Almers et al., 1981; Cota and Stefani, 1985) can be suppressed by various CCA (Almers et al., 1981; Almers and McCleskey, 1984; Ildefonse, Jacquemond, Rougier, Fosset and Lazdunski, 1985; Lamb, 1986 a, b). Graf and Schatzmann (1984) have proposed a model for the role of Ca\(^{2+}\) in the activation process that integrates external Ca\(^{2+}\) into the mechanism of voltage-dependent charge movement. A Ca\(^{2+}\) component of nonlinear intramembrane charge movement has also been described in rabbit T-tubule membranes. This Ca\(^{2+}\) component which may be in the form of slow Ca\(^{2+}\) gating current, was suppressed by CCA and may play a role in the EC coupling mechanism (Ildefonse et al., 1985; Lamb, 1986 a, b).

Rapid Ca\(^{2+}\) efflux from the SR is a necessary step in EC coupling in skeletal muscle and is thought to be mediated by Ca\(^{2+}\) channels (Ebashi, 1976; Endo, 1977; Bianchi and Frank, 1982). Several investigators have described the characteristics of the putative Ca\(^{2+}\) release channel of the SR. Miyamoto and Racker (1982) proposed the existence of two Ca\(^{2+}\) channels in the terminal cisternae of the SR. One channel is voltage operated, while the second contains a Ca\(^{2+}\) receptor, which upon binding Ca\(^{2+}\), causes the channel to open and initiate Ca\(^{2+}\) release. This channel is blocked by ruthenium-red and is described as the ruthenium-red sensitive Ca\(^{2+}\) channel.
Morii and Tonomura (1983) observed adenine nucleotide activated Ca\(^{2+}\) channels in fragmented SR membrane preparations. They described the gating behaviour of this channel in relation to the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism and suggested that the Ca\(^{2+}\) channel gate opens when the external Ca\(^{2+}\) concentration exceeds a characteristic threshold. Recently, Ca\(^{2+}\) channels in the terminal cisternae membrane fraction of the SR were found to be nucleotide activated, blocked by ruthenium-red, selective for divalent cations and voltage-dependent (Smith, Coronado and Meissner, 1985). These channels also exhibited larger conductances for certain divalent cations in comparison with T-tubule membrane Ca\(^{2+}\) channels.

Ca\(^{2+}\)-dependent ATPase is uniformly distributed throughout the longitudinal tubules and much of the terminal cisternae of the SR, but is seemingly absent from the region of the cisternal membrane which forms the triad with the T-tubule membrane (Martonosi, 1984). The unique association between the mechanism of Ca\(^{2+}\) release and the membrane elements derived from the triadic junction indicate that the Ca\(^{2+}\) channels responsible for initiating Ca\(^{2+}\) release in stimulated muscle cells are distinct from Ca\(^{2+}\)-dependent ATPase and from other regions of the SR membrane (Martonosi, 1984). Observations by Kirino and Shimizu (1983) and Morii and Tonomura (1983) also indicate that the subcellular distribution of
Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release channels differs from that of Ca\textsuperscript{2+}-dependent ATPase. Although Ca\textsuperscript{2+} permeability is increased when Ca\textsuperscript{2+}-dependent ATPase is incorporated into bilayer membranes prepared from phospholipids (Jilka, Martonosi and Tillack, 1975; Jilka and Martonosi 1977), increased permeability of vesicles to sucrose, sodium ions and choline is also observed. This implies that Ca\textsuperscript{2+}-dependent ATPase does not act as a specific Ca\textsuperscript{2+} channel, but forms non specific hydrophilic channels across the membrane (Jilka et al., 1975; Jilka and Martonosi, 1977).

While Ca\textsuperscript{2+} channels may exist in the SR membrane, particularly in the junctional region of the terminal cisternae which forms the triad, specific binding sites for CCA have yet to be identified. While high affinity binding sites for \textsuperscript{3}H-nitrendipine have been observed in the membrane elements of the triadic junction from rabbit skeletal muscle, and associated with Ca\textsuperscript{2+}-channels of the SR (Fairhurst, Thayer, Colker and Beatty, 1983), the binding sites could also be attributed to the presence of T-tubule membranes (Martonosi, 1984). Thus, whilst it is possible that CCA may block Ca\textsuperscript{2+} release from the SR, their primary sites of action in skeletal muscle appear to be the Ca\textsuperscript{2+} channels of the T-tubule membrane system.
3.1.3 Calcium Channel Antagonists and Malignant Hyperpyrexia

The precise site of the lesion in MH skeletal muscle is yet to be defined, but evidence suggests a dysfunction in the mechanism of EC coupling (Chapter 2, Section 2.3). Suppression of EC coupling by dantrolene inhibits the MH response (Chapter 2, Section 2.3). Dantrolene exerts its effects primarily on elements of the triadic junction to lower myoplasmic Ca\(^{2+}\) levels (Chapter 2, Section 2.3).

In skeletal muscle CCA bind preferentially to Ca\(^{2+}\) channels located in the T-tubular membrane to inhibit the influx of Ca\(^{2+}\) (Borsotto et al., 1984; Curtis and Catterall, 1984). While the role of Ca\(^{2+}\) inflow through these channels during EC coupling is not fully understood it may play a functional role (Graf and Schatzmann, 1984; Ildefonse et al., 1985). CCA may therefore be able to specifically modify events of EC coupling at the level of the T-tubule membrane and perhaps inhibit the MH response.

Preliminary investigations on the effects of CCA on the hypercontractility of MH muscle have been conducted. A single observation in isolated MH susceptible human muscle showed that diltiazem was able to suppress halothane and halothane-caffeine induced muscle contractures (Iwatsuki, Koga and Amaha, 1983). Ilias, Williams, Fulfer
and Dozier (1985) also demonstrated that diltiazem inhibited the hypercontractility of isolated MH susceptible porcine skeletal muscle. Bikhazi, Thomas and Foldes (1979) investigated the effects of verapamil on the contractility of the rat phrenic nerve-hemidiaphragm preparation, and suggested that verapamil together with relatively small doses of dantrolene may be superior to dantrolene administration alone in the treatment of MH. Verapamil was also shown to prevent the characteristic hypercontractility of isolated muscle strips from patients susceptible to MH (Gruener and Blank, 1979).

3.2 EFFECT OF CALCIUM CHANNEL ANTAGONISTS ON CONTRACTILITY

The results of experiments designed to assess the effects of CCA on the hypercontractility of isolated MH susceptible porcine skeletal muscle fibres are described in this chapter. The effects of verapamil, diltiazem and nifedipine on the contractile responses of MH susceptible and control gracilis muscle to the diagnostic agents, halothane, caffeine, and potassium chloride were determined. The effect of dantrolene on the contractile responses to these agents was also investigated. The effect of diltiazem and dantrolene on contractile responses to caffeine was investigated also in detubulated muscles fibres in an attempt to define their sites of action.
3.3 MATERIALS AND METHODS

3.3.1 Experimental Animals

Malignant hyperpyrexic susceptible swine were bred from crosses of MH susceptible Large White and Landrace breeds. Control pigs were obtained from either external breeding programmes or were non affected siblings of MH susceptible stock.

3.3.2 Muscle Biopsy Procedures

Pigs were premedicated by intramuscular injection of the neuroleptic, Stresnil, at a dose of 1.5-2 mg/kg of body weight. General anaesthesia was induced by injection of thiopentone into the ear vein at a dose of 6-8 mg/kg of body weight. Pigs then underwent endotracheal intubation, and anaesthesia was maintained by the delivery of 66% nitrous oxide in oxygen.

During anaesthesia, gracilis muscle strips measuring 4cm x 1cm x 0.5cm were clamped under tension and dissected free from the hind limb. Biopsy specimens were then immediately immersed into Ringer solution, which had been maintained at 37°C and pH 7.4. The Ringer solution was continuously aerated with 5% carbon dioxide in oxygen (carbogen).
3.3.3 The Isolated Skeletal Muscle Preparation and the Diagnosis of Susceptibility to Malignant Hyperpyrexia

Biopsy specimens were dissected free of surrounding fat and fascia. Bundles of muscle fibres were then cut into strips of approximately 2mm in diameter and 2.5cm long and mounted vertically under tension in organ bath chambers. Muscle strips were continuously bathed in 20ml of Ringer solution which was maintained at 37°C and bubbled with carbogen. Changes in isometric force were monitored with a Watson Victor force displacement transducer coupled to a preamplifier (San-EI 6M51) and a chart recorder (Perkin-Elmer 56). Muscle strips were initially suspended under 2g of tension and the viability of the specimen tested by electrically stimulating the preparation with 30V. Fibres which did not elicit a twitch response equivalent to 1g or more were discarded. After muscle strips had equilibrated to a constant resting base line tension of approximately 1.5g, representative fibres were tested for MH susceptibility. Responses to diagnostic agents were made in duplicate before a diagnosis was made. The remaining fibres were used for other experiments.

MH susceptibility was identified by using the established isolated contracture test devised by Okumura et al. (1979). These investigations showed a correlation
between MH susceptibility in vivo and the presentation of abnormal contracture responses to certain chemical stimuli in skeletal muscle fibres in vitro. In this study MH susceptible pigs were defined as swine whose isolated contracture responses to 3% halothane and 2mM caffeine and 80mM potassium chloride were higher than the normal range defined by Okumura et al. (1979). Hypercontractility observed in response to KCl only was not taken solely as indicative of MH susceptibility (Galloway, 1982). Only animals which gave unequivocal contracture responses were used. A contractile response to only one agent was obtained with each preparation.

3.3.4 Isometric Twitch Experiments

Muscle strips were electrically driven (30V, 1msec duration) until a constant twitch response was obtained. Fibres were then challenged with the required drug and the effect on twitch height observed. The effect of a drug on twitch response was measured as a percentage increase or decrease of the twitch height before the addition of that drug.

3.3.5 Calcium Channel Antagonists and Dantrolene

Pretreated Fibres

In experiments to determine the effect of a CCA or dantrolene on contractile responses, muscle strips
were first equilibrated with the appropriate drug for 15-20 minutes. Contractile responses were then obtained. In some experiments contractile responses were induced first (after a constant level of tension was obtained), then the effect of a particular CCA or dantrolene was investigated.

3.3.6 Glycerol Pretreated Fibres

Bathing muscle fibres in hypertonic Ringer's solution prepared with 400mM glycerol followed by re-exposure to normal Ringer's solution results in detubulation and the uncoupling of contraction from excitation (Gage and Eisenberg, 1969a, b; Howell, 1969).

In this study fibres were detubulated by immersing the muscle strip under tension for 2 hours in 400mM glycerol Ringer solution. Preparations were then repeatedly washed in normal Ringer solution (10min) and electrically stimulated (30V) until the twitch response was abolished. Concentration-effect responses were then constructed under the required conditions.

3.3.7 Concentration-effect Responses

Concentration-effect curves were constructed for caffeine by the addition of cumulative concentrations to the organ bath. A maximum of one concentration-effect
curve was constructed in each muscle strip. Halothane, and KCl were added at a single concentration to the organ bath. Results are reported in grams tension + or - the standard error of the mean (s.e.m.).

3.3.8 Solutions

3.3.8.1 Drug Solutions

Thymol free halothane was added to the organ bath by passing carbogen at a constant rate of flow through a Dragewick halothane vapouriser. Stock solutions of drugs were prepared either in Ringer solution (caffeine, KCl and diltiazem) or dimethylsulphoxide (DMSO) (dantrolene, nifedipine and verapamil). These were added directly to the organ bath. Both dantrolene and nifedipine were stored protected from light and experiments involving these drugs were conducted in a darkened laboratory.

3.3.8.2 Ringer Solutions

Ringer solution was used as the bathing medium in all experiments. This contained (mM) NaCl 121; KCl 5.4; NaHCO₃ 15; NaH₂PO₄ 1.2; MgSO₄ 1.2; CaCl₂ 2.5 and D-glucose 11.5. Glycerol-Ringer solution was normal Ringer solution with glycerol added to make a final concentration of 400mM.
3.3.9 Drugs

Drugs used were halothane (ICI), caffeine (Sigma), verapamil (Knoll AG.), diltiazem (Marion), nifedipine (Bayer), dantrolene (Norwich-Eaton Pharmaceuticals) and glycerol (BDH). KCl and other reagents (Ajax chemicals) were of analytical grade.

3.3.10 Statistics

Significant differences in mean values were determined using Students non-paired t-test.

3.4 RESULTS

3.4.1 Identification of Malignant Hyperpyrexia

Susceptible and Control Swine

Contractile responses to 2mM caffeine, 3% halothane and 80mM KCl were used to distinguish control from MH susceptible muscle. MH susceptible preparations displayed a characteristic hypercontractility to these agents: contracture responses were significantly greater than those in control tissue (Table 3.1). Typical tracings of contractile responses to these agents in both control and MH susceptible muscle are shown in Figure 3.1.
TABLE 3.1 Contractile Responses of Isolated Malignant Hyperpyrexia Susceptible and Control Porcine Skeletal Muscle Induced by Halothane, Caffeine and KCl

<table>
<thead>
<tr>
<th>Agent</th>
<th>MH susceptible tissue</th>
<th>Control tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% Halothane</td>
<td>0.93 +/- 0.07*</td>
<td>0.09 +/- 0.03</td>
</tr>
<tr>
<td></td>
<td>(103)</td>
<td>(91)</td>
</tr>
<tr>
<td>2mM Caffeine</td>
<td>0.40 +/- 0.03*</td>
<td>0.03 +/- 0.01</td>
</tr>
<tr>
<td></td>
<td>(120)</td>
<td>(89)</td>
</tr>
<tr>
<td>80mM KCl</td>
<td>1.08 +/- 0.11*</td>
<td>0.14 +/- 0.09</td>
</tr>
<tr>
<td></td>
<td>(78)</td>
<td>(51)</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m grams tension. Numbers in parentheses indicate the number of observations. Responses in MH susceptible preparations were significantly greater than in controls, p < 0.001*.
FIGURE 3.1 Typical Contractile Responses of Malignant Hyperpyrexia Susceptible and Control Porcine Skeletal Muscle to Caffeine, KCl and Halothane.

Contractile responses to halothane (HAL), caffeine (CAFF) and KCl are shown in MH susceptible (MHS) and control (C) preparations.
1.0 g TENSION

MHS C

2 min

2 mM CAFF

MHS C

80 mM KCL

3% HAL
3.4.2 Cumulative Concentration-Effect Curves to Caffeine

Cumulative concentration-effect curves to caffeine were obtained from both control and MH susceptible muscle preparations. Caffeine caused concentration-dependent contractions of MH susceptible fibres that were significantly greater than in control tissue, from 0.5 to 16mM (Fig. 3.2). At higher concentrations of caffeine (24 and 32mM) contractile responses were not significantly different.

3.4.3 The Effect of DMSO on Contractile Responses to Halothane and Caffeine.

Since DMSO was used as a solvent for some drugs, it was necessary to establish whether DMSO itself had any effect on muscle contractility. The concentration of DMSO in the organ bath in any experiment did not exceed 0.50% v/v. As shown in Table 3.2 DMSO (0.50% v/v) did not alter significantly the contractile responses of MH susceptible muscle to 3% halothane or 2mM caffeine. Responses to 4mM caffeine in control tissue were also unaltered.
FIGURE 3.2 Cumulative Concentration-Effect Curves to Caffeine in Isolated Malignant Hyperpyrexia Susceptible and Control Porcine Skeletal Muscle.

Results from MH susceptible muscle are represented by a circular symbol. Control data is represented as squared symbols.

Results are expressed as mean +/- s.e.m grams tension. The numbers in parentheses represent the number of observations.

The number of observations vary as full dose response curves were not obtained in all preparations.

x significantly greater than MH p < 0.05
xxx significantly greater than control p < 0.001
† no significant difference between MH susceptible and control responses.
TABLE 3.2 The Effect of Dimethylsulphoxide on Contractile Responses to Halothane and Caffeine in Malignant Hyperpyrexia Susceptible and Control Muscle.

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>In the presence of DMSO 0.5%(v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH Susceptible</td>
<td>Control</td>
</tr>
<tr>
<td>3% HAL</td>
<td>0.93+/-0.07</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(103)</td>
<td></td>
</tr>
<tr>
<td>2mM CAFF</td>
<td>0.40+/-0.03</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(120)</td>
<td></td>
</tr>
<tr>
<td>4mM CAFF</td>
<td>-</td>
<td>0.18+/-0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100)</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e. mean.

Numbers in parentheses represent the number of observations

No significant difference, P > 0.05 (non-paired t-test).
3.4.4 The Effect of Calcium Channel Antagonists on the Resting Tension of Isolated Muscle Preparations.

At concentrations of up to 100uM, verapamil and diltiazem did not alter the resting tension of control (n = 20) or MH susceptible (n = 20) preparations. While nifedipine did not alter the resting tension of control preparations (n = 20), a contracture (mean = 0.28g) was induced in 9 out of 44 MH susceptible fibres.

3.4.5 The Effect of Calcium Channel Antagonists and Dantrolene on Isometric Twitch.

Twitch responses could be obtained in both MH susceptible (n = 6) and control (n = 6) isolated muscle preparations which were constant, or decreased by only 10%, over a 2 hour period. Dantrolene (6uM) reduced twitch height in both MH susceptible (n = 4) and control (n = 4) preparations by 86% in less than 2 hours. Increasing the concentration of dantrolene to 12uM failed to have any further effect on twitch height. Diltiazem (80uM) and 100uM verapamil abolished the twitch response in less than 2 hours while 100uM nifedipine reduced twitch height by 80% in MH susceptible preparations (n = 4 per drug). Typical tracings of twitch response from MH susceptible muscle in the presence of dantrolene and the CCA are shown in Figure 3.3. Similar responses were obtained in control tissue (n = 4 per drug).
FIGURE 3.3 Representative Tracings of Isometric Twitch Responses in Isolated Malignant Hyperpyrexia Susceptible Muscle in the Absence of any Additions and in the Presence of Dantrolene and Calcium Channel Antagonists.

Twitch responses are shown in the absence of any additions (A) and in the presence of 80uM diltiazem (B), 6uM dantrolene (C), 100uM verapamil (D) and 100uM nifedipine (E).
A. MHS

B. DILT (80 μM)

C. DAN (6 μM)

D. VER (100 μM)

E. NIF (100 μM)
3.4.6. The Effect of Calcium Channel Antagonists and Dantrolene on the Contractile Responses Induced by Halothane, and KCl

Contractile responses induced by 3% halothane, and 80mM KCl were examined in fibres pretreated with either 100μM nifedipine, 100μM verapamil, 80μM diltiazem or 6μM dantrolene. The concentrations of CCA or dantrolene used represent a concentration that produced a significant effect on contractility. Control preparations (n = 6) did not contract in response to halothane or KCl in the presence of dantrolene, diltiazem, verapamil or nifedipine. These experiments were conducted to show that these agents did not potentiate responses to halothane or KCl. Pretreatment of MH susceptible fibres with dantrolene or diltiazem significantly inhibited the contractile responses to halothane and KCl (Fig. 3.4.). Verapamil and nifedipine also significantly reduced KCl induced contractures (Fig. 3.4.). In contrast to the effects of diltiazem and dantrolene, verapamil and nifedipine significantly increased the contractile sensitivity of MH muscle fibres to halothane (Fig. 3.4.).

3.4.7 The Effect of Calcium Channel Antagonists and Dantrolene on Cumulative Concentration-Effect Curves to Caffeine.

Contractile responses induced by caffeine in control
FIGURE 3.4. The Effects of Dantrolene and Calcium Channel Antagonists on Contractile Responses of Malignant Hyperpyrexia Susceptible Muscle to Halothane and KCl.

The effects of 6μM dantrolene (I) 80μM diltiazem (II) 100μM verapamil (III) and 100μM nifedipine (IV) on contractile responses of MH susceptible muscle to 3% halothane (A) and 80mM KCl (B) are shown. Contractile responses to 3% halothane and 80mM KCl in the absence of any additions are also shown (V).

Each column represents the mean +/- s.e.m grams tension. Numbers in parentheses indicate the number of observations.

xxx significantly less than V p< 0.001
* significantly greater than V p< 0.05
*** significantly greater than V p< 0.001
preparations in the presence of dantrolene or a CCA are shown in Figure 3.5. All contractile responses to caffeine (2 to 64mM) were significantly reduced in preparations pretreated with 100uM verapamil or 6uM dantrolene. Diltiazem (80uM) also significantly reduced caffeine-induced contractures at 2 to 24mM, but failed to alter significantly the tension generated by higher concentrations (32 and 48mM). Nifedipine (100uM) had no effect on the tension generated by caffeine at 2, 4 or 8mM, but significantly reduced the tension generated at higher concentrations (16 to 64mM).

The effect of 80uM diltiazem or 6uM dantrolene on caffeine-induced contractile responses in MH susceptible preparations was similar to that observed in control preparations (Fig.3.6). Diltiazem significantly decreased contractile responses to caffeine (0.5 to 24mM) but failed to alter contractures at higher concentrations (32mM). A significant decrease in all contracture responses to caffeine was observed in preparations pretreated with dantrolene. In MH muscle 100uM verapamil failed to affect caffeine induced contractile activity at 0.5 to 8mM, but significantly reduced the tension generated at higher concentrations (16, 24, 32mM). In contrast, 100uM nifedipine potentiated the caffeine-induced hypercontractility of MH susceptible muscle, increasing contractile responses at 1, 2 and 4mM. Nifedipine significantly reduced the tension generated by 16mM
Cumulative concentration-effect curves to caffeine alone are shown (C) and in the presence of 6μM dantrolene (DAN), 100μM nifedipine (NIF), 80μM diltiazem (DILT) and 100μM verapamil (VER).

Results are expressed as mean +/- s.e.m grams tension. Numbers in parentheses indicate the number of experiments.

At concentrations of 2 and 4mM caffeine, VER (n = 10 and n = 15), DAN (n = 10 and n = 11) and DILT (n = 16 and n = 18) significantly inhibited contractile responses (p<0.001). Contractile responses were not significantly affected at these concentrations of caffeine in the presence of NIF (n = 9 and n = 9).

+ significantly less than control response p< 0.05
++ significantly less than control response p<0.01
+++ significantly less than control response p< 0.001
† not significantly different from control response.
Cumulative concentration-effect curves to caffeine are shown (MHS) and in the presence of 6uM dantrolene (DAN), 80uM diltiazem (DILT), 100uM verapamil (VER) and 100uM nifedipine (NIF).

Results are expressed as mean +/- s.e.m grams tension. In the presence of DAN or a CCA the number of observations ranged from 5 to 16 for any one concentration.

+ significantly less than MHS response p<0.05
++ significantly less than MHS response p< 0.01
+++ significantly less than MHS response p< 0.001
† no significant difference

*** significantly greater than MHS response p<0.001
caffeine, but failed to affect higher concentrations.

3.4.8 The Relaxant Effects of Calcium Channel Antagonists and Dantrolene on Contractures Induced by Halothane, and Caffeine in MH Susceptible and Control Muscle

Dantrolene (6uM) reversed contractile responses in MH muscle to 3% halothane (Fig. 3.7A). Higher concentrations of diltiazem (640uM) were required to effectively reverse contracture responses to halothane (Fig. 3.7A). In the presence of halothane 100uM verapamil and 100uM nifedipine failed to reverse the induced contracture (n = 4, in all cases). In some preparations verapamil and nifedipine caused a contracture (Fig. 3.7A).

Dantrolene (6uM) and 640uM diltiazem completely reversed 8mM caffeine-induced contractures in MH susceptible and control preparations (Results not shown). At higher concentrations of caffeine (16mM), 6uM dantrolene and 640uM diltiazem partially reversed induced tension (Fig. 3.7B). However, 6uM dantrolene still reversed 16mM caffeine-induced (n = 8) contractures after 640uM diltiazem-induced relaxation and vice versa (Fig. 3.7B). Increasing the concentration of diltiazem (>640uM) or dantrolene (>6uM) failed to induce any further relaxation. This was observed in both MH susceptible and control preparations.
FIGURE 3.7 Effect of Dantrolene and Diltiazem on Contractile Responses of Malignant Hyperpyrexia Susceptible Isolated Pig Skeletal Muscle to Halothane and Caffeine. The Effects of Nifedipine and Verapamil on Halothane-induced Contractures are also shown.

The effect of 6uM dantrolene (DAN) 640uM diltiazem (DILT), 100uM verapamil (VER) and 100uM nifedipine (NIF) in the presence of halothane are shown (A). The effect of 6uM DAN and 640uM DILT on caffeine-induced contractures is also shown (B).
3.4.9 The Effect of Glycerol-induced Detubulation on Contractile Responses to Caffeine in Malignant Hyperpyrexia and Control Preparations

Twitch response was abolished in all detubulated fibres before contracture responses were superimposed. All caffeine contractures were significantly reduced in MH susceptible and control detubulated muscle fibres (Fig.3.8). The hypercontractility of MH susceptible preparations to caffeine was abolished.

3.4.10 The Effect of Dantrolene and Diltiazem on Contracture Responses to Caffeine in Detubulated Malignant Hyperpyrexia Susceptible and Control Preparations

After twitch responses were abolished in detubulated fibres, dantrolene or diltiazem was added to the bathing medium. Dantrolene 6uM significantly reduced while 80uM diltiazem had no effect on contractile responses induced by caffeine in detubulated MH susceptible and control preparations (Fig.3.9).

Diltiazem (640uM) also failed to reverse contractile responses to 16mM caffeine in MH susceptible (n = 6) or control (n = 6) detubulated fibres while 6uM dantrolene induced relaxation in both preparations (Fig.3.10).
FIGURE 3.8. Effect of 400mM Glycerol-induced Detubulation on Cumulative Concentration-Effect Curves to Caffeine in Malignant Hyperpyrexia Susceptible and Control Pig Skeletal Muscle.

Contractile responses in untreated MH susceptible (MHS) and control (C) preparations are shown. Contractile responses in pretreated MH susceptible (filled circle) and control (filled square) are shown. Results are expressed as mean +/- s.e.m grams tension. The number of experiments ranged from 14 - 18 separate fibre preparations for both MH susceptible and control pretreated strips.

+ significantly less than untreated MHS response
  p< 0.001

++ significantly less than untreated control response
  p< 0.001.
FIGURE 3.9. The Effect of Dantrolene and Diltiazem on Cumulative Concentration-Effect Curves to Caffeine in Glycerol Detubulated Malignant Hyperpyrexia Susceptible and Control Pig Skeletal Muscle.

The effect of 6μM dantrolene (DAN) and 80μM diltiazem (DILT) on responses to caffeine in 400mM glycerol pretreated MH susceptible (MHS) (A) and control (C) (B) are shown.

Results are expressed as mean +/- s.e.m grams tension. For both MH susceptible and control preparations the number of observations ranged from 14 - 18 separate fibre preparations for untreated strips and 8 - 10 for DAN or DILT pretreated strips.

+ significantly less than untreated p< 0.05
++ significantly less than untreated p< 0.01
FIGURE 3.10 The Effect of Diltiazem and Dantrolene on Contractures Induced by Caffeine in Glycerol Detubulated Control and Malignant Hyperpyrexia Susceptible Isolated Pig Skeletal Muscle.

The effect of 6uM dantrolene (DAN) and 640uM diltiazem (DILT) on caffeine-induced contractures in control (A) and MH susceptible (B) glycerol pretreated preparations.
[A] CONTROL.  
DAN (6μM)  
CAFF 16mM

[B] MHS.  
DAN (6μM)  
CAFF 16mM

DILT (640μM)  
CAFF 16mM
3.5 DISCUSSION

Contractile responses to 3% halothane, 2mM caffeine and 80mM KCl were used to distinguish control from MH susceptible muscle. Fibres diagnosed as MH susceptible produced contractures which were significantly greater than control responses to these agents (Table 3.1; Fig. 3.1, 3.2). Contractile responses fell within the respective ranges defined for control or MH susceptible pig muscle (Okumura et al., 1979).

The calcium channel antagonist diltiazem (80μM) inhibited the characteristic hypercontractility of MH susceptible muscle. Like the skeletal muscle relaxant dantrolene (6μM), diltiazem inhibited or reversed contractile responses induced by 3% halothane, and 80mM KCl (Fig. 3.4, 3.7). Contractile responses to caffeine (Fig. 3.5, 3.6) and electrical stimulation (Fig. 3.3) were also depressed by diltiazem or dantrolene in control and MH susceptible muscle. These results are consistent with the view that these agents act to inhibit contractile activity by lowering myoplasmic Ca^{2+} levels.

The effects of dantrolene on contractile responses in this study were similar to those observed by other investigators. Dantrolene has been shown to inhibit contractile responses to the variety of agents used to identify MH susceptibility in isolated muscle fibres.
(Anderson and Jones, 1976; Austin and Denborough, 1977; Okumura et al., 1980) and reduce twitch responses by up to 70% (Ellis, 1980; Tamai, 1982). Caffeine-induced contractures in normal muscle are also inhibited by dantrolene (Nelson and Denborough, 1977; Halsall and Ellis 1979). Dantrolene modifies contractile responses in skeletal muscle by lowering myoplasmic Ca^{2+} levels. The skeletal muscle relaxant may act to displace trigger Ca^{2+} into the extracellular fluid (Oba and Hotta, 1978) depleting the amount available to initiate release of activator Ca^{2+} from the SR (Ellis and Wessels, 1977; Yamamoto et al., 1977; Nelson, 1983b). A number of studies on isolated SR membranes suggest that dantrolene may not only depress the mechanism that couples T-tubular depolarization with Ca^{2+} release from the SR, but also directly inhibit Ca^{2+} release from the SR (Homma, Kurihara and Sakai, 1976; Van Winkle, 1976; Morgan and Bryant, 1977).

The effect of diltiazem on contractile responses is also consistent with suppression of EC coupling. CCA preferentially bind to VOCC located in the T-tubular membranes of skeletal muscle (Fosset et al., 1983). These Ca^{2+} channels may be activated during contractile activity and may be important in EC coupling (Idlefonse et al., 1985). Therefore, like dantrolene, diltiazem may inhibit contractile responses by modifying processes of EC coupling, possibly by inhibiting Ca^{2+} influx at the level
of the T-tubular membrane.

In the present study diltiazem modified contractile responses to a variety of agents including caffeine. Caffeine may act on elements of the triadic junction, such as the coupling process between the T-tubules and terminal cisternae to induce contractile activity (Bianchi, 1975; Ebashi, 1976). In skinned skeletal muscle fibres, where a functional connection is maintained between the T-tubule and SR system, caffeine enhances Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Endo et al., 1970; Endo, Kakuta and Kitazawa, 1981), while in detubulated fibres responses to caffeine are often suppressed (Marco and Nastuk, 1968; Sandow, 1970). Further evidence indicating a role for T-tubules in caffeine-induced Ca\(^{2+}\) release comes from studies on frog and rat skeletal muscles, where caffeine modified contractile mechanisms at the level of the T-tubule membrane (Luttgau and Oetliker, 1968; Anwyl, Bruton and McLoughlin, 1984). Modification of caffeine-induced contractile activity by diltiazem is therefore consistent with the CCA acting at the level of the T-tubule membrane. However, experiments with skeletal muscle fibres exposed to Ca\(^{2+}\) free solutions (Buss and Frank, 1969) and Ca\(^{2+}\) release studies from isolated SR membranes (Weber and Herz, 1968; Ogawa, 1970; Thorpe and Seeman, 1971) suggest that caffeine may also act directly on the SR to release Ca\(^{2+}\) into the myoplasm. Diltiazem therefore may also inhibit Ca\(^{2+}\) channels involved in the
release of Ca$^{2+}$ from the terminal cisternae. However, in detubulated muscle fibres responses to caffeine could be modified by dantrolene but not by diltiazem (Fig. 3.9, 3.10). Diltiazem (640uM) could also reverse caffeine-induced contractures in intact muscle fibres in the presence of maximal effective concentrations of dantrolene and the converse was also true (Fig. 3.7). These results suggest that diltiazem modifies caffeine-induced contractures at the level of the T-tubule membrane, probably by inhibiting the inward flow of extracellular Ca$^{2+}$, while dantrolene has an additional different site of action. Results with dantrolene are consistent with those of other investigators, who suggest that the skeletal muscle relaxant may displace trigger Ca$^{2+}$ into the extracellular fluid as well as directly antagonising SR calcium release (Ellis and Wessels, 1977; Yamamoto et al., 1977; Oba and Hotta, 1978; Nelson, 1983b).

Uncoupling of excitation from contraction by glycerol treatment inhibits the hypercontractility of MH susceptible muscle induced by caffeine (Fig. 3.8). This result is consistent with the observations of Okumura et al. (1980), and supports the view that EC coupling is abnormal in MH tissue. Suppressed caffeine responses in detubulated fibres have been reported by other investigators and support the view that caffeine does not solely act on the SR to increase myoplasmic Ca$^{2+}$ levels
A reduction in the tension generated by caffeine in diltiazem pretreated (Fig. 3.5, 3.6) or detubulated (Fig. 3.8) fibres may indicate a role for extracellular Ca\(^{2+}\) in the caffeine-induced contractile response. Although the role of extracellular Ca\(^{2+}\) in the activation of contractile activity remains unknown, a number of studies have demonstrated an active inward Ca\(^{2+}\) current during contractile activity in skeletal muscle (Sanchez and Stefani 1978; Almers and Palade, 1981; Chiarandini and Stefani, 1983; Cota and Stefani, 1985). During sustained activity this current enters the muscle cell through Ca\(^{2+}\) channels located in T-tubules, and can be blocked by CCA such as nitrendipine and verapamil and detubulation (Beaty and Stefani, 1976; Siri et al., 1980; Almers et al., 1981; Thompson and Dryden, 1981; Almers and McCleskey 1984; Ildefonse et al., 1985). Ca\(^{2+}\) entering from the external solution can significantly contribute to the development of contracture (Idlefonse et al., 1985; Potreau and Raymond, 1980 a, b) and may be required during contractile activity when Ca\(^{2+}\) release from the SR becomes inadequate (Lamb, 1986b). Such Ca\(^{2+}\) currents may be activated during the construction of a caffeine concentration-effect curve. While caffeine may act by directly releasing Ca\(^{2+}\) from the SR, the degree of tension generated may be dependent on the activation of an extracellular Ca\(^{2+}\) component. Nelson and Denborough
(1977) demonstrated that halothane and halothane-caffeine-induced contractures of MH muscle may be associated with the influx of Ca\(^{2+}\) from the extracellular fluid. Moulds and Denborough (1974c) also showed that extracellular Ca\(^{2+}\) was required to maintain abnormal drug-induced contractures in MH susceptible muscle. Nelson and Chausmer (1981) suggested that caffeine may induce contractile activity by affecting two different calcium sites, one of which is in equilibrium with the extracellular Ca\(^{2+}\). Halothane-induced contractile responses of MH susceptible muscle may also depend on the involvement of an extracellular Ca\(^{2+}\) pool (Nelson and Chausmer, 1981).

Unlike diltiazem or dantrolene, 100uM verapamil and 100uM nifedipine did not inhibit the hypercontractility of MH susceptible muscle. Although 100uM verapamil inhibited twitch responses (Fig. 3.3) and contractile responses to KCl (Fig. 3.4) this drug did not affect contractures induced by up to 8mM caffeine in MH susceptible muscle (Fig. 3.6). Verapamil also increased the sensitivity of MH muscle to halothane (Fig. 3.4). Nifedipine (100uM) also inhibited twitch responses and KCl-induced contractures, but potentiated contractile responses to halothane and caffeine (1 to 4mM) in MH susceptible muscle. These results are not consistent with these CCA solely acting to inhibit the influx of Ca\(^{2+}\) through T-tubules. Like dantrolene, verapamil and
nifedipine were dissolved in DMSO. However, DMSO failed to induce contractures in MH susceptible muscle in the presence of halothane or caffeine. High concentrations of verapamil and nifedipine were required to produce significant effects on contractility. While these CCA and diltiazem bind with nanomolar affinity to CCA receptors of isolated skeletal muscle membranes (Dehpour et al., 1982; Reynolds et al., 1983; Galizzi et al., 1984a, b) higher concentrations (>20μM) of these CCA are often required to induce an effect on skeletal muscle contractility (Godfraind, Miller and Wibo, 1986). In fact in voltage clamp experiments using frog muscle, Almers and McCleskey (1984) reported a IC₅₀ of 80μM for diltiazem. Thus a poor correlation between binding studies and in vitro pharmacological studies is often observed. However high concentrations may not only indicate poor diffusion into the muscle but also nonspecific effects.

At high concentrations (10⁻⁴ M or greater), CCA such as verapamil have been shown to depress surface membrane excitability, inhibiting potassium-induced contractile activity and twitch response (Bondi, 1978; Van Der Kloot and Kita, 1975; Erdreich and Rahamimoff, 1984). At these concentrations CCA have also been shown to induce contractile activation by releasing Ca²⁺ from intracellular stores (Bondi, 1978; Marwahu and Treffers, 1980; Frank, 1982,). An increased level of Ca²⁺ in the myoplasm in response to verapamil or nifedipine in MH.
muscle would account for the potentiation of halothane and caffeine-induced contractures. Another possibility is that verapamil and nifedipine may affect the affinity of binding of Ca$^{2+}$ to intracellular stores, so that release of Ca$^{2+}$ by halothane or caffeine is facilitated.

Nifedipine not only blocks but can also activate Ca$^{2+}$ channels (Schramm et al., 1983; Garcia, Sala, Reig, Viniegra, Frias, Fonteriz and Gandia, 1984). Bean (1984) has suggested that inhibition of Ca$^{2+}$ channels by CCA such as the dihydropyridines depends on the functional state of the Ca$^{2+}$ channel. In the heart dihydropyridines appear to act selectively on the inactivated state (Bean, 1984). If elevated resting Ca$^{2+}$ levels in MH muscle cells are due to more activated than inactivated channels this also may explain the different effect of nifedipine in MH susceptible versus control tissue. In view of these observations it is unlikely that verapamil or nifedipine would be effective therapeutic agents for the treatment of MH.

3.6 SUMMARY

Like the skeletal muscle relaxant dantrolene, diltiazem inhibited and reversed the drug-induced hypercontractility of MH susceptible muscle. Diltiazem and dantrolene also inhibited contractility in control muscle. In detubulated MH susceptible and control muscle
Diltiazem failed to affect contracture responses to caffeine while dantrolene inhibited responses. Furthermore, diltiazem could induce relaxation in caffeine contracted fibres in the presence of maximal effective concentrations of dantrolene and the converse was true. These results suggest that dantrolene and diltiazem have different sites of action. Diltiazem probably acts to inhibit the influx of \( \text{Ca}^{2+} \) through T-tubules while dantrolene may directly inhibit \( \text{Ca}^{2+} \) release from the SR as well as depressing some other aspect of EC coupling associated with the T-tubular membrane. Results with diltiazem and detubulated muscle suggests that \( \text{Ca}^{2+} \) entering the muscle via T-tubules may be important in the aetiology of the MH syndrome.

While verapamil and nifedipine inhibited twitch response and KCl-induced contractures in MH susceptible muscle, these drugs failed to inhibit the hypercontractility response to caffeine and halothane.
CHAPTER 4

THE EFFECT OF CALCIUM CHANNEL ANTAGONISTS AND DANTROLENE ON THE CONTRACTILITY OF HUMAN ISOLATED SKELETAL MUSCLE

4.1 INTRODUCTION

Dantrolene and the CCA diltiazem effectively inhibited and reversed the drug-induced hypercontractility of isolated MH susceptible porcine skeletal muscle (Chapter 3). Verapamil and nifedipine did not inhibit the abnormal contractile responses (Chapter 3). This chapter describes experiments designed to assess the effects of these CCA and dantrolene on the contractile responses of isolated MH susceptible and control human skeletal muscle to the diagnostic agents 3% halothane, 2mM caffeine and 80mM KCl.

4.2 MATERIALS AND METHODS

4.2.1 Source of Human Skeletal Muscle

Vastus lateralis muscle specimens were obtained from patients undergoing biopsies for the diagnosis of MH susceptibility. Control vastus lateralis muscle was also obtained from patients undergoing orthopaedic surgery. General anaesthesia was induced by sodium pentothal and maintained with nitrous oxide in patients undergoing
muscle biopsies. Orthopaedic surgery was predominantly performed after epidural block. However, anaesthesia using a muscle relaxant (gallamine or pancuronium), sodium pentothal and nitrous oxide was used on occasions. Tissue from individuals who received muscle relaxants was repeatedly washed for 2 hrs before being challenged with a diagnostic agent. No differences in the contractile activity was observed between muscle taken under epidural block and after the administration of a skeletal muscle relaxant.

4.2.2 Diagnosis of Susceptibility to Malignant Hyperpyrexia

Susceptibility of human Vastus lateralis muscle to MH was determined by the methods previously described in Chapter 3, Section 3.3.3. Like MH porcine muscle, MH human muscle contracts to a greater extent than control muscle when challenged with 3% halothane, 2mM caffeine or 80mM KCl (Moulds and Denborough, 1974c; Denborough, 1980). In this study contractile responses to halothane and caffeine were always obtained. Contractile responses to these agents were obtained at least 3 times before a positive diagnosis was made. Muscle diagnosed as MH susceptible or control produced contractures which fell within the respective ranges defined for MH susceptible or control human muscle (Sim, 1985).
The methods for preparing fibres and measuring changes in isometric tension have been previously described (Chapter 3, Section 3.3.3). The procedures used for examining the effect of a particular CCA or dantrolene on contractile responses are also as described in Chapter 3 (Sections 3.3.4, 3.3.5, 3.3.7). Responses to caffeine were obtained by the cumulative addition of caffeine to the organ bath. All observations are reported as the number of fibres tested. However these results include at least 4 responses from fibres of different individuals. Results shown were obtained from fibres from 10 MH susceptible and 24 control individuals.

4.2.3 Drugs and Solutions

All solutions and drugs used in this study have been described previously in Chapter 3 (Sections 3.3.8, 3.3.9).

4.2.4 Statistics

The students non-paired t-test was used to determine statistical significance. Results are expressed as mean +/− s.e.m grams tension.
4.3 RESULTS

4.3.1 Identification of Control and Malignant Hyperpyrexia Susceptible Muscle Fibres

Contractile responses to 3% halothane, 2mM caffeine and 80mM KCl were used to distinguish between MH susceptible and control muscle. MH susceptible preparations displayed the characteristic contractility to these agents that was significantly greater than responses in control tissue (Table 4.1).

4.3.2. The Effect of Calcium Channel Antagonists and Dantrolene on Isometric Twitch

Twitch responses were obtained in both control (n = 4) and MH susceptible (n = 5) muscle preparations which were constant, or decreased by less than 10%, over a 30 minute period. Dantrolene (6uM) reduced twitch height in both MH susceptible (n = 4) and control (n = 9) fibres by 90% in less than 30 minutes. Diltiazem (10uM) and 10uM verapamil abolished or reduced the twitch response by 90% in control and MH preparations (n = 9 per drug). In two control preparations 100uM nifedipine abolished twitch response within 40 minutes. Typical tracings of twitch responses from control muscle in the presence of dantrolene and the CCA are shown in Figure 4.1. Similar responses were obtained in MH susceptible tissue (n = 3 per drug).
### TABLE 4.1 Contractile Responses of Isolated Malignant Hyperpyrexia Susceptible and Control Human Vastus Lateralis Muscle Induced by Halothane, Caffeine and KCl.

<table>
<thead>
<tr>
<th>Agent</th>
<th>MH Susceptible tissue</th>
<th>Control tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% Halothane</td>
<td>1.25 +/- 0.48*</td>
<td>0.09 +/- 0.28</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(24)</td>
</tr>
<tr>
<td>2mM Caffeine</td>
<td>0.44 +/- 0.11*</td>
<td>0.01 +/- 0.01</td>
</tr>
<tr>
<td></td>
<td>(52)</td>
<td>(24)</td>
</tr>
<tr>
<td>80mM KCl</td>
<td>0.77 +/- 0.24*</td>
<td>0.11 +/- 0.03</td>
</tr>
<tr>
<td></td>
<td>(51)</td>
<td>(35)</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m grams tension. The numbers in parentheses indicate the number of observations. Observations were on fibres obtained from 10 MH susceptible and 24 control individuals. Responses of MH susceptible fibres were significantly greater than those of control, p < 0.001*
FIGURE 4.1 Representative Tracings of Isometric Twitch Responses in Isolated Control Skeletal Muscle.

Results are shown in the absence of any additions (A) and in the presence of verapamil (B, 10uM), diltiazem (C, 10uM), nifedipine (D, 100uM) and dantrolene (E, 6uM). The arrow indicates when the drugs were added. Muscle strips were stimulated at 30V for a duration of 1 msec.
The effect of nifedipine was only examined in control tissue, owing to a limited supply of MH susceptible tissue.

4.3.3 The Effect of Calcium Channel Antagonists and Dantrolene on the Contractile Responses Induced by Halothane

Contractile responses induced by 3% halothane were examined in fibres pretreated for 15 - 20 minutes with either 10uM diltiazem, 6uM dantrolene, 10uM verapamil or 100uM nifedipine. Preliminary experiments showed no effect of lower concentrations. Control preparations failed to contract in response to halothane in the presence of diltiazem, dantrolene or verapamil. (These experiments were conducted to show that these agents did not potentiate responses to halothane or KCl). However, 100uM nifedipine significantly increased the contractile sensitivity of control tissue to halothane (Fig.4.2). Pretreatment of MH susceptible fibres with dantrolene, diltiazem or verapamil significantly inhibited contractile responses to halothane (Fig.4.2). Owing to a limited supply of MH tissue the effect of nifedipine was not examined.
FIGURE 4.2 The Effect of Calcium Channel Antagonists and Dantrolene on Contractile Responses Induced by Halothane in MH Susceptible and Control Muscle.

Each column represents the mean +/- s.e.m grams tension. Contractile responses in the presence of 10uM diltiazem (II), 6uM dantrolene (III), 10uM verapamil (IV) and 100uM nifedipine (V) are shown. The mean contractile response to halothane in the absence of any other additions is also shown (I).

The number of observations ranged between 4 and 6. + significantly less than I p < 0.001
* significantly greater than I p < 0.001
4.3.4 The Effect of Calcium Channel Antagonists and Dantrolene on Contractile Responses Induced by Caffeine

Contractile responses induced by 2mM caffeine (in MH susceptible preparations) and 4mM caffeine (in control muscle) in the presence of dantrolene or a CCA are shown in Figure 4.3. Dantrolene (6uM), 10uM diltiazem and 10uM verapamil abolished the hypercontractility of MH susceptible fibres. These agents also significantly reduced contractile responses induced by 4mM caffeine in control fibres. However, 100uM nifedipine significantly potentiated contractile responses induced by 4mM caffeine in control fibres (Fig. 4.3). Owing to a limited supply of MH tissue the effect of nifedipine was not be examined.

4.3.5 The Effect of Calcium Channel Antagonists and Dantrolene on the Contractile Responses Induced by KCl

Diltiazem (10uM) (n = 4), 10uM verapamil (n = 1) and 6uM dantrolene (n = 4) abolished 80mM KCl-induced contractile responses in MH susceptible fibres (Fig. 4.4). Control fibres failed to contract in the presence of 10uM diltiazem (n = 2), 10uM verapamil (n = 2), 100uM nifedipine (n = 6) or 6uM dantrolene (n = 2), in response to 80mM KCl.
FIGURE 4.3 The Effect of Calcium Channel Antagonists and Dantrolene on Contractile Responses Induced by Caffeine in MH Susceptible and Control Skeletal Muscle.

Each column represents the mean +/- s.e.m grams tension. Contractile responses in the presence of 10uM diltiazem (II), 6uM dantrolene (III), 10uM verapamil (IV) and 100uM nifedipine (V) are shown. Contractile responses to caffeine in the absence of any other additions were also shown (I).

The number of observations ranged between 4 and 5 for MH susceptible and between 8 and 10 for control preparations.

+ significantly less than 1, p < 0.001
* significantly greater than 1, p < 0.01
A. MHS
(2 mM CAFF)

B. Control
(4 mM CAFF)

TENSION (g)

- 1.0
- 0.5
- 0.0

I II III IV

I II III IV V

*
FIGURE 4.4 Effect of Calcium Channel Antagonists and Dantrolene on Contractile Responses Induced by KCl in MH Susceptible and Control Skeletal Muscle.

The tracings represent typical responses of MH Susceptible (A) and control muscle (B) induced by 80mM KCl. Dantrolene (6uM), 10uM diltiazem, 10uM verapamil and 100uM nifedipine were added (filled symbols) prior to KCl (open symbol).
A. MHS

\[ \Delta \]

KCl 80 mM (Δ)

B. Control

\[ \Delta \]

KCl 80 mM (Δ)
4.3.6 The Relaxant Effects of Calcium Channel Antagonists and Dantrolene on Contractures Induced by Halothane and Caffeine

Diltiazem (10µM) and 6µM dantrolene completely reversed contractile responses in MH susceptible muscle to 3% halothane (n = 11) (Fig. 4.5) and 2mM caffeine (n = 5) (Fig. 4.6). Diltiazem (n = 8) and dantrolene (n = 4) also effectively reversed contractile responses induced by 4mM caffeine in control fibres (Fig. 4.6). Contractile responses to a higher concentration of caffeine (16mM) were only partially reversed by diltiazem (40µM) or dantrolene (12µM). The effects of the two drugs in combination at maximally effective concentrations were additive (Fig. 4.7). This was observed in both control and MH susceptible fibres. Results are shown for MH susceptible muscle. The effects of nifedipine and verapamil were not investigated in preparations already contracted with caffeine or halothane. However in control tissue exposed to 3% halothane, 100µM nifedipine caused contraction.

4.4 DISCUSSION

Contractile responses to 3% halothane, 2mM caffeine and 80mM KCl were used to distinguish control from MH susceptible vastus lateralis muscle. Fibres diagnosed as MH susceptible produced contractures to these agents
FIGURE 4.5 The Effect of Dantrolene and Diltiazem on MH Susceptible Skeletal Muscle and Nifedipine on Control Skeletal Muscle after Exposure to 3% Halothane.

Responses to 6uM dantrolene and 10uM diltiazem are shown in MH susceptible preparations (A) after exposure to 3% halothane. The effect of 100uM nifedipine in control preparations (B) after exposure to 3% halothane is also shown.
The relaxant effects of 10uM diltiazem and 10uM
dantrolene are shown on MH susceptible (A) and control
(B) muscle preparations in the presence of caffeine.
A. MHS

DILT ($\mu$M) 10

DAN ($\mu$M) 6

CAFF (mM) 2

10 mins

B. Control

DILT ($\mu$M) 10

DAN ($\mu$M) 6

CAFF (mM) 4

10 mins
FIGURE 4.7 The Relaxant Effects of Diltiazem and
Dantrolene in MH Susceptible Muscle Exposed to Caffeine

The relaxant effects of diltiazem in the presence of maximally effective concentrations of dantrolene in a caffeine contracted preparation is shown (A). The relaxant effects of dantrolene in the presence of maximally effective concentrations of diltiazem in a caffeine contracted preparation is shown (B).
A. DAN (μM) 6

DILT (μM)

12

10

20

40

B. DILT (μM) 10

DAN (μM) 6

20

40

12

TENSION (g)

0

5

10 mins

CAFF (mM)

2

4

8

16

24

CAFF (mM)

2

4

8

16

24
which were significantly greater than than control responses (Table 4.1). Contractile responses fell within the respective ranges defined for control or MH susceptible human tissue (Sim, 1985).

The calcium channel antagonists diltiazem (10uM) and verapamil (10uM) inhibited the characteristic hypercontractility of isolated MH susceptible muscle to 3% halothane, 2mM caffeine and 80mM KCl (Figs. 4.2 4.3, 4.4). Contractile responses induced by 4mM caffeine (control tissue) (Fig.4.3) and electrical stimulation (MH susceptible and control tissue) (Fig.4.1) were also inhibited by these CCA. Diltiazem not only inhibited but also reversed contractile responses induced by halothane and caffeine (Fig.4.5, 4.6). These results support preliminary published investigations with skeletal muscle strips from patients susceptible to MH which demonstrate that diltiazem (Iwatsuki et al., 1983) and verapamil (Gruener and Blank, 1979) could prevent abnormal contractile responses to 1% halothane and 1mM caffeine.

In MH susceptible and control detubulated porcine skeletal muscle, 6uM dantrolene modified contractile responses to caffeine, while diltiazem had no effect (Chapter 3, Section 3.4.10). This suggests that dantrolene and diltiazem had different sites of action. In this study diltiazem and dantrolene completely reversed caffeine-induced contractures in MH susceptible (2mM
caffeine) and control preparations (4mM caffeine) (Fig. 4.6). At higher caffeine concentrations (16mM) (Fig. 4.7) diltiazem only partially decreased the contracture, this was further decreased by 6uM dantrolene and no further change was observed in the presence of 12uM dantrolene. Similarly, after partial reversal of the caffeine induced contracture by 6uM dantrolene, 10uM diltiazem caused a further reduction in tension and no further change was observed in the presence of 40uM diltiazem. However, in neither case was the contracture reversed completely. These results also suggest that diltiazem and dantrolene have different sites of action. Dantrolene acts to modify contractile responses in skeletal muscle by lowering myoplasmic Ca\(^{2+}\) levels. The muscle relaxant may achieve this by directly inhibiting Ca\(^{2+}\) release from the SR and/or suppressing some other aspect of EC coupling (Homma et al., 1976; Ellis and Wessels, 1977; Morgan and Bryant, 1977; Yamamoto et al., 1977; Oba and Hotta, 1978; see also Chapter 2, Section 2.3). The effect of diltiazem and verapamil on contractile responses in control and MH susceptible muscle is also consistent with the lowering of myoplasmic Ca\(^{2+}\) levels. Specific binding sites on VOCC, located primarily in the T-tubular membranes of skeletal muscle, have been observed for CCA (Fosset et al., 1983; Schmid, Renaud, Fosset, Meaux and Lazdunski, 1984). Recently, CCA-sensitive Ca\(^{2+}\) channels have been identified in isolated human skeletal muscle membranes which have binding characteristics similar to VOCC from T-tubular
membranes of other species (Desnuelle, Renaud, Delpont, Serratrice and Lazdunski, 1986). Diltiazem and verapamil may therefore modify contractile activity by inhibiting Ca\(^{2+}\) fluxes through these channels. Diltiazem and verapamil may inhibit Ca\(^{2+}\) influx associated with VOCC of the T-tubular membrane while dantrolene may inhibit Ca\(^{2+}\) release directly from the SR. However, these sites of action still remain to be defined.

The effects of nifedipine on contractile responses were examined only in control tissue. High concentrations of nifedipine (100uM) were required to observe a significant effect. Nifedipine potentiated contractile responses to 3% halothane (Fig. 4.2) and 4mM caffeine (Fig.4.3), while inhibiting twitch response (Fig.4.1) and having no effect on responses to KCl (Fig.4.4). Nifedipine also induced contractures in the presence of 3% halothane (Fig.4.5) in control muscle. Nifedipine (100uM) has also been shown to increase the magnitude of caffeine and caffeine-halothane-induced contractures in isolated rat skeletal muscle (Carter, Gergis, Carter, Davies and Sokoll, 1983). At a high concentration verapamil (>100uM) has been shown to induce contractile activity by releasing Ca\(^{2+}\) from intracellular stores (Bondi, 1978; Marwahu and Treffers, 1980; Frank, 1982). An increased level of myoplasmic Ca\(^{2+}\) in response to nifedipine would account for the potentiation of halothane-and caffeine-induced contractures. High
concentrations of nifedipine may cause Ca$^{2+}$ release or inhibit the uptake of Ca$^{2+}$ by the SR. Pretreatment of control tissue with nifedipine causes hypercontractility to caffeine and halothane, similar to that observed in MH muscle. In view of these observations it is unlikely that nifedipine would inhibit the hypercontractility of MH susceptible muscle.

4.5 **SUMMARY**

The CCA diltiazem and verapamil inhibited the hypercontractility of MH susceptible muscle to 3% halothane, 2mM caffeine and 80mM KCl. Like the skeletal muscle relaxant dantrolene, diltiazem not only prevented but reversed contractile responses to halothane and caffeine. Diltiazem and verapamil also inhibited contractile responses in control tissue. Verapamil and diltiazem may modify contractile responses by inhibiting Ca$^{2+}$ influx through VOCC in T-tubular membranes. Nifedipine potentiated halothane- and caffeine-induced contractures in control muscle and induced contractile activity in the presence of halothane.
CHAPTER 5

THE BIOCHEMICAL CHARACTERISATION AND THE MEASUREMENT OF CALCIUM FLUXES OF SARCOPLASMIC RETICULUM PREPARATIONS FROM MALIGNANT HYPERPYREXIA SUSCEPTIBLE AND CONTROL PORCINE SKELETAL MUSCLE

5.1 INTRODUCTION

The defect in MH susceptible muscle leads to an abnormally high myoplasmic Ca\(^{2+}\) concentration (Denborough, 1980). The intracellular distribution of Ca\(^{2+}\) in skeletal muscle is primarily controlled by the SR. The myoplasmic Ca\(^{2+}\) concentration reflects the balance between the rate of Ca\(^{2+}\) transport from the myoplasm into the lumen of the SR, the binding of Ca\(^{2+}\) to elements within the SR, and the rate of Ca\(^{2+}\) release from the SR into the myoplasm (Martonosi, 1984). A defect in SR function could therefore explain the raised myoplasmic Ca\(^{2+}\) concentration in MH skeletal muscle.

Investigations on the uptake and release of Ca\(^{2+}\) by isolated SR membrane vesicles from humans susceptible to MH have produced conflicting results. No difference in the rate of Ca\(^{2+}\) uptake by isolated MH susceptible human SR vesicles was observed when compared to control SR preparations (Kalow et al., 1970; Dhalla, Sulakhe, Clinch, Wade and Naimark, 1972; Blanck et al., 1981). However,
a reduction in the rate of Ca\(^{2+}\) uptake has been reported (Britt, Kalow, Gordon, Humphrey and Newcastle, 1973; Isaacs and Hefferon, 1975). Similar studies on the rate of Ca\(^{2+}\) uptake by isolated SR vesicles from MH susceptible pigs have also yielded varied results. Ca\(^{2+}\) uptake has been reported to be increased (Berman and Kench, 1973; Brucker, Williams, Popinigis, Galvez, Vail and Taylor, 1973; Britt et al., 1975), normal (Nelson et al., 1972; Denborough, Hird, King, Marginson, Mitchelson, Naylor, Rex, Zapf and Condron, 1973; White et al., 1983a) or reduced (McIntosh, Berman and Kench, 1977; Nelson, 1978; Gronert et al., 1979) in comparison to control rates. The observed effect of halothane on the rate of Ca\(^{2+}\) uptake also varies. In the presence of halothane, Ca\(^{2+}\) uptake by MH susceptible and control human and porcine SR vesicles was unaffected (Isaacs and Hefferon, 1975), inhibited to the same extent (Gronert et al., 1979; Blanck et al., 1981), increased (Nelson et al., 1972) and MH susceptible inhibited to a greater degree than control (Kalow et al., 1970; Britt et al., 1973; Brucker et al., 1973).

The rates of passive Ca\(^{2+}\) exchange and efflux were found to be similar in MH susceptible and control fragmented SR preparations (White et al., 1983a). However, Nelson (1983a) and Ohnishi et al. (1983), studying Ca\(^{2+}\)-induced Ca\(^{2+}\) release from SR vesicles, observed a lower Ca\(^{2+}\) concentration threshold for Ca\(^{2+}\) release in MH
susceptible fractions when compared with controls. Ohnishi et al. (1983), also demonstrated halothane-induced Ca\(^{2+}\) release from MH and control SR fractions, which was inhibited by dantrolene. Nelson (1983a) observed that caffeine decreased the Ca\(^{2+}\) threshold for Ca\(^{2+}\)-induced Ca\(^{2+}\) release to a greater degree in MH than in control SR preparations. These observations support the findings in skinned muscle fibres from a patient susceptible to MH that Ca\(^{2+}\)-induced Ca\(^{2+}\) release was activated at a lower Ca\(^{2+}\) concentration and achieved a higher rate at the optimal Ca\(^{2+}\)-concentration when compared to control fibres (Endo et al., 1983).

However, as yet, no definitive conclusion can be made on whether an abnormality exists in the ability of the SR isolated from MH susceptible muscle to sequester or release Ca\(^{2+}\).

5.1.1 Characterisation of the Sarcoplasmic Reticulum

The SR of mammalian muscle consists of two morphologically distinct regions, the terminal cisternae and the longitudinal tubules. The isolated SR membrane can be separated into vesicular fractions rich in the membrane elements of these regions by differential and sucrose-gradient centrifugation. Vesicles representing the terminal cisternae and longitudinal tubules are known as the heavy SR (HSR) and light SR (LSR) fractions.
respectively. Functional differences exist between isolated fractions, which are related to their fundamental roles in Ca\textsuperscript{2+} transport in vivo (Campbell and Shamoo, 1980; Campbell, Franzizi-Armstrong and Shamoo, 1980). HSR vesicles represent the primary site of Ca\textsuperscript{2+} release and storage while the LSR fraction represents those SR membrane elements primarily involved in the uptake of Ca\textsuperscript{2+} from the myoplasm.

The various membrane elements of these fractions have been extensively characterised (Meissner, 1975; Caswell, Lau and Brunschwig, 1976; Lau, Caswell and Brunschwig, 1977; Campbell et al., 1980; Caswell, Brandt and Brunschwig, 1980; Louis, Nash-Adler, Fudyma, Shigekawa, Akowitz and Katz, 1980; Hidalgo, Gonzalez and Lagos, 1983; Mitchell, Palade and Fleischer, 1983). Essentially, these fractions differ in protein composition and Ca\textsuperscript{2+}-transport activities. HSR vesicles are primarily characterised by the presence of Ca\textsuperscript{2+} dependent ATPase (100,000 daltons), calsequestrin (55,000 daltons) and the Ca\textsuperscript{2+} binding proteins of 45,000 and 42,000 daltons. HSR vesicles contain electron-dense material in their interiors, which is also observed in the terminal cisternae of intact muscle, and which is believed to represent the calcium binding protein calsequestrin (Caswell et al., 1976). LSR vesicles are also rich in Ca\textsuperscript{2+}-dependent ATPase but have less visible content (electron dense material) in their lumens. HSR
fractions have a higher Ca\(^{2+}\) content than LSR vesicles (McIntosh et al., 1977). This corresponds to the observed higher Ca\(^{2+}\) content of the terminal cisternae in comparison to other regions of the SR (Somlyo, Shuman and Somlyo, 1977). Both membrane fractions contain little or no Na\(^{+}\)-K\(^{+}\)-ATPase or cholesterol.

A detailed biochemical characterisation of the SR membrane from MH susceptible skeletal muscle has not been conducted. This chapter describes the characterisation and calcium fluxes of the HSR and LSR fractions of control and MH susceptible porcine skeletal muscle. Ca\(^{2+}\) uptake and release and Ca\(^{2+}\)-dependant ATPase activities from these functionally distinct regions will be compared between MH susceptible and control muscle in an attempt to clarify whether SR function is abnormal in MH. The effects of dantrolene, CCA and caffeine on these Ca\(^{2+}\)-dependent processes will also be examined with a view to defining their site of action in skeletal muscle.

5.2 MATERIALS AND METHODS

5.2.1 Surgical Techniques

Muscle was removed for the diagnosis of MH susceptibility under general anaesthesia, as described in Chapter 3. While still under anaesthesia animals were killed by captive bolt and exsanguinated. The biceps
femoris and semitendinosus muscles were immediately removed from the hind limb, minced finely and rapidly frozen in liquid nitrogen (-180°C). Muscle was then stored at -70°C until required.

5.2.2. Preparation of Crude or Fragmented Sarcoplasmic Reticulum

Frozen muscle samples (100-150g) were allowed to equilibrate to 0°C in 5 volumes of 0.25M sucrose / 10mM Tris. HCl buffer, pH 7.4. The muscle suspension was then homogenized in 3 x 30 second bursts with 30 second intervals in a Waring Blender (Low Speed Setting). After filtration through coarse gauze, connective tissue and myofibrillar proteins were removed from the homogenate by centrifugation at 3000g for 5 minutes. The supernatant was then taken and centrifuged at 10,000g for 20 minutes to remove mitochondria. The supernatant was then centrifuged at 100,000g for 20 minutes to obtain the microsomal pellet. The microsomal pellet was washed once in 0.6M KCl, recentrifuged at 100,000g for 20 minutes and the final pellet resuspended in a minimum volume of 0.25M sucrose 10mM Tris. HCl buffer, pH 7.4 using a glass / Teflon homogenizer. This membrane suspension was called the fragmented sarcoplasmic reticulum (FSR) preparation and was prepared by the method of White et al. (1983a). The entire isolation procedure was performed at 0 - 4°C.
5.2.3 Isolation of Heavy and Light Sarcoplasmic Reticulum Vesicles

The procedure for obtaining light and heavy SR vesicles was essentially the same as the method described by Campbell et al. (1980). FSR vesicles were placed on a linear sucrose gradient 8 - 44% sucrose (w/v) (30ml) with a 60% (w/v) sucrose cushion and centrifuged at 23,000 rev / min in a Beckman SW27 rotor. After 4 hrs at 4°C protein migration was complete and the rotor was allowed to decelerate without braking assistance. The gradient was then unloaded and the percentage of sucrose in the effluent fractions continuously monitored using a refractometer. Fractions from 29 - 33% sucrose (light) or 34 - 40 % sucrose (heavy) were combined and diluted in 0.25M sucrose 10mM Tris. HCl buffer, pH 7.4. The purified SR vesicles were then centrifuged at 100,000g for 1 hr. The resultant pellets were then gently resuspended using a glass / Teflon homogenizer in a minimum volume of 0.25M sucrose 10mM Tris. HCl buffer, pH 7.4. Fractions were then immediately used or frozen in liquid nitrogen (-180°C) and stored at -70°C.

5.2.4 Isolation of Mitochondria

The mitochondrial pellet obtained during the isolation procedure for FSR vesicles was washed once in 0.25M sucrose 10mM Tris. HCl buffer, pH 7.4. The resulting
pellet was then suspended in the washing buffer (0.5 - 1ml) with a glass / Teflon homogenizer and used for marker enzyme analysis.

5.2.5 Proteins and Phospholipid Estimations

Protein was determined using a modification of the Lowry method (Peterson, 1977). The phospholipid content of membranes was measured after HClO₄ hydrolysis of the phospholipid to inorganic phosphate (Dittmer and Wells, 1969).

5.2.6 Marker Enzyme Assays

5.2.6.1 5'-Nucleotidase Activity

Plasma membrane contamination of FSR, HSR and LSR preparations was determined by estimation of 5'-nucleotidase activity by the method of White et al. (1983a). The amount of inorganic phosphate (Pᵢ) liberated was determined according to the method of Heinonen and Lahti (1981).

A buffer (0.5ml) consisting of 50mM 5'-AMP, 0.5M glycine/NaOH, 0.01M MgCl₂, pH 9.1 was warmed to 37°C. The reaction was initiated by the addition of approximately 100µg of protein and the liberation of Pᵢ from AMP was measured after 20 minutes. The reaction was terminated by
adding 4mls of an acetone/molybdate/acid colour reagent and within 2 minutes full colour development took place. While standing in an ice slurry, 0.5ml of 1M citric acid was added to the test tubes to stop colour development and the absorbance read at 355nm. Citric acid is added in excess to chelate all unused molybdate, so that unstable organic phosphates cannot react further (Heinonen and Lathi, 1981).

5.2.6.2 Na⁺-K⁺-ATPase Activity

Plasma membrane and T-tubule contamination of various SR membrane fractions was determined by estimation of Na⁺-K⁺-ATPase activity. Activity was measured in the same assay medium as described for Ca²⁺-independent ATPase activity (Section 5.2.7). Na⁺-K⁺-ATPase activity was taken as the difference between the Ca²⁺-independent ATPase activity in the presence and absence of 10mM ouabain.

5.2.6.3 Succinate Dehydrogenase Activity

The degree of mitochondrial contamination in FSR, HSR and LSR membrane fractions was determined by measuring succinate dehydrogenase activity. The method was the same as that described by King (1963). The activity observed in isolated SR preparations is presented as a percentage of the activity of the mitochondrial fractions obtained after the 10,000g centrifugation step.
during the isolation of the FSR.

5.2.6.4 Acid Phosphatase Activity

Acid phosphatase activity of FSR, HSR and LSR preparations was determined to estimate the degree of lysosomal contamination. The method was the same as that described by White et al. (1983a).

Acid phosphatase activity was determined at 37°C in 0.5ml of a medium containing 0.1M citrate and 1mM p-nitrophenyl phosphate, pH 5.0. The reaction was initiated by the addition of approximately 100ug of protein and stopped after 20 minutes by the addition of 0.2ml of 10% (w/v) sodium dodecyl sulphate and 5ml of 20mM NaOH. The amount of p-nitrophenol formed was determined at 400nm.

5.2.7 Calcium - Dependent ATPase Activity

Total ATPase activity was determined in 0.5ml of a solution consisting of 100mM NaCl, 10mM MgCl₂, 10mM KCl, 1mM ATP, 10mM Tris. HCl, pH 7.4 (White and Denborough, 1984), and up to 0.5mM CaCl₂. Ca²⁺-independent activity was measured in the presence of up to 1mM EGTA instead of CaCl₂. Ca²⁺-dependent ATPase activity was the difference between the total ATPase activity and the Ca²⁺-independent activity. During titration experiments to determine
various levels of endogenous and exogenous Ca$^{2+}$-dependent ATPase activities, CaCl$_2$ and EGTA were added to the reaction medium at the concentrations indicated in the text. Endogenous activity was measured in the absence of any added CaCl$_2$, while exogenous activity was measured in the presence of EGTA by adding CaCl$_2$ to the reaction medium. The reaction was initiated by the addition of 20 - 50ug of protein (HSR, LSR or FSR vesicles) to 0.5ml of the reaction buffer which had been warmed to 37°C. After 15 minutes the reaction was terminated by immersing reaction test tubes in an ice slurry and adding 4ml acetone/molybdate/acid colour reagent. After 2 minutes, colour development was stopped by the addition of 0.4ml of 1M citric acid, and absorbance was measured at 355nm (Heinonen and Lathi, 1981).

When the effect of various drugs on Ca$^{2+}$-dependent ATPase activity was examined, the drug was made up to the required concentration in the reaction medium.

5.2.8 Calcium Transport Studies

5.2.8.1 Calcium Uptake

Calcium uptake by FSR, HSR and LSR preparations was measured by a Millipore filtration method. The reaction buffer consisted of 100mM KCl, 5mM MgCl$_2$, 10mM Tris. HCl pH 7.4, 5mM potassium-oxalate and 20uM CaCl$_2$ (including
In some uptake studies potassium-oxalate was omitted from the buffer. Vesicles (50 - 100ug protein) were diluted 10-fold in 0.5ml of buffer pre-warmed to 37°C. Calcium uptake was initiated by the addition of ATP to a final concentration of 2mM. Aliquots (20ul) of the vesicle suspensions were removed at various intervals, diluted into 3ml of ice cold uptake buffer (excluding $^{45}\text{Ca}^{2+}$) and filtered through 0.22um microporous filters (Zetapour) using an Amicon VFMI vacuum manifold. Vesicles were then quickly washed with a further 3ml of ice cold uptake buffer. Filtration and washing was complete within 15 seconds. Filters were counted for radioactive calcium after drying and immersion in scintillant (Xylene/Triton X-114, 2:1 (v/v) containing 0.5% (w/v) 2,5 diphenyloxazole) using a Packard Tri-carb liquid scintillation counter.

Calcium uptake in the presence of drugs was only examined in the LSR fraction as this fraction represents the SR membrane primarily involved in Ca$^{2+}$ uptake. Drugs were made up to the required concentration in uptake buffer. Vesicles were incubated for 5 minutes at 37°C in the presence of a drug before uptake was initiated. When the effect of a drug was examined, it was present in all buffers.
5.2.8.2 Calcium Release Studies

5.2.8.2.1. Equilibrium Calcium Exchange Experiments

Ca$^{2+}$ movements across FSR, HSR and LSR membranes were also measured by the millipore filtration method, using 0.22μm filters. Vesicles were passively loaded by incubating 1 - 2mg of protein in 1ml of reaction buffer consisting of 100mM KCl, 5mM MgCl$_2$, 100mM Tris. HCl, pH 7.4 and 1mM CaCl$_2$ (containing $^{45}$Ca$^{2+}$, 21.78 uCi/ml), overnight at 0°C (White et al., 1983a). Loaded vesicles (50 ul) were preincubated to 37°C before calcium exchange was initiated by a 50-fold dilution into tracer free reaction buffer containing 1mM CaCl$_2$. Transport was terminated by taking 500ul aliquots of the vesicle suspension at various intervals and filtering the sample in the presence of 3ml of ice-cold, tracer-free reaction buffer containing 1mM CaCl$_2$. The filter was then washed again in 3ml of the same buffer. Filters were then counted for radioactivity under the same conditions as those described for calcium uptake studies.

5.2.8.2.2. Ca$^{2+}$ Efflux Experiments

After passive loading of vesicles in 1mM $^{45}$CaCl$_2$, Ca$^{2+}$-free buffers were used facilitating the examination of Ca$^{2+}$ release down a concentration gradient.
Ca²⁺ release in the presence of drugs was examined on only the HSR fraction (as this fraction represents the region of the SR membrane which is primarily involved in Ca²⁺ release). Loaded vesicles were preincubated for 15 minutes at 37°C in the presence of the drug before the reaction was initiated. The drug was also present in all washing buffers.

5.2.9 Polyacrylamide Gel Electrophoresis (PAGE)

Sodium dodecylsulphate (SDS) PAGE was performed using 7.5% - 15% (w/v) continuous polyacrylamide gradient separating gels containing 0.1% SDS (w/v), with 4.5% polyacrylamide stacking gels containing 0.1% SDS. Gels were run in the discontinuous buffer system described by Laemmli (1970) and at a constant current of 40mA/gel. Protein bands on gels were developed by fixation in a solution containing 25% isopropanol and 10% acetic acid (v/v), followed by staining with Coomassie blue in 10% isopropanol/acetic acid solution (v/v) and destained in 10% isopropanol/acetic acid solution (v/v). Gels were then scanned at 520nm in a Beckman DU-8 spectrophotometer.

Before electrophoresis, proteins were treated with a solution containing 2.5% SDS, 1% mercaptoethanol, 1 mg sucrose, 20mM Tris. HCl, pH 7.4 and approximately 1mg of bromophenol blue tracking dye, and boiled for 5 minutes. Proteins (50ug) were then loaded onto stacking gels for
electrophoresis. The apparent molecular weights of various protein bands were obtained by calibration of the gel with proteins of known molecular weights (Sigma Chemical company).

5.2.10 Atomic Absorbtion Spectroscopy (AAS)

AAS was carried out in the Research School of Chemistry, Australian National University. HSR and LSR membranes were analysed for their Ca$^{2+}$ content. A membrane suspension of 10ug of protein in a 1ml solution of 0.1M KCl and 10mM Tris. HCl, pH 7.4 was used for analysis.

5.2.11 Electron Microscopy

Samples of FSR, HSR and LSR fractions for transmission electron microscopy and freeze fracture studies were prepared by the method of Campbell et al., 1980. Electron micrographs and freeze fracture replicas were prepared by Mr. K. Hopkinson of the MH muscle group, Department of Medicine and Clinical Science, John Curtin School of Medical Research.

5.2.12 Isolation of Calsequestrin

Calsequestrin was isolated according to the method White, Thomas and Denborough, 1983b.
5.2.13 Drugs and Reagents

The sources of all drugs were as described in Chapter 3. All reagents were of analytical grade.

5.3 RESULTS

5.3.1 Fractionation Profile of Crude Sarcoplasmic Reticulum

In order to compare the Ca\(^{2+}\)-transport characteristics of MH susceptible and control pig muscle, the FSR preparation was further fractionated into two functionally distinct regions of the SR membrane, the HSR and LSR fractions. The fractionation profile of the SR membrane proteins from MH susceptible muscle is shown in Figure 5.1. Protein was concentrated in regions of the profile which represented the light (fractions 8 to 11), intermediate (fraction 7) and heavy (fractions 3 to 6) SR fractions (Campbell et al., 1980). The intermediate SR fraction was discarded, as it is not clear whether the region of the SR membrane represented by this fraction is primarily involved in Ca\(^{2+}\) uptake or release. The intermediate fraction primarily serves to distinctly separate the HSR from the LSR regions of the SR membrane. Approximately 90-95\% of the total applied protein was always recovered after centrifugation. The protein fraction profiles of control and MH susceptible fragmented
FIGURE 5.1 Protein Fractionation Profile of the
Fragmented Sarcoplasmic Reticulum Membrane from Porcine
MH Susceptible Muscle

MH susceptible FSR preparations were applied to a continuous sucrose gradient and centrifuged for 4 hours at 100,000g.
Some 80% of the total protein was recovered in fractions 3 to 11. Heavy SR were obtained by pooling fractions 3, 4 and 5; light SR were obtained from fractions 8 to 11.
Protein recovered (%) Total

Sucrose (M)

Fraction

Top

Bottom
SR preparations following continuous sucrose gradient centrifugation were similar.

5.3.2 Analysis of Enzymatic Activities of Sarcoplasmic Reticulum Fractions

The activity of Ca\(^{2+}\)-ATPase, the primary protein constituent of the SR membrane, was high in all SR fractions (Table 5.1). Contamination of FSR, HSR and LSR preparations by extraneous membranes was also investigated (Table 5.1). Succinate dehydrogenase activity in all SR preparations was low, indicating low levels of mitochondrial contamination. The presence of plasma and T-tubule membrane impurities was also low, as evidenced by the low levels of 5' -nucleotidase and Na\(^{+}\)-K\(^{+}\)-ATPase activities. Acid phosphatase activity was also low, indicating low levels of lysosomal contamination.

HSR and LSR membrane preparations had consistently less extraneous enzyme and higher Ca\(^{2+}\)-ATPase activities than the crude FSR preparation.

5.3.3 Phospholipid/Protein Ratios of Sarcoplasmic Reticulum Fractions

Phospholipid to protein ratios did not significantly vary between SR fractions isolated from control or MH susceptible muscle, or between the same fractions isolated
TABLE 5.1 Endogenous Ca\textsuperscript{2+}-ATPase and Other Enzyme Activities of Isolated Control and Malignant Hyperpyrexia Susceptible Pig Skeletal Muscle SR Fractions

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control</th>
<th>MH Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FSR</td>
<td>HSR</td>
</tr>
<tr>
<td>Endogenous Ca\textsuperscript{2+}-ATPase (umole P\textsubscript{i}/mg protein/min)</td>
<td>0.58+/−0.06</td>
<td>0.98+/−0.07</td>
</tr>
<tr>
<td>Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (umole P\textsubscript{i}/mg protein/min)</td>
<td>0.20+/−0.07</td>
<td>0.03+/−0.01</td>
</tr>
<tr>
<td>Acid phosphatase (nmol p-nitrophenol/mg protein/min)</td>
<td>41.22+/−12.83</td>
<td>3.29+/−1.04</td>
</tr>
<tr>
<td>Succinate dehydrogenase (%)</td>
<td>6.20+/−0.34</td>
<td>1.93+/−0.34</td>
</tr>
<tr>
<td>5'-nucleotidase (umole P\textsubscript{i}/mg protein/min)</td>
<td>0.18+/−0.03</td>
<td>0.05+/−0.01</td>
</tr>
</tbody>
</table>

Succinate dehydrogenase activity is presented as a percentage of the activity of the purified mitochondrial preparation. Results are expressed as mean +/- s.e.m. The number of determinations ranged from 4 to 9.
from control and MH susceptible muscle (Table 5.2). LSR had a slightly higher phospholipid content than the HSR.

5.3.4 The Calcium Content of Sarcoplasmic Reticulum Fractions

The calcium content (mean +/- s.e.m, nmoles/mg membrane protein) of control (71.66 +/- 5.3, n = 10) and MH susceptible (70.13 +/- 4.8, n = 6) HSR preparations were not significantly different. Similarly, the calcium contents of LSR preparations from MH susceptible (45.40 +/- 6.32, n = 6) and control (43.45 +/- 7.3, n = 10) were not significantly different. However, HSR membrane fractions contained significantly (p < 0.05) more calcium than LSR preparations.

5.3.5 Morphological Studies on Sarcoplasmic Reticulum Preparations

Typical electron micrographs of control FSR, HSR and LSR vesicles are shown in Figure 5.2. Micrographs of MH susceptible SR fractions showed essentially the same characteristics. Closed membranous vesicles of various shapes and sizes were observed in all three fractions. All fractions contained vesicles of various shapes and sizes, however HSR (Fig.5.2.A) and LSR (Fig.5.2.B) fractions appeared to have a more homogeneous population
TABLE 5.2 Phospholipid to Protein Ratios of Isolated Sarcoplasmic Reticulum Membrane Fractions from Control and Malignant Hyperpyrexia Susceptible Pig Skeletal Muscle.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>MH Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSR</td>
<td>0.77 +/- 0.01</td>
<td>0.74 +/- 0.05</td>
</tr>
<tr>
<td>HSR</td>
<td>0.69 +/- 0.04</td>
<td>0.73 +/- 0.04</td>
</tr>
<tr>
<td>LSR</td>
<td>0.75 +/- 0.03</td>
<td>0.77 +/- 0.04</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m.
The number of determinations ranged from 5 - 6.
FIGURE 5.2 Electron Micrographs of Sarcoplasmic Reticulum Fractions from Control Pig Skeletal Muscle.

Micrographs of Heavy (A), Light (B) and Fragmented (C) preparations are shown (magnifications x 16000). A micrograph of a mitochondrial fraction (D) is also shown at the same magnification.
of vesicles than the FSR (Fig. 5.2.C) fraction. HSR fractions contained more electron dense material than LSR vesicles. This is likely to represent the greater calsequestrin content of the HSR fraction (Section 5.3.1.5). Micrographs did not reveal any contamination with mitochondria, although biochemical analysis detected low levels of mitochondrial enzyme activity. Contamination was probably in the form of submitochondrial vesicles. Electron micrographs of purified mitochondria showed that mitochondrial vesicles were larger than SR vesicles, with numerous invaginations (Fig. 5.2.D).

Representative freeze-fracture replicas of control HSR and LSR vesicles are shown in Figure 5.3. Replicas from MH susceptible SR fractions showed essentially the same features. All fractions show spherical vesicles. In most vesicles the cytoplasmic leaflet (concave) is occupied by particles while the luminal leaflet (convex) is smoother. These particles are thought to represent the calcium pump protein, Ca\textsuperscript{2+}-dependent-ATPase (MacLennan, Seeman, Iles and Yip, 1971). It was difficult to distinguish any differences between the replicas from HSR and LSR fractions.
FIGURE 5.3 Freeze Fracture Replicas of Heavy and Light Sarcoplasmic Reticulum Vesicles from Control Pig Skeletal Muscle

Replicas of Heavy (A) and Light (B) preparations are shown (magnification x 7,500).
5.3.6 Protein Polyacrylamide Gel Electrophoresis of Sarcoplasmic Reticulum Fractions

SDS-PAGE protein patterns of LSR and HSR membrane fractions from MH susceptible and control muscle are illustrated in Figure 5.4. Spectrophotometric scans of protein patterns are also shown (Fig. 5.5). Protein patterns or scans from the same SR fraction did not differ between MH susceptible and control preparations. The differences observed between membrane fractions, which characterised them as HSR or LSR, were the same for control as MH susceptible membrane preparations. The major protein band from both HSR and LSR fractions corresponded to the calcium pump, Ca\(^{2+}\)-ATPase (approximately 100,000 MW). The percentage of Ca\(^{2+}\)-ATPase protein ranged between 41.5 - 47.5 (n = 6) and 52 - 53 (n = 6) for HSR and LSR fractions respectively. The major difference in the protein composition between HSR and LSR fractions was the content of calsequestrin (approximately 66,000 MW). HSR fractions were enriched in the Ca\(^{2+}\)-binding protein. Of the total membrane protein LSR fractions contained 5.0% while HSR fractions contained 10% calsequestrin (n = 6, per fraction). Thus, LSR fractions were characterised by a higher Ca\(^{2+}\)-ATPase/calsequestrin ratio than HSR fractions. This is consistent with the greater amount of electron dense material in HSR preparations. Both LSR and HSR fractions were relatively free of higher molecular weight proteins (> 120,000 MW,
FIGURE 5.4 SDS-PAGE of Heavy and Light Sarcoplasmic Reticulum Proteins from Malignant Hyperpyrexia Susceptible and Control Porcine Skeletal Muscle.

MH susceptible protein patterns are shown in the lanes marked 1. Control protein patterns are shown in lanes marked 2. The migration of purified calsequestrin is shown in lane 3.
The gels shown in figure 5.4 were scanned at 520nm in a Beckman DU-8 spectrophotometer. Heavy (HSR) and Light (LSR) fractions are indicated. Peak 1 corresponds to Ca$^{2+}$-dependent ATPase (MW approximately 100,000), while Peak 2 represents calsequestrin (MW approximately 66,000).
(A) HSR

Control

MHS

(B) LSR

Control

MHS
contractile proteins) but contained a substantial amount of minor protein components (< 66,000 MW). These minor protein components did not vary between the same SR membrane fraction from MHS and control muscle, but the relative amounts did vary between HSR and LSR fractions. These protein components of the SR membrane are yet to be identified.

5.3.7 Calcium-Dependent ATPase Activities of Sarcoplasmic Reticulum Membrane Fractions

The specific activity of the Ca^{2+}-ATPase was consistently higher in HSR and LSR preparations than in FSR preparations. Ca^{2+}-ATPase specific activity was similar in both HSR and LSR fractions. CCA (verapamil and diltiazem), dantrolene and caffeine had no effect on the specific enzyme activity of HSR and LSR vesicles from MH susceptible or control preparations.

5.3.7.1 EGTA Titration of Endogenous Ca^{2+}-dependant ATPase Activity

The use of unphysiologically high concentrations of Ca^{2+} may not be suitable for the study of drug-induced changes on Ca^{2+} ATPase activity. When no exogenous Ca^{2+} is added to the assay medium (Fig. 5.6), Ca^{2+}-ATPase activity is similar to that observed in the presence of high Ca^{2+} concentrations (Fig. 5.7) in both HSR and LSR
Endogenous Ca$^{2+}$-dependent ATPase activity was determined in the presence of various concentrations of EGTA in control (A) and MH susceptible (B) HSR and LSR preparations. 

Results are expressed as the mean of 6 determinations. Results were not significantly different between HSR and LSR preparations or between the same preparation from MH susceptible and control muscle.
Calcium-ATPase activity

**Calcium-ATPase activity (μmoles P_i/mg protein/min)**

**A.**

**B.**

1.0
0.8
0.6
0.4
0.2
0

EGTA (μM)

0 10 20 30 40 50 60 80

HSR

LSR
FIGURE 5.7. Exogenous Ca\textsuperscript{2+}-dependent ATPase Activity of Sarcoplasmic Reticulum Fractions

Exogenous Ca\textsuperscript{2+}-dependent ATPase activity was measured at various concentrations of Ca\textsuperscript{2+} in the presence of EGTA (80\textmu M) in control (A) and MH susceptible (B) HSR and LSR preparations.

Results are expressed as the mean of 6 determinations. There was no significant difference between HSR and LSR preparations or between the same preparation from MH susceptible and control muscle.
vesicles. This indicates that the levels of endogenous Ca\(^{2+}\) found in these fractions is sufficient to obtain maximum Ca\(^{2+}\)- ATPase activities. By adding increasing amounts of EGTA to an assay medium containing no exogenous Ca\(^{2+}\), the Ca\(^{2+}\)-ATPase activity of HSR and LSR vesicles was reduced in a concentration dependent manner to very low levels (Fig. 5.6). EGTA titration curves of Ca\(^{2+}\)-ATPase activity did not significantly differ between different fractions, or between the same fractions in MH susceptible and control preparations (Fig.5.6). The reduction of Ca\(^{2+}\)-dependent ATPase activity by EGTA allowed the examination of Ca\(^{2+}\)-ATPase activity over a range of exogenous Ca\(^{2+}\) concentrations. These conditions are more suitable to examine differences in Ca\(^{2+}\)-ATPase activities between MH susceptible and control muscle and drug-induced changes in Ca\(^{2+}\)-ATPase activities.

5.3.7.2 Exogenous Ca\(^{2+}\)-dependent ATPase Activities of Sarcoplasmic Reticulum Membrane Fractions

EGTA (80uM) reduced Ca\(^{2+}\)-dependent ATPase activity in all HSR and LSR vesicles to low levels (Fig.5.7). The residual Ca\(^{2+}\)-ATPase activity which was still present indicated that there was no excess EGTA available to chelate exogenous Ca\(^{2+}\). The addition of exogenous Ca\(^{2+}\) to the assay medium at various concentrations restored Ca\(^{2+}\) ATPase activity. Activity was initiated by the addition of 2uM Ca\(^{2+}\) and was essentially linear with respect to Ca\(^{2+}\)
concentration up to 10μM. Maximal activation was obtained at 80μM Ca$^{2+}$ (Fig. 5.7). No significant differences in Ca$^{2+}$-ATPase activities were observed at any Ca$^{2+}$ concentration, between the same fractions or between different fractions, from MH susceptible and control muscle (Fig. 5.7).

Dantrolene (20μM), 80μM diltiazem, 100μM verapamil and 10mM caffeine failed to significantly increase or decrease Ca$^{2+}$-dependent ATPase activity from any fraction from MH susceptible or control muscle, over the Ca$^{2+}$ concentration range examined (2 to 80μM). The effect of various drugs on Ca$^{2+}$-ATPase activities is only shown at 8μM Ca$^{2+}$ (representing low levels of activity and where activity is still linear) and 50μM Ca$^{2+}$ (representing the Ca$^{2+}$ concentration that induces near maximal activity) (Table 5.3 and 5.4).

5.3.8 Calcium Uptake by Isolated Membrane Fractions in the Presence and Absence of Oxalate

In experiments designed to test the effect of oxalate on Ca$^{2+}$ uptake by HSR and LSR, the uptake of $^{45}$Ca$^{2+}$ was initiated by the addition of ATP to the incubating medium. In the presence of oxalate uptake proceeded rapidly in all SR fractions and was essentially complete within 2 mins. The rate of accumulation of Ca$^{2+}$ and the loading capacity for Ca$^{2+}$ was greater in HSR and LSR.
### TABLE 5.3 The Effect of Various Agents on the Ca\(^{2+}\)-dependent ATPase Activity of Sarcoplasmic Reticulum

Membrane Fractions from Control Pig Skeletal Muscle in the Presence of EGTA (80uM)

<table>
<thead>
<tr>
<th>Exogenous Ca(^{2+}) (uM)</th>
<th>untreated</th>
<th>dantrolene (20uM)</th>
<th>diltiazem (80uM)</th>
<th>verapamil (100uM)</th>
<th>caffeine (10mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSR FRACTION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.31+/−0.03</td>
<td>0.29+/−0.02</td>
<td>0.33+/−0.03</td>
<td>0.27+/−0.01</td>
<td>0.28+/−0.01</td>
</tr>
<tr>
<td>8</td>
<td>0.54+/−0.02</td>
<td>0.55+/−0.03</td>
<td>0.55+/−0.02</td>
<td>0.53+/−0.04</td>
<td>0.54+/−0.02</td>
</tr>
<tr>
<td>50</td>
<td>0.97+/−0.05</td>
<td>0.95+/−0.01</td>
<td>0.94+/−0.04</td>
<td>0.94+/−0.05</td>
<td>0.93+/−0.43</td>
</tr>
<tr>
<td>LSR FRACTION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.27+/−0.01</td>
<td>0.34+/−0.01</td>
<td>0.27+/−0.02</td>
<td>0.26+/−0.01</td>
<td>0.33+/−0.01</td>
</tr>
<tr>
<td>8</td>
<td>0.54+/−0.03</td>
<td>0.58+/−0.03</td>
<td>0.55+/−0.03</td>
<td>0.60+/−0.03</td>
<td>0.61+/−0.06</td>
</tr>
<tr>
<td>50</td>
<td>0.94+/−0.03</td>
<td>0.93+/−0.03</td>
<td>0.92+/−0.03</td>
<td>0.91+/−0.03</td>
<td>0.98+/−0.05</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m. of 6 observations.

The differences between untreated and drug treated preparations were not significant.
TABLE 5.4 The Effect of Various Agents on the Ca^{2+}-dependent ATPase Activity of Sarcoplasmic Reticulum Fraction from Malignant Hyperpyrexia Susceptible Pig Skeletal Muscle in the Presence of EGTA (80uM)

<table>
<thead>
<tr>
<th>Exogenous Ca^{2+} (uM)</th>
<th>untreated</th>
<th>dantrolene (20uM)</th>
<th>diltiazem (80uM)</th>
<th>caffeine (10mM)</th>
<th>verapamil (100uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSR FRACTION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.34+-/0.02</td>
<td>0.37+-/0.01</td>
<td>0.34+-/0.02</td>
<td>0.36+-/0.02</td>
<td>0.32+-/0.01</td>
</tr>
<tr>
<td>8</td>
<td>0.59+-/0.03</td>
<td>0.62+-/0.04</td>
<td>0.58+-/0.08</td>
<td>0.57+-/0.03</td>
<td>0.56+-/0.03</td>
</tr>
<tr>
<td>50</td>
<td>1.00+-/0.03</td>
<td>1.00+-/0.03</td>
<td>0.97+-/0.02</td>
<td>0.97+-/0.03</td>
<td>0.97+-/0.03</td>
</tr>
<tr>
<td>LSR FRACTION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.29+-/0.02</td>
<td>0.31+-/0.02</td>
<td>0.29+-/0.02</td>
<td>0.31+-/0.02</td>
<td>0.30+-/0.02</td>
</tr>
<tr>
<td>8</td>
<td>0.59+-/0.03</td>
<td>0.61+-/0.03</td>
<td>0.57+-/0.03</td>
<td>0.56+-/0.03</td>
<td>0.58+-/0.03</td>
</tr>
<tr>
<td>50</td>
<td>0.91+-/0.06</td>
<td>0.93+-/0.06</td>
<td>0.94+-/0.03</td>
<td>0.89+-/0.06</td>
<td>0.90+-/0.06</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m. of 6 observations.

The differences between untreated and drug treated preparations were not significant.
vesicles than in FSR preparations from both MH susceptible and control muscle (Fig. 5.8). The initial uptake of Ca$^{2+}$ by HSR and LSR fractions was extremely rapid and no distinctions could be made between the capacities of these fractions or between the same fractions from MH susceptible and control preparations to accumulate Ca$^{2+}$ after 15 and 30 secs. However, HSR membrane vesicles accumulated significantly greater amounts of Ca$^{2+}$ than LSR preparations (Fig. 5.8). When the uptake capacities at various time intervals were examined between the same fraction from MH susceptible and control muscle no differences were observed (Fig. 5.8).

In the absence of oxalate in the incubation medium Ca$^{2+}$ uptake was much more rapid and essentially complete within 15 to 30 seconds (Fig. 5.8) in all SR fractions. The capacity of all preparations to accumulate Ca$^{2+}$ in the absence of oxalate was also lower, probably due to the simultaneous efflux of calcium from the vesicles.

The effect of 20uM dantrolene, 80uM diltiazem, 100uM verapamil and 10mM caffeine on Ca$^{2+}$ uptake by LSR vesicles from MH susceptible and control preparations is shown in Tables 5.5 and 5.6. All agents failed to alter significantly the rate of calcium accumulation by the LSR fraction in the presence or absence of oxalate after 15, 30, 60 or 120 seconds. For simplicity only the amount of Ca$^{2+}$ accumulated in the presence of a drug after
FIGURE 5.8 Calcium Uptake by Isolated Sarcoplasmic Reticulum Fractions

The time course of Ca^{2+} uptake in the presence (filled symbols) and absence (open symbols) of oxalate by control (A) and MH susceptible (B) membrane vesicles is shown.

Results are expressed as mean +/- s.e.mean or mean values.

The number of observations was 10 for control and 5 for MH susceptible preparations.

+ significantly less than LSR value p < 0.001
++ significantly greater than control LSR value p < 0.01
+++ significantly greater than MH susceptible LSR value p < 0.001
CALCIUM UPTAKE (nmoles/mg protein)

A.

TIME (min)

HSR

LSR

FSR

0 1 2 3

B.

TIME (min)

HSR

LSR

FSR

0 1 2 3
TABLE 5.5 The Effect of Various Agents on Calcium Accumulation by Light Sarcoplasmic Reticulum Vesicles from Malignant Hyperpyrexia Susceptible and Control Pig Skeletal Muscle in the Absence of Oxalate

<table>
<thead>
<tr>
<th></th>
<th>Untreated (nmoles/mg/protein)</th>
<th>DAN (20μM)</th>
<th>DILT (80μM)</th>
<th>VER (100μM)</th>
<th>CAFF (10mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.97 ± 0.12</td>
<td>13.98 ± 0.05</td>
<td>14.25</td>
<td>14.33</td>
<td>14.71</td>
</tr>
<tr>
<td>MHS</td>
<td>14.76 ± 0.21</td>
<td>14.21 ± 0.39</td>
<td>14.82 ± 0.53</td>
<td>14.35 ± 0.27</td>
<td>14.02 ± 0.39</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e.m. of 4 experiments.

There was no significant difference between treated and untreated values.
TABLE 5.6 The Effect of Various Agents on the Accumulation of Calcium by the Light Sarcoplasmic Reticulum Fraction from Malignant Hyperpyrexia Susceptible and Control Pig Skeletal Muscle in the Presence of Oxalate

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>DAN (20μM)</th>
<th>DILT (80μM)</th>
<th>VER (100μM)</th>
<th>CAFF (10mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>151.97</td>
<td>141.48</td>
<td>142.37</td>
<td>149.49</td>
<td>157.8</td>
</tr>
<tr>
<td></td>
<td>+/- 3.13</td>
<td>+/- 5.75</td>
<td>+/- 5.50</td>
<td>+/- 8.47</td>
<td>+/- 13.7</td>
</tr>
<tr>
<td>MHS</td>
<td>145.95</td>
<td>140.19</td>
<td>145.64</td>
<td>141.86</td>
<td>135.12</td>
</tr>
<tr>
<td></td>
<td>+/- 4.33</td>
<td>+/- 5.33</td>
<td>+/- 3.45</td>
<td>+/- 2.60</td>
<td>+/- 4.99</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m. of 5 experiments

There was no significant difference between treated and untreated values.
30 seconds (absence of oxalate) and 2 minutes (presence of oxalate) is shown.

5.3.9 Calcium Release by Isolated Membrane Fractions from Malignant Hyperpyrexia Susceptible and Control Pig Muscle

5.3.9.1 Equilibrium Exchange

The rate of $^{45}\text{Ca}^{2+}$ exchange from passively loaded HSR, LSR and FSR membrane vesicles was compared at 1, 2, 3, 6 and 12 minute periods. HSR and LSR fractions significantly released more $\text{Ca}^{2+}$ than FSR vesicles after 1 and 2 minutes, from both MH susceptible and control preparations (Fig. 5.9). $\text{Ca}^{2+}$ release was also more rapid from HSR than LSR vesicles when measured at the same time interval (1 and 2 minutes) (Fig. 5.9). The amount of $\text{Ca}^{2+}$ released by all fractions was similar at 3, 6 and 12 minutes. When the amount of $\text{Ca}^{2+}$ exchanged at different intervals was compared between the same fraction obtained from MH susceptible and control muscle, no significant differences were observed (Fig. 5.9).

5.3.9.2 Calcium Efflux

The rate of $^{45}\text{Ca}^{2+}$ efflux from passively loaded HSR, LSR and FSR membrane fractions was also compared at 1, 2, 3, 6 and 12 minute periods. Efflux of $\text{Ca}^{2+}$ was
FIGURE 5.9 Equilibrium Exchange of Ca$^{2+}$ Across Sarcoplasmic Reticulum Membrane Fractions

The exchange of Ca$^{2+}$ across HSR (circular symbols), LSR (square symbols) and FSR (diamond symbols) membrane vesicles derived from control (A) and MH susceptible (B) pig muscle is shown. Exchange was initiated when vesicles were diluted into a medium containing 1mM Ca$^{2+}$ (see Materials and Methods section). Results are expressed as mean or mean +/- s.e.m. The number of observations ranged from 5 - 9.

+ Exchange from FSR was significantly less than from HSR $p < 0.01$

++ Exchange from LSR was significantly less than from HSR $p < 0.05$
significantly greater from HSR vesicles in comparison to FSR vesicles over the first 3 minutes of release, from both MH susceptible and control preparations (Fig. 5.10). Efflux of Ca\(^{2+}\) from LSR preparations was similar to that of HSR vesicles from both MH susceptible and control muscle (Fig. 5.10). The amount of Ca\(^{2+}\) released at 6 minutes was similar in all fractions (Fig. 5.10). No significant difference in the amount of Ca\(^{2+}\) released over 6 minutes was observed when similar fractions from MH susceptible and control muscle were compared.

5.3.9.3 The Effect of Various Agents on Equilibrium Calcium Exchange from Heavy Sarcoplasmic Reticulum Vesicles

The effects of dantrolene, diltiazem and verapamil were investigated on the rate of Ca\(^{2+}\) exchange from the terminal cisternae membrane of MH susceptible and control muscle. Calcium exchange was monitored at 1, 2, 3, 6 and 12 minute intervals. Drug-induced changes in the exchange rate were usually observed at the end of 1 minute intervals (Table 5.7). At later sampling intervals the exchange rate was slower (Fig. 5.9) and the effects of drugs were not apparent. Verapamil 100uM and 80uM diltiazem did not affect the amount of Ca\(^{2+}\) exchanged by HSR membrane vesicles from either control or MH susceptible preparations (Table 5.7). Diltiazem (640uM) at a concentration that reversed drug-induced contractures
The efflux of Ca$^{2+}$ from HSR (circular symbols), LSR (square symbols) and FSR (diamond symbols) membrane vesicles derived from control (A) and MH susceptible (B) pig muscle. Efflux was initiated when vesicles were diluted into a Ca$^{2+}$-free medium (see Materials and Methods section). Results are expressed as mean or mean +/- s.e.m. The number of observations ranged from 5 - 8.

+ Efflux from control FSR was significantly less than from control HSR $p < 0.01$

++ Efflux from MH susceptible FSR was significantly less than from MH susceptible HSR $p < 0.05$
CALCIUM EXCHANGE

A. CONTROL

B. MHS

EXCHANGE (% calcium retained)

CALCIUM

TIME (min)

1 2 3

0 50 100

0 50 100

+ + +

+ + +

+ + +

0 1 2 3 6 12

0 50 100
### TABLE 5.7 The Effect of Various Agents on Equilibrium Ca^{2+} Exchange from Heavy Sarcoplasmic Reticulum Vesicles from Control and Malignant Hyperpyrexia Susceptible Porcine Skeletal Muscle

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>60.55 +/- 2.37</td>
<td>59.03 +/- 5.99</td>
</tr>
<tr>
<td>dantrolene (20μM)</td>
<td>74.31 +/- 1.57 ***</td>
<td>71.65 +/- 1.83 +</td>
</tr>
<tr>
<td>diltiazem (80μM)</td>
<td>65.41 +/- 3.75</td>
<td>64.71 +/- 1.33</td>
</tr>
<tr>
<td>diltiazem (640μM)</td>
<td>63.21 +/- 1.86</td>
<td>64.70 +/- 2.01</td>
</tr>
<tr>
<td>verapamil (100μM)</td>
<td>60.05 +/- 1.57</td>
<td>67.78 +/- 3.23</td>
</tr>
<tr>
<td>caffeine (10mM)</td>
<td>63.98 +/- 1.95</td>
<td>62.40 +/- 1.83</td>
</tr>
<tr>
<td>caffeine (32mM)</td>
<td>40.27 +/- 3.25 ***</td>
<td>39.23 +/- 1.72 ***</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m.

The number of observations was 5 for each agent.

+ significantly greater than untreated MHS value p < 0.05
+++ significantly greater than untreated value p < 0.001
in isolated muscle fibres also failed to affect \( \text{Ca}^{2+} \) exchange. However, 20uM dantrolene significantly
decreased the amount of \( \text{Ca}^{2+} \) exchanged across the HSR
membrane at 1 and 2 minutes in control and at 1 minute in
MH susceptible preparations (Table 5.7). Caffeine at 10mM
failed to affect the amount of \( \text{Ca}^{2+} \) exchanged, but
significantly increased exchange at 32mM (Table 5.7).

5.3.9.4 The Effect of Various Agents on Calcium
Efflux from the Heavy Sarcoplasmic Reticulum
Membrane

\( \text{Ca}^{2+} \) efflux was monitored over a period of 12 minutes
at the same intervals described in the previous section.

Drug induced changes were only observed at the 1
and 2 minute sampling intervals. Only results at the 1
minute period are presented (Table 5.8).

Dantrolene (20uM) significantly decreased the
amount of \( \text{Ca}^{2+} \) released from HSR vesicles at 1 minute in
control and 1 and 2 minutes in MH susceptible preparations.
Diltiazem (80 and 640uM), 100uM verapamil and 10mM caffeine
failed to affect \( \text{Ca}^{2+} \) efflux (Table 5.8). However, a
higher concentration of caffeine (32mM) significantly
increased the amount of \( \text{Ca}^{2+} \) released from vesicles in both
MH susceptible and control preparations (Table 5.8).
<table>
<thead>
<tr>
<th></th>
<th>% $Ca^{2+}$ retained at 1 minute</th>
<th>Control</th>
<th>MHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>60.88 +/- 2.04</td>
<td>60.56 +/- 1.75</td>
<td></td>
</tr>
<tr>
<td>Dantrolene (20uM)</td>
<td>74.70 +/- 4.55 +</td>
<td>78.66 +/- 3.44 ++</td>
<td></td>
</tr>
<tr>
<td>Diltiazem (80uM)</td>
<td>64.74 +/- 5.02</td>
<td>62.78 +/- 2.11</td>
<td></td>
</tr>
<tr>
<td>Diltiazem (640uM)</td>
<td>63.91 +/- 3.20</td>
<td>64.20 +/- 1.72</td>
<td></td>
</tr>
<tr>
<td>Verapamil (100uM)</td>
<td>61.30 +/- 3.00</td>
<td>62.17 +/- 6.08</td>
<td></td>
</tr>
<tr>
<td>Caffeine (10mM)</td>
<td>58.13 +/- 3.32</td>
<td>61.47 +/- 3.44</td>
<td></td>
</tr>
<tr>
<td>Caffeine (32mM)</td>
<td>42.60 +/- 1.83 +++</td>
<td>43.27 +/- 1.85 +++</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m.

The number of observations was 5 for each drug

+ significantly greater than untreated control $p < 0.05$

++ significantly greater than untreated MHS $p < 0.01$

+++ significantly greater than untreated $p < 0.001$
5.4 DISCUSSION

5.4.1. Characterisation of Sarcoplasmic Reticulum Fractions

The SR membrane from control and MH susceptible skeletal muscle was separated by sucrose gradient centrifugation into two vesicular fractions enriched in the membrane elements of the terminal cisternae and longitudinal tubules respectively (Fig. 5.1). These membrane preparations were highly purified, containing little contaminating enzymic activity. HSR and LSR preparations had consistently less mitochondrial, lysosomal, plasma and T-tubule membrane contamination in comparison with crude FSR preparations (Table 5.1). Furthermore, while the activity of Ca\(^{2+}\)-dependent ATPase was high in all membrane fractions, the LSR and HSR preparations had consistently higher specific activities than FSR preparations (Table 5.1). Electron micrographs showed that HSR and LSR vesicles were more homogeneous in shape and size than FSR preparations while LSR vesicles were larger in volume than HSR vesicles. (Fig.5.2). Both fractions consisted of essentially closed membranous vesicles. Electron micrographs did not reveal any mitochondrial contamination, suggesting that the low levels of activity detected by chemical analysis probably originated from submitochondrial vesicles.
The two purified SR fractions had distinctive features which were associated with their origins in the SR membranous network. Calsequestrin is primarily localized in the terminal cisternae of intact muscle cells (Caswell et al., 1976). Electron micrographs of HSR vesicles showed more electron dense material than LSR preparations (Fig. 5.2). This visible content is believed to represent calsequestrin with a large amount of bound calcium (Ikemoto, Nagy, Bhatnagar and Sergely, 1974; Ostwald, MacLennan and Dorrington, 1974; Meissner, 1975). The calcium content of the membrane preparations also indicated that the fractions were from different regions of the SR. HSR vesicles contained significantly more Ca$^{2+}$ than LSR preparations (Section 5.3.4). These results are consistent with those of other investigators (McIntosh et al., 1977; Campbell et al., 1980) and support electron probe studies which indicate that Ca$^{2+}$ is primarily stored in the terminal cisternae of the SR (Somlyo et al., 1977).

The freeze fracture replicas of HSR and LSR vesicles showed basically the same asymmetric patterns indicating that vesicles are right side out, having the same orientation as the SR membrane in vivo (Campbell et al., 1980) (Fig. 5.3). Particles on the cytoplasmic leaflet of vesicles are believed to represent the Ca$^{2+}$-pump protein (MacLennan et al., 1971). The morphological data in this study were similar to those which have been
described by other investigators, which were considered to indicate that light vesicles are derived from the longitudinal reticulum and heavy vesicles from the terminal cisternae of the SR (Meissner, 1975; Caswell et al., 1976; Caswell, Lau, Garcia and Brunschwig, 1979; Louis et al., 1980; Campbell et al., 1980; Mitchell et al., 1983).

Analysis of the membrane preparations by SDS-PAGE indicated that the protein composition of both HSR and LSR fractions was complex (Fig. 5.4). The major protein component of each preparation was the Ca$^{2+}$-transport ATPase, with the apparent MW of 100,000. This result is consistent with a number of studies (Martonosi, 1969; MacLennan, 1971; Campbell et al., 1980; White et al., 1983b) that the Ca$^{2+}$-ATPase is uniformly distributed throughout the longitudinal and much of the terminal cisternae membranes, and is only absent from those regions of the SR membrane which directly form the triad with the T-tubule membrane (Martonosi, 1984). The major difference in the membrane elements of the LSR and HSR preparations was the content of calsequestrin (66,000 MW protein). Calsequestrin was enriched in the HSR fraction, in agreement with electron micrographs and the suggestion that this membrane fraction originates from the terminal cisternae (Meissner, 1975; Lau et al., 1977). In this study LSR fractions were characterised by a higher Ca$^{2+}$-ATPase/calsequestrin ratio than HSR fractions.
In addition to these major proteins, the SR fractions contained a large number of other proteins which represent elements of the SR membrane which are yet to be defined. The phospholipid/protein ratios of SR fractions did not vary (Table 5.2). This result is different from that obtained by McIntosh et al. (1977) for pig muscle, and may reflect differences in the method of preparation and muscle selected for isolation of the SR preparations.

Further evidence that HSR and LSR membranes were from two functionally distinct regions of the SR comes from studies on Ca\(^{2+}\) fluxes across the membrane preparations. While the initial rate of Ca\(^{2+}\) uptake (in the presence of oxalate) did not appear to vary between fractions (which would not be expected as both fractions have the same Ca\(^{2+}\)-dependent ATPase activities (Fig. 5.7)), the HSR membrane had a greater total capacity to store Ca\(^{2+}\) (Fig. 5.8). This may reflect the binding of Ca\(^{2+}\) to calsequestrin which is more concentrated in HSR vesicles. Ca\(^{2+}\) exchange was more rapid from HSR than LSR preparations (Fig. 5.9), while Ca\(^{2+}\) efflux was similar (Fig. 5.10). Passive Ca\(^{2+}\) release has been shown to be more rapid from HSR vesicles by Campbell et al. (1980). This increased release of Ca\(^{2+}\) is believed to be associated with the unique area of HSR membrane which is in contact with the feet of the triadic junction and is the primary Ca\(^{2+}\) release site. The fact that initial Ca\(^{2+}\) release was
rapid with approximately 50% of internal $^{45}\text{Ca}^{2+}$ exchanged within 2 minutes supports this view (Campbell et al., 1980). Greater contamination by membranes originating from the mitochondria, sarcolemma and lysosomes may contribute to the diminished Ca$^{2+}$-transport activity of the FSR preparations (Fig. 5.8, 5.9 and 5.10).

5.4.2. Comparisons Between MH Susceptible and Control SR Preparations.

After the extensive characterisation of the SR membrane, comparisons were made between the same membrane fractions derived from MH susceptible and control muscle. Characteristics of membrane fractions derived from MH susceptible muscle did not differ from those of control preparations. Morphological studies and SDS-PAGE (Fig. 5.4) showed the same membrane elements in corresponding fractions. Ca$^{2+}$ fluxes associated with HSR vesicles (Fig. 5.10) did not vary nor did Ca$^{2+}$ uptake by the longitudinal tubules (Fig. 5.9). Ca$^{2+}$ ATPase activities were also similar in all membrane fractions (Fig. 5.6 and 5.7). SR fractions also had similar Ca$^{2+}$ content (Section 5.3.4) and phospholipid to protein ratios (Fig. 5.2). In this study no distinction could be made between the membrane elements or Ca$^{2+}$ fluxes associated with these functionally distinct regions of MH susceptible and control SR membranes.
5.4.3. The Effect of Various Agents on Ca\(^{2+}\) Fluxes and Ca\(^{2+}\) ATPase Activity.

Diltiazem has been shown to abolish the hypercontractility of MH susceptible muscle (Chapters 3 and 4). However, between 80 and 640 uM diltiazem failed to affect contractile responses to caffeine in detubulated muscle fibres (Chapter 3), suggesting a T-tubule site of action. Results in this study also support the view that diltiazem acts to modify contractile responses by inhibiting Ca\(^{2+}\)-fluxes associated with T-tubular membranes, rather than acting directly on the SR. Ca\(^{2+}\) release and uptake by the HSR and LSR membrane preparations were not affected by diltiazem (Table 5.6). Diltiazem also failed to affect Ca\(^{2+}\)-ATPase activities of HSR and LSR preparations (Table 5.3). Verapamil (100uM) also failed to modify Ca\(^{2+}\)-uptake, Ca\(^{2+}\)-release or Ca\(^{2+}\)-ATPase activities (Tables 5.3 and 5.6).

It is not surprising that the CCA failed to affect the accumulation of Ca\(^{2+}\) by LSR vesicles. Ca\(^{2+}\)-ATPase is not considered to function as a specific Ca\(^{2+}\) channel and CCA have been shown to bind preferentially to T-tubular membranes in comparison to isolated SR membrane fractions (Fosset et al., 1983). Ca\(^{2+}\)-ATPase functioning as a Ca\(^{2+}\) channel during Ca\(^{2+}\) release has been considered (Jilka et al., 1975; Endo, 1977) but no firm conclusions have been drawn. It is probable that Ca\(^{2+}\) ATPase is distinct
from the gated Ca\(^{2+}\) channels that are primarily involved in Ca\(^{2+}\) release from the SR (Morii and Tonomura, 1983). While passive Ca\(^{2+}\) release may occur through nonspecific hydrophilic channels formed by Ca\(^{2+}\)-ATPase across HSR membrane preparations, it is speculated that a component of this efflux is derived from the area of the membrane which is associated with Ca\(^{2+}\) release in intact muscle cells (Campbell et al., 1980). The greater efflux of Ca\(^{2+}\) from HSR preparations (Fig.5.9) and the increased sensitivity of this efflux to dantrolene in comparison to LSR preparations (Campbell et al., 1980) supports this view. The fact that diltiazem failed to affect Ca\(^{2+}\)-release across the terminal cisternae membrane (Tables 5.7, 5.8) further suggests a site of action distant from the SR.

Dantrolene modified contractile responses in detubulated muscle fibres (Chapter 3) and decreased Ca\(^{2+}\) exchange and efflux across the terminal cisternae membrane (Tables 5.7, 5.8), suggesting that the muscle relaxant acts directly on the SR as well as other elements of the triadic junction. Dantrolene in similar concentrations (20uM) failed to affect Ca\(^{2+}\)-ATPase activities and the accumulation of Ca\(^{2+}\) by the LSR vesicles. Similar results were obtained by White and Denborough (1984) with FSR preparations from the pig and are consistent with the observation that dantrolene does not affect the sequestration of Ca\(^{2+}\) during relaxation (Desmedt and
Hainaut, 1977). High affinity binding sites exist in skeletal muscle SR for dantrolene that are approximately 1000 times less than the concentration of Ca\textsuperscript{2+}-ATPase (Sengupta, Meyer and Carafoli, 1980). It is unlikely that dantrolene would bind to Ca\textsuperscript{2+}-transport ATPase with high affinity (Martonosi, 1984) and these sites probably represent gated Ca\textsuperscript{2+} channels at the junctional region of the SR (Martonosi, 1984).

High concentrations of caffeine (32mM) increased the amount of Ca\textsuperscript{2+} efflux and exchange across the terminal cisternae membrane (Tables 5.7, 5.8). Caffeine has been shown to potentiate Ca\textsuperscript{2+} release by isolated SR preparations, but under different experimental conditions. Caffeine increases the sensitivity of the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism to Ca\textsuperscript{2+} (Endo 1975, 1977; Morii and Tonomura, 1983) and causes rapid efflux of Ca\textsuperscript{2+} (10-40\%) from HSR vesicles after loading in the presence of 0.1mM ATP and 1mM Mg\textsuperscript{2+} (Morii and Tonomura, 1983; Nagasaki and Kasai, 1983). Results in this study with 32mM caffeine support the view that caffeine may also increase passive Ca\textsuperscript{2+} permeability of the SR membrane which leads to a rapid efflux of Ca\textsuperscript{2+} (Wood, 1978).

Caffeine 10mM failed to affect the amount of Ca\textsuperscript{2+} taken up by LSR membrane preparations (Table 5.6). Caffeine concentrations of 10mM or less also failed to affect the amount of Ca\textsuperscript{2+} taken up by isolated SR
preparations from rabbit (Weber, 1968; Weber and Herz, 1968) and pig (Collins 1984). These results support the suggestion that caffeine acts selectively at the junctional region of the SR (Martonosi, 1984).

5.4 SUMMARY

The SR membrane from control and MH susceptible muscle was separated into two vesicular fractions enriched in the membrane elements of the terminal cisternae and longitudinal tubules. The two membrane preparations were highly purified and had distinctive features which were associated with their origins in the SR membranous network. Calsequestrin and calcium enriched in the HSR fraction in comparison to LSR preparations. The HSR membrane also had a greater total capacity to store Ca$^{2+}$ and Ca$^{2+}$ release was more rapid than from LSR preparations. No distinction could be made between the membrane elements or Ca$^{2+}$ fluxes associated with these functionally distinct regions of MH susceptible and control preparations.

Diltiazem and verapamil failed to affect Ca$^{2+}$-uptake, Ca$^{2+}$-release or Ca$^{2+}$-dependent ATPase activity of the respective regions of the SR membrane. These results support the view that diltiazem and verapamil act at a site distant to the SR to modify contractile responses, probably at the level of the T-tubule membrane. Dantrolene failed to affect Ca$^{2+}$-ATPase activities and Ca$^{2+}$-uptake
by LSR vesicles. However, dantrolene decreased the rate of Ca^{2+} efflux from HSR preparations suggesting that the muscle relaxant acts directly on the SR as well as other elements of the triadic junction. Caffeine failed to modify Ca^{2+}-uptake by LSR membranes but increased Ca^{2+}-efflux and exchange across the terminal cisternae membrane suggesting that caffeine acts selectively at the junctional region of the SR to release Ca^{2+}.
6.1 INTRODUCTION

Porcine MH can be induced by succinylcholine and/or halothane (Hall et al., 1966; Harrison et al., 1968; Berman et al., 1970; Gronert and Theye, 1976 a, b; Gronert, Milde and Theye, 1976). In the presence of a precipitating agent the clinical features of the porcine MH syndrome are similar to those described in man (Chapter 1). Essentially, MH in swine is characterised by a rapid and sustained rise in core body temperature and gross muscle rigidity. Biochemical changes such as metabolic acidosis and increased blood CO$_2$ levels precede the development of these features (Berman et al., 1970). Tachycardia, decreased blood O$_2$ levels and a falling blood pressure are also observed. Elevations in serum electrolytes and CPK levels are detected during and after the syndrome (Berman et al., 1970; Denborough, 1980).

Elevated levels of Ca$^{2+}$ in the myoplasm of skeletal muscle is considered to be the major factor in the development of a MH episode (Denborough, 1980; Gronert, 1980). Dantrolene sodium, the drug of choice for the treatment of MH, acts to lower myoplasmic Ca$^{2+}$ levels.
by suppressing EC coupling. One of the rationales for the original use of dantrolene in the treatment of MH was based on the demonstration that the skeletal muscle relaxant could prevent and reverse the contracture responses to the diagnostic agents in isolated muscle fibres (Austin and Denborough, 1977, Chapters 3 and 4).

The CCA diltiazem has also been shown to inhibit and reverse the drug-induced hypercontractility of isolated MH susceptible human and porcine muscle preparations (Chapters 3 and 4) (Iwatsuki et al., 1983; Ilias et al., 1985). The therapeutic value of diltiazem in the treatment of MH would depend on its site of action. Diltiazem does not affect Ca$^{2+}$ release or Ca$^{2+}$ uptake by isolated porcine skeletal muscle SR membrane preparations (Chapter 5). It would appear that diltiazem acts to inhibit Ca$^{2+}$ fluxes associated with the transverse tubular membrane (Chapter 3).

This chapter describes experiments designed to investigate the effectiveness of diltiazem in inhibiting and reversing the porcine MH syndrome.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Protocol

Six MH susceptible and four control pigs with a
body weight ranging from 25 - 47 kg were selected from mixed litters. Pigs were of either sex and undiagnosed as MH susceptible or control. All swine were premedicated and anaesthesia induced as previously described (Section 3.3.2). Muscle tissue for the diagnosis of susceptibility was then removed and anaesthesia maintained with 66% nitrous oxide in oxygen. Physiological parameters (Section 6.2.3) were then recorded every minute for approximately 15 minutes and blood (10mls) taken for biochemical analysis (Section 6.2.4). Succinylcholine and halothane were then administered in the presence (Inhibition experiment) or absence (Reversal experiment) of diltiazem.

6.2.1.1 Inhibition Experiments

Increasing doses of diltiazem were administered intravenously (as described in Section 6.2.5) to a total dose of 2, 5 or 10 mg/kg (in 1, 1 and 4 pigs respectively). After physiological parameters had stabilized, succinylcholine was administered intravenously (ear vein) in 4 doses (to reduce fasciculations) to a total dose of 2mg / kg. If required, pigs were manually ventilated at a constant rate until respiratory function returned. After the administration of the muscle relaxant, nitrous oxide in oxygen was discontinued and 1% halothane anaesthesia initiated. Halothane anaesthesia was maintained for approximately 90 minutes. Physiological parameters
were monitored every minute for the duration of the challenge. Blood for biochemical analyses was then again taken and anaesthesia terminated.

6.2.1.2 Reversal Experiments

MH susceptible pigs were allowed to recover for no less than two weeks before again being challenged in the absence of diltiazem. After vital signs had stabilized under nitrous oxide / oxygen anaesthesia, succinylcholine was again administered in 4 doses to a total concentration of 2mg/kg and 1% halothane administered. After the initiation of a MH syndrome (Section 6.2.2), halothane was discontinued and increasing doses of diltiazem were administered intravenously (as described in Section 6.2.5) to a total concentration of 7 to 10mg/kg in 5 pigs and 2mg/kg in one other. Sodium bicarbonate was also administered (I.V.) and anaesthesia was maintained with 66% nitrous oxide in oxygen. Blood samples for biochemical analyses were taken (1) immediately before the administration of succinylcholine and halothane, (2) when the syndrome was established and (3) after administration of diltiazem. Vital signs were monitored every minute throughout the challenge.

The same protocol was followed with control pigs, with blood being taken for biochemical analysis under nitrous oxide / oxygen anaesthesia and at two periods
during halothane anaesthesia. Halothane anaesthesia was maintained for 90 minutes.

6.2.2 Criteria for Establishing the Initiation of a MH Episode

In this study a MH episode was defined to be in progress when (1) body temperature increased by at least 1°C, (2) a clinical score of at least 3 was obtained for extensor rigidity, and (3) tachycardia and a falling blood pressure were observed. Often abnormal ECG patterns were also observed. While metabolic changes such as increased levels of blood CO$_2$ and lactate precede the development of muscular rigidity and hyperpyrexia during a MH crisis, it was not possible to monitor continuously these parameters throughout the duration of the challenge. The syndrome was therefore primarily characterised by muscular rigidity and elevated body temperature. The development of these later features of MH was considered enough to render any observed response to diltiazem unequivocal.

An increase in rectal temperature of 1°C was also considered sufficient to indicate the expression of an MH reaction as elevated body temperature is not only a late feature of an MH episode but also occurs against a tendency of hypothermia under halothane anaesthesia. Furthermore, death has occurred at rectal temperatures as low as 40°C
in MH pigs (Hall, Lucke and Lister, 1975).

In two pigs which had been used for inhibition and reversal experiments, MH was again induced under identical conditions. Halothane anaesthesia was discontinued and sodium bicarbonate administered at stages in the reaction which were similar to those of previous reversal experiments. These experiments were conducted with a view to establishing that withdrawal of the triggering agent would not prevent the development of the hyperpyrexic response.

6.2.3 Physiological Measurements

Blood pressure was monitored via an arterial line which was placed in the artery supplying the hind limb vasculature (Fig.6.1.A) which was connected to a pressure transducer (Fig.6.1.B) (Bently Physiological Transducer) and coupled to a cardiac monitor (Telectronics HSG) (Fig.6.1.C). Patency of the catheter was maintained by a constant infusion of (approximately 10ml/hour) heparinized saline solution (1000 units/l). Electrodes were also placed for the monitoring of heart rate and E.C.G. Rectal temperature was measured using a thermometer (Fig.6.1.C). Muscular rigidity of the front extensor was estimated and given a clinical score ( 0, flaccid extensor; 1, initiation of rigidity; 2, mild rigidity ; 3, pronounced rigidity of extensor ; 4, severe rigidity, difficulty in bending
FIGURE 6.1 Equipment Used to Measure Physiological Parameters.

Blood pressure was monitored via an arterial line which was placed in the artery supplying the hind limb vasculature (A). The line was connected to a pressure transducer (B) and coupled to a cardiac monitor (C). Rectal temperature was measured using a thermometer (C).
extensor; 5, rigor, unable to bend extensor). All physiological parameters were recorded at intervals of 1 minute throughout the challenge. An overview of the experimental equipment is shown in Figure 6.2.

6.2.4 Biochemical Measurements

Arterial blood samples were taken and analysed for pH, PaCO₂, PaO₂, potassium, inorganic phosphate, calcium, lactate and CPK. Blood gas analysis was conducted within 30 minutes of sampling. Estimations of plasma electrolytes and CPK were obtained within 24 hours. All biochemical measurements were conducted in the Department of Clinical Biochemistry at Royal Canberra Hospital.

6.2.5 Administration of Diltiazem

Diltiazem (Marion Laboratories) was dissolved in sterile water (ABBOT Laboratories) to make a final concentration of 1mg/ml. Diltiazem was administered intravenously through a catheter placed in the ear. The rate of administration varied but did not exceed 1mg/kg/minute. This prevented dramatic and sustained decreases in blood pressure. In between the administration of diltiazem, the catheter was kept patent by regular flushing with heparinized saline.
FIGURE 6.2 An Overview of the Experimental Equipment.

Equipment used for the delivery of anaesthesia is shown (A) as well as equipment used for physiological recording (B).
6.3 RESULTS

6.3.1 Reversal Experiments

Exposure of MH susceptible pigs to 2 mg/kg succinylcholine and 1% halothane in the absence of diltiazem resulted in the development of the MH syndrome (Table 6.1.B and 6.2.B). Pigs developed pronounced muscular rigidity (clinical score of 3 to 4) followed by an elevation in rectal temperature. Heart rate doubled and blood analysis showed an increase in PaCO₂ levels by 17.3 mmHg and a subsequent decrease in PaO₂. Plasma lactate concentrations increased from 3.8 to 6.9 mmol/l while blood pH and base excess (BE) decreased by 0.16 units and 4.4 mEq/l respectively, consistent with metabolic acidosis (Table 6.2.B). Plasma potassium and inorganic phosphate levels were elevated during and after the challenge, while calcium levels remained virtually unchanged. CPK levels also increased approximately 2 fold (Table 6.2.B). In 3 of the six pigs challenged blotchy cyanosis of the skin was also observed.

Once the clinical features of MH had been identified with muscular rigidity and an increase in body temperature of at least 1°C, 2 to 10mg/kg of diltiazem was administered intravenously. Diltiazem caused a rapid decrease in both systolic and diastolic blood pressure. However administration of small doses with careful
TABLE 6.1 The Effect of Diltiazem in Malignant Hyperpyrexia Pigs in the Presence of Succinylcholine and Halothane: Physiological Parameters.

<table>
<thead>
<tr>
<th>Anaesthetic Conditions</th>
<th>Temperature °C</th>
<th>Rigidity (Clinical Score)</th>
<th>Heart Rate beats/min</th>
<th>Blood Pressure SBP/DBP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Inhibition of MH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. NO₂/O₂</td>
<td>36.0</td>
<td>0</td>
<td>80.3</td>
<td>99/58 +/− 2.8/2.3</td>
</tr>
<tr>
<td>2. DILT (2-10mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35.0</td>
<td>0</td>
<td>97</td>
<td>93/48 +/− 2.9/2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Reversal of MH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. NO₂/O₂</td>
<td>37.2</td>
<td>0</td>
<td>77 +/− 1.94</td>
<td>105/54 +/− 2.6/2.5</td>
</tr>
<tr>
<td>2. SUCC (2mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>38.3</td>
<td>3-4</td>
<td>163 +/− 21.52</td>
<td>74/42 +/− 5.9/3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. After the adminstration of DILT (2-10mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.2</td>
<td>0</td>
<td>114 +/− 11.97</td>
<td>86/46 +/− 3.4/2.2</td>
</tr>
</tbody>
</table>

Results are expressed as mean values obtained from 6 MH susceptible swine. Swine were premedicated with diltiazem before the administration of succinylcholine and halothane (A). After a recovery period of at least two weeks MH episodes were precipitated in these swine by succinylcholine and halothane and the effect of diltiazem observed (B).
TABLE 6.2 The Effect of Diltiazem in Malignant Hyperpyrexia
Pigs in the Presence of Succinylcholine and Halothane:

Biochemical Parameters.

<table>
<thead>
<tr>
<th>Anaesthetic Conditions</th>
<th>CPK (units/1)</th>
<th>pH</th>
<th>PaCO₂ mmHg</th>
<th>PaO₂ mmHg</th>
<th>BE mEq/1</th>
<th>lactate mmol/1</th>
<th>K mmol/1</th>
<th>PO₄ mmol/1</th>
<th>Ca mmol/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Inhibition of MH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. NO₂/O₂ +/-</td>
<td>1410</td>
<td>7.41</td>
<td>43.4</td>
<td>305.6</td>
<td>3.12</td>
<td>2.8</td>
<td>3.1</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.01</td>
<td>2.4</td>
<td>21.5</td>
<td>0.34</td>
<td>0.51</td>
<td>0.21</td>
<td>0.51</td>
<td>0.25</td>
</tr>
<tr>
<td>2. DILT (2-10mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>followed by 2mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUCC and 1% HAL</td>
<td>2048</td>
<td>7.36</td>
<td>45.2</td>
<td>251.5</td>
<td>1.9</td>
<td>1.9</td>
<td>3.7</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>+/-</td>
<td>287</td>
<td>0.03</td>
<td>2.6</td>
<td>30.2</td>
<td>0.44</td>
<td>0.63</td>
<td>0.31</td>
<td>0.21</td>
<td>0.32</td>
</tr>
<tr>
<td>B. Reversal of MH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. NO₂/O₂ +/-</td>
<td>1670</td>
<td>7.42</td>
<td>46.1</td>
<td>342.1</td>
<td>5.03</td>
<td>3.8</td>
<td>3.3</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>0.01</td>
<td>2.39</td>
<td>26.56</td>
<td>1.24</td>
<td>0.51</td>
<td>0.12</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>2. SUCC (2mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and 1% HAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(precipitation of MH)</td>
<td>3515</td>
<td>7.26</td>
<td>63.4</td>
<td>213.9</td>
<td>0.62</td>
<td>6.9</td>
<td>4.5</td>
<td>3.0</td>
<td>2.3</td>
</tr>
<tr>
<td>+/-</td>
<td>186</td>
<td>0.01</td>
<td>2.9</td>
<td>18.42</td>
<td>0.59</td>
<td>0.41</td>
<td>0.55</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>3. After the</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>administration of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DILT (2-10mg/kg) HAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>off NO₂/O₂ on</td>
<td>3782</td>
<td>7.44</td>
<td>40.5</td>
<td>255.4</td>
<td>6.3</td>
<td>-</td>
<td>4.8</td>
<td>3.1</td>
<td>2.3</td>
</tr>
<tr>
<td>i.v.NaHCO₃ +/-</td>
<td>577</td>
<td>0.02</td>
<td>3.26</td>
<td>40.09</td>
<td>0.46</td>
<td>0.46</td>
<td>0.235</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e.m values obtained from 6 MH susceptible swine. Swine were premedicated with diltiazem before the administration of succinylcholine and halothane (A). After a recovery period of at least two weeks MH episodes were precipitated in these swine by succinylcholine and halothane and the effect of diltiazem observed (B).
monitoring of blood pressure prevented sustained hypotension. Blood pressure recovered to pre-administration levels within 5 to 10 minutes, (allowing further administration if required). The administration of diltiazem caused a rapid loss of extensor rigidity followed by a progressive decrease in rectal temperature. Heart rate fell, while blood pressure in the presence of diltiazem remained low. Blood pH, B.E., PaCO₂ and PaO₂ (255.4 mmHg) returned to values approaching prechallenge levels (Table 6.1.B and 6.2.B). A total concentration of 7 to 10 mg/kg of diltiazem was required to reverse the MH syndrome in 5 pigs and 2mg/kg in one other. The only ancillary treatment pigs received was the administration of sodium bicarbonate to all 6 pigs, and active cooling of one pig after body temperature had increased above 40°C. All pigs survived.

6.3.2 Inhibition Experiments

Pretreatment of MH susceptible pigs with diltiazem effectively blocked the initiation of the MH syndrome by 2 mg/kg succinylcholine and 1% halothane (Table 6.1.A and 6.2.A) over a 90 minute period. In the absence of diltiazem all reactor pigs developed the syndrome within 15 to 30 minutes. In the presence of diltiazem, extensor muscles remained flaccid and body temperature decreased by approximately 1°C. Blood pressure dropped due to the administration of diltiazem but heart rate did not exceed
100 beats/minute. Blood pH and B.E values dropped slightly, but not to the same degree as observed during a MH episode. Similar observations were made during anaesthesia in control swine (Table 6.3). Furthermore, plasma lactate concentration did not increase, and blood gas analysis indicated that PaCO₂ and PaO₂ did not markedly change in diltiazem pretreated pigs. Plasma concentrations of calcium, potassium and inorganic phosphate also remained essentially unchanged. CPK levels did increase in pretreated animals during anaesthesia by 638 units/l (Table 6.1.A). However, this increase was substantially less than in untreated pigs (2112 units/l) (Table 6.1.B) and was probably due to the muscular fasciculations induced by succinylcholine.

In two MH susceptible swine which had been previously used for inhibition and reversal experiments, MH was again induced under identical conditions. Halothane anaesthesia was discontinued and sodium bicarbonate administered at stages in the reaction which were similar to previous reversal experiments. Both pigs developed fulminant MH and died. This suggests that metabolism had been stimulated sufficiently and withdrawal of the triggering agent did not prevent the development of the hyperpyrexic response.
### TABLE 6.3 The Effect of Succinycholine and Halothane on the Physiological and Biochemical Parameters of Control Swine

<table>
<thead>
<tr>
<th>Anaesthetic conditions</th>
<th>NO\textsubscript{2}/O\textsubscript{2}</th>
<th>SUCC + HAL (A)</th>
<th>SUCC + HAL (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temp (°C)</strong></td>
<td>36.3</td>
<td>35.7</td>
<td>35.6</td>
</tr>
<tr>
<td><strong>Rigidity</strong> (clinical score)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>CPK (units/l)</strong></td>
<td>953.5 +/- 64.76</td>
<td>1059 +/- 20</td>
<td>1119 +/- 27</td>
</tr>
<tr>
<td><strong>Heart rate</strong> (beats/min)</td>
<td>86 +/- 3.17</td>
<td>90 +/- 4.4</td>
<td>95 +/- 6.25</td>
</tr>
<tr>
<td><strong>Blood Pressure</strong> (SBP/DBP) (mmHg)</td>
<td>112/65 +/- 5.7</td>
<td>105/65 +/- 5.2</td>
<td>103/61 +/- 4.7</td>
</tr>
<tr>
<td><strong>pH (units)</strong></td>
<td>7.37 +/- 0.03</td>
<td>7.4 +/- 0.03</td>
<td>7.35 +/- 0.02</td>
</tr>
<tr>
<td><strong>PaCO\textsubscript{2}</strong> (mmHg)</td>
<td>48.4 +/- 4.1</td>
<td>47.1 +/- 4.6</td>
<td>53.0 +/- 3.0</td>
</tr>
<tr>
<td><strong>PaO\textsubscript{2}</strong> (mmHg)</td>
<td>298.5 +/- 58</td>
<td>416.0 +/- 43</td>
<td>417.0 +/- 56</td>
</tr>
<tr>
<td><strong>BE(mEq/l)</strong></td>
<td>2.02 +/- 0.42</td>
<td>3.38 +/- 0.83</td>
<td>2.84 +/- 0.85</td>
</tr>
<tr>
<td><strong>lactate</strong> (mmol/l)</td>
<td>3.50 +/- 0.2</td>
<td>2.67 +/- 0.21</td>
<td>2.84 +/- 0.3</td>
</tr>
<tr>
<td><strong>K (mmol/l)</strong></td>
<td>3.1 +/- 0.11</td>
<td>3.6 +/- 0.24</td>
<td>3.4 +/- 0.12</td>
</tr>
<tr>
<td><strong>PO\textsubscript{4}</strong> (mmol/l)</td>
<td>2.0 +/- 0.19</td>
<td>1.9 +/- 0.17</td>
<td>2.1 +/- 0.09</td>
</tr>
<tr>
<td><strong>Ca (mmol/l)</strong></td>
<td>2.3 +/- 0.02</td>
<td>2.1 +/- 0.08</td>
<td>2.1 +/- 0.05</td>
</tr>
</tbody>
</table>

Results are expressed as mean or mean +/- s.e.m values obtained from 4 control swine.

A. After 30 minutes of anaesthesia
B. After 90 minutes of anaesthesia
6.3.3 Succinylcholine and Halothane Anaesthesia in Control Pigs

Temperature decreased by approximately 1°C and extensors remained flaccid during 2 mg / kg succinylcholine and 1% halothane induced anaesthesia in control swine (Table 6.3). Physiological and biochemical features remained essentially constant.

6.4 DISCUSSION

The calcium channel antagonist diltiazem effectively inhibited and reversed the porcine MH syndrome. In the absence of diltiazem MH susceptible pigs developed the characteristic clinical features of MH. These included hyperpyrexia, muscular rigidity, metabolic acidosis, elevated plasma potassium, inorganic phosphate and CPK levels and increased arterial blood PaCO₂. Tachycardia, arrhythmia and hypotension were also observed.

The clinical features of the porcine MH syndrome result from an increase in the level of myoplasmic Ca²⁺, in response to succinylcholine and halothane administration (Denborah, 1979, 1980). An excess of Ca²⁺ in the myoplasm activates phosphorylase, stimulating glycolysis and the production of lactic acid (Heilmeyer et al., 1970). Metabolic acidosis results from the accelerated muscle metabolism. An increased level of myoplasmic Ca²⁺ also
serves to stimulate and maintain muscular contraction, observed as extensor rigidity (Denborough, 1980; Gronert, 1980). The continued synthesis and utilization of ATP by glycolysis, gluconeogenesis and muscle contraction during a MH crisis, results in the liberation of heat and body temperature rises.

Myocardial function was also altered during the MH episodes, as evidenced by tachycardia, arrhythmias and hypotension. The observed cardiac dysfunction may have been a result of the accelerated muscle metabolism and a subsequent increase in sympathetic nervous system activity, leading to elevated levels of circulating catecholamines (Gronert and Theye, 1976b, Gronert et al., 1976; Lucke, Hall and Lister, 1976). Catecholamines may also contribute to heat production by accelerating a number of metabolic processes and inducing vasoconstriction (Denborough, 1979). Blotchy cyanosis of the skin (observed during 3 MH episodes) may have been due to vasoconstriction, resulting in reduced circulation and regional hypoxia (Harrison, Saunders, Biebuyck, Hickman, Dent, Weaver and Terblanche, 1969).

Active MH also results in elevated plasma levels of potassium, inorganic phosphate and CPK (Lucke et al., 1976; Jones, Nelson, Anderson, Kerr and Burnap, 1972; Berman et al., 1970). These results indicate a shift of these substances from the intracellular to the extracellular
fluid, which may reflect an increase in the permeability of the sarcolemma (Lucke et al., 1976). Elevated inorganic phosphate may result from the hydrolysis of intracellular high energy phosphates (Berman et al., 1970; Nelson et al., 1972; Lucke et al., 1976). Increased CPK levels may be due in part to muscular fasciculations induced by succinylcholine. However, in the absence of diltiazem, MH pigs had consistently greater CPK levels than control or diltiazem pretreated MH swine. No change in the plasma Ca^{2+} concentration was observed after MH was induced in this study. While increased plasma Ca^{2+} concentrations after porcine MH episodes have been observed by some investigators (Berman et al., 1970; Lucke et al., 1976), others have found no increase (Jones et al., 1972).

Blood gas analysis in unpretreated swine was also consistent with the expression of the MH syndrome. In this study all pigs were continuously or intermittently ventilated at a constant rate. Alterations in blood gases occurred in the absence of any obvious respiratory cause (eg. underventilation) and reflected metabolic acidosis and increased CO_2 production. A rapid fall in B.E corresponding to the rise in PaCO_2 was also observed. Furthermore, despite relatively high arterial PaO_2 (> 200 mmHg) at all times, lactate production reflecting anaerobic glycolysis was always observed during halothane anaesthesia in the absence of diltiazem in MH pigs. By comparison, arterial blood PaCO_2, PaO_2, pH and B.E measurements
remained essentially constant during halothane anaesthesia in control and diltiazem pretreated MH pigs.

The effect of pretreating MH susceptible pigs with diltiazem was dramatic. Succinylcholine and halothane did not trigger the MH syndrome. Skeletal muscle metabolism was not accelerated, plasma lactate and arterial blood pH and blood gases remained essentially unchanged. Subsequently, extensors remained flaccid and cardiac dysfunction and increased plasma concentrations of inorganic phosphate and potassium were not observed. Also, rectal temperature over the duration of the challenge decreased. Plasma CPK levels did rise, but not to the same degree as observed in unpretreated MH susceptible pigs. This elevation probably reflected increased muscular activity, observed as fasciculation after the administration of succinylcholine, as CPK levels were also very slightly elevated in control swine after the administration of succinylcholine. The clinical features of halothane anaesthesia in pretreated MH susceptible pigs were essentially similar to those observed in control swine.

Furthermore, two MH susceptible pigs in which MH was initiated by halothane (but which did not receive diltiazem), developed fulminant MH and died. This suggests that metabolism had been stimulated sufficiently and withdrawal of halothane did not prevent
the development of the MH response. Similar observations have been made by other investigators who have shown that once metabolism has been stimulated sufficiently the MH syndrome can continue unabated with further increases in temperature in the absence of the triggering agent (Berman et al., 1970; Harrison, 1975; Lucke et al., 1976).

The experiments reported in this study indicate that diltiazem may be an effective therapeutic agent in the treatment of MH if administered early in the onset of a MH crisis. Like dantrolene sodium, diltiazem prevents the onset and further expression of an initiated MH syndrome, probably by lowering myoplasmic Ca\(^{2+}\).

Biochemical (Chapter 5) and pharmacological experiments (Chapter 3) indicate that diltiazem may modify Ca\(^{2+}\) fluxes at the level of the T-tubule membrane. Ca\(^{2+}\) entering the muscle via T-tubules may therefore be important in the aetiology of the MH syndrome. Diltiazem not only acts on skeletal muscle but also has cardiovascular effects. Although diltiazem induces hypotension this was not an adverse effect in this study during the MH episode. Diltiazem may also prevent arrhythmias and protect the heart from ischaemia (Antman et al., 1980; Naylor and Horowitz, 1983). The release of catecholamines from the adrenal medulla is a Ca\(^{2+}\)-dependent process (Douglas and Rubin, 1963). Increased circulating catecholamines in MH may also be mediated by Ca\(^{2+}\) (Denborough, 1980) and diltiazem may inhibit this Ca\(^{2+}\)-dependent process.
6.5 SUMMARY

The ability of the calcium channel antagonist diltiazem to inhibit and reverse the porcine MH syndrome was investigated. In the presence of diltiazem (2 – 10 mg/kg) six MH susceptible pigs did not express the clinical features of MH, after being challenged with the precipitating agents succinylcholine and halothane. The same six pigs all developed MH in response to succinylcholine and halothane in the absence of diltiazem. The MH response was characterised by extensor rigidity, elevated body temperature, metabolic acidosis, cardiovascular dysfunction, altered plasma electrolytes, CPK and arterial blood gas composition. Administration of diltiazem during a MH crisis dramatically prevented any further expression of the syndrome. The biochemical and physiological features of halothane anaesthesia in diltiazem pretreated MH susceptible pigs were similar to those that were observed in control pigs.

Diltiazem may be an effective therapeutic agent in the treatment of MH if administered early in the onset of a crisis or before the initiation of anaesthesia.
CHAPTER 7

OBSERVATIONS ON THE METABOLIC PROFILES OF MALIGNANT HYPERPYREXIC SUSCEPTIBLE AND CONTROL PORCINE SKELETAL MUSCLE USING $^{31}$PHOSPHORUS NUCLEAR MAGNETIC RESONANCE.

7.1. INTRODUCTION

The finding that isolated MH susceptible skeletal muscle fibres produce abnormal isometric contracture responses to certain chemical stimuli has proved to be the most reliable diagnostic test for the identification of MH susceptible individuals. Other tests designed to identify susceptibility have not been definitive. These include the measurement of ATP (Harrison et al., 1969; Nelson et al., 1972; Isaacs and Hefferon, 1975; Isaacs, Hefferon and Badenhorst, 1975), and myophosphorylase A levels (Willner, Wood, Cerri and Britt, 1980) in excised MH muscle, other investigations on blood include serum CPK levels (King and Zapf, 1972; Denborough, 1975; McPherson and Taylor, 1982; Ellis, 1973; Britt, Endrenyi, Peters, Kwong and Kadijevic, 1976; Ellis, Clarke, Modgill, Currie and Harriman, 1975; Amaranath, Lavin, Trussor and Boutros, 1983), platelet function (Solomans, McDermott and Mahowald, 1980; Zsigmond, Penner and Kothay, 1980; Rosenberg, Fisher, Reed and Addonizio, 1981; Giger and Kaplan, 1983), erythrocyte fragility (Ellis et al., 1975;
Zsigmond et al., 1980) and plasma cholinesterase variants (Whittaker, Spencer and Searle, 1977).

While the testing of skeletal muscle contractility is the most reliable diagnostic test, the disadvantage of this method is its invasive nature. $^{31}$P nuclear magnetic resonance ($^{31}$P-NMR) spectroscopy can be used as a non-invasive probe of tissue metabolism (Gordon, Hanley and Shaw, 1982).

7.1.1 Application of $^{31}$P-NMR to Malignant Hyperpyrexia

$^{31}$P-NMR has become an important technique for investigating metabolism in living tissue. The application of $^{31}$P-NMR to metabolic studies in muscle is well documented (Hoult, Busby, Gadian, Radda, Richards and Seeley, 1974; Dawson, Gadian and Wilkie, 1977; Brown, Ugurbil and Shulman, 1977; Gadian, Radda, Brown, Chance, Dawson and Wilkie, 1981; Meyer, Kushmerick and Brown, 1982). The principle advantage of $^{31}$P-NMR is that tissue metabolism can be examined non-invasively. $^{31}$P-NMR can be used to map intracellular changes in the group of key phosphorus intermediates involved in energy metabolism in skeletal muscle as well as intracellular pH. Phosphorus resonances in skeletal muscle can be identified on the basis of chemical shift dependence from known organic phosphates (Hoult et al., 1974). Resonances from creatine phosphate and ATP are easily identified as they
are present in high concentrations in healthy muscle tissue and resonate upfield from phosphoric acid. The intracellular concentration of inorganic phosphate is usually lower relative to the high energy intermediates and chemical shifts in the resonance can be used to measure intracellular pH (Moon and Richards, 1973). Sugar phosphates (if present in high enough concentrations) resonate near inorganic phosphate, but are usually separated sufficiently to identify easily.

The purpose of this study is to investigate the phosphate metabolite profiles of isolated MH susceptible and control muscle with a view to defining an abnormality in the MH muscle which might permit the development of a specific non-invasive test using topical NMR. The effect of halothane and caffeine on muscle metabolism is also investigated.

7.2 MATERIALS AND METHODS

Biopsy samples of gracilis muscle were obtained from MH susceptible and control swine using the methods described in Chapter 3 (Section 3.3.2). Susceptibility to MH was assessed in biopsy samples by pharmacological methods (Okumura et al., 1979). Muscle samples for NMR studies were dissected into 30mm strips of 1mm diameter, four or five of which were attached to a platinum wire holder and placed into a 10mm NMR tube (Fig.7.1A). Tissue
integrity was maintained by a recirculating superfusion system of oxygenated Ringer solution maintained at 37°C and pH 7.4. Approximately 500ml of the Ringer solution was continuously aerated with 5% carbon dioxide in oxygen and pumped to and from the NMR tube at 6mls/min using a peristaltic pump. The capillary tubing which carried the buffer was covered with a rubber insulating material to reduce heat loss between the buffer reservoir and the NMR tube. The probe region of the NMR spectrometer was maintained at 37°C and a towel placed over the top of the magnet in order to keep the full length of the magnet bore at 37°C. This method proved to be successful in maintaining the temperature of the buffer. An overview of the experiment is shown in Figure 7.1B.

$^{31}$P-NMR spectra were recorded in the Fourier transform mode at 80.96 MHz on a Bruker CXP-200 spectrometer, with an Oxford Instruments/Spectrospin 4.6T superconducting magnet. The homogeneity of the magnetic field was optimised while observing the free-induction decay of the H$_2$O resonance. Spectra were then recorded over 50 minutes, using 3000 pulses at a repetition rate of 1 second with a 90° pulse (unless otherwise stated). Access to the spectrometer was limited and these recording conditions allowed for maximum utilization of time. A line broadening factor of 5 Hz was applied to the free induction decay before Fourier Transform. Samples were not spun. Chemical shifts were measured from a reference
FIGURE 7.1 An Overview of Spectrometer, Superfusion System and Muscle Preparation

A. NMR tube containing muscle preparation
B. The magnet of the NMR spectrometer and superfusion system
of 1M methylene diphosphonic acid (pH 9) contained in a capillary tube which was placed in the NMR tube. For presentation, the chemical shifts were measured relative to the primary $^{31}$P reference, 85% phosphoric acid. Under anoxic conditions chemical shifts were measured relative to creatine phosphate.

The muscle spectrum was measured twice and the resulting spectra compared to ensure that the phosphate metabolite profile was stable (in all figures the second spectra is shown), rendering the effects of drugs or anoxia on the profile unequivocal. After the addition of a drug to the bathing medium, a period of 10 min elapsed before spectral accumulation continued. Drug-induced changes of spectra were demonstrated by subtracting the spectrum obtained before drug addition from that obtained after. Halothane was added to the muscle buffer by passing the carbogen through a calibrated vapouriser before bubbling through the reservoir of Ringer. All other drugs were added directly to the reservoir. Spectra were also observed under conditions of anoxia. During these experiments the flow of oxygenated Ringer to the NMR tube was stopped for various periods as indicated in the text while spectral accumulation continued. Changes in the spectra under these conditions were also demonstrated by subtracting the spectrum obtained before from that obtained after periods of anoxia. The Ringer solution was modified for anoxic experiments. A phosphate
free Ringer solution which contained 1mM Tris. HCl buffer, pH 7.4 was used as it was intended to measure intracellular pH. All experiments were reproduced five times.

7.3 RESULTS

7.3.1 Metabolite Profiles of Control and MH Susceptible Porcine Skeletal Muscle

There was no difference between the phosphate metabolite profiles of MH susceptible and control muscle (Fig. 7.2). In both preparations resonances derived from creatine phosphate (CP) (-3.2 ppm), inorganic phosphate (Pᵢ) (2.2 ppm) and three from ATP corresponding to the gamma (-5.6 ppm), alpha (-11.0 ppm) and beta (-19.0 ppm) phosphates. Creatine phosphate was present in high concentrations relative to ATP and inorganic phosphate. The relative concentrations of these metabolites remained essentially stable over 300 minutes of spectral accumulation (Fig. 7.3). A small resonance (3.7 ppm) down field from inorganic phosphate was observed in spectra obtained from control and MH susceptible muscle which consisted of 10,000 pulses (Fig. 7.2), probably deriving from sugar phosphates.
FIGURE 7.2 $^{31}$P-NMR Spectra of MH Susceptible and Control Porcine Skeletal Muscle

A. Spectrum of MH Susceptible Muscle
B. Spectrum of Control Muscle

Spectra were recorded over 167 minutes using 10,000 pulses at a repetition rate of 1 second. Spectra showed resonances from creatine phosphate (CP), inorganic phosphate ($P_i$) and from the gamma ($\gamma$) alpha (a) and beta (B) phosphates of ATP. The reference signal (REF) was from methylene diphosphoric acid. A small resonance (3.7ppm) which probably derived from sugar phosphates was also observed.
FIGURE 7.3 $^{31}$P-NMR Spectra of MH Susceptible Porcine Skeletal Muscle

The spectra (1 - 6) were taken consecutively from the same muscle preparation. Metabolite levels remained stable over 300 minutes.
methylenediphosphonic acid
7.3.2 The Effect of Halothane and Caffeine on the Metabolite Profiles of Control and MH Susceptible Muscle

The spectra obtained from control muscle before and after the addition of 3% halothane or 2mM caffeine are shown in Figures 7.4 and 7.5 respectively. There was no change in the relative concentrations of any of the metabolites. However, 3% halothane (Fig. 7.6) and 2mM caffeine (Fig. 7.7) induced a reduction in the level of intracellular creatine phosphate and an increase in the level of inorganic phosphate while ATP concentrations remained unchanged in MH susceptible muscle. Dantrolene (6uM) reversed this effect returning creatine phosphate and inorganic phosphate to prechallenge levels (Fig. 7.7).

Higher concentrations of caffeine (10mM) induced a change in the metabolite profile of control muscle (Fig. 7.8). In the presence of 10mM caffeine creatine phosphate levels fell and the inorganic phosphate concentration increased. In one out of the five preparations studied a small decrease in the concentration of intracellular ATP was also observed (Fig. 7.8). In MH susceptible muscle in the presence of 10mM caffeine the level of phosphocreatine fell rapidly while a large increase in the concentration of inorganic phosphate was observed. In all preparations studied a large decrease in the level of ATP was also observed (Fig. 7.9).
FIGURE 7.4 $^{31}$P-NMR Spectra of Control Porcine Skeletal Muscle Before and After the addition of 3% Halothane

A. No additions
B. After 3% Halothane
B - A. Difference Spectrum
FIGURE 7.5 $^{31}$P-NMR Spectra of Control Porcine Skeletal Muscle Before and After the Addition of 2mM Caffeine

A. No addition

B. After 2mM Caffeine

B - A. Difference Spectrum
B-A

B

CP

A

Pi

γ

α

β

10  0  -10  -20

PPM
FIGURE 7.6 \( ^{31} \text{P-NMR} \) Spectra of MH Susceptible Porcine Skeletal Muscle Before and After the Addition of 3% Halothane

A. No addition

B. After 3% Halothane

B - A. Difference Spectrum
FIGURE 7.7 $^{31}$P-NMR Spectra of MH Susceptible Porcine Skeletal Muscle Before and After the Addition of 2mM Caffeine and 6uM Dantrolene

A. No addition
B. After the addition of 2mM Caffeine
C. After the addition of 6uM dantrolene in the presence of 2mM caffeine

B - A. The difference spectrum between spectra B and A
C - B. The difference spectrum between spectra C and B
C-B

B-A

C

B

CP

A Pi

ATP

\gamma \alpha \beta

10 0 -10 -20

PPM
FIGURE 7.8 $^{31}$P-NMR Spectra of Control Porcine Skeletal Muscle Before and After the Addition of 10mM Caffeine

A. No additions

B. After the addition of 10mM Caffeine

B - A. Difference spectrum
FIGURE 7.9 $^{31}$P-NMR Spectra of MH Susceptible Porcine Skeletal Muscle Before and After the Addition of 10mM Caffeine

A. No addition

B. After the addition of 10mM Caffeine

B - A. Difference Spectrum
7.3.3 The Effect of Anoxia on the Metabolite Profiles of Control and MH Susceptible Muscle

During the various periods of anoxia (50, 100, 150 minutes) a progressive change in the relative intracellular concentrations of creatine phosphate, ATP and inorganic phosphate were observed in MH susceptible tissue (Fig. 7.10). During the first period of anoxia (50 min) (Fig. 7.10B) creatine phosphate levels fell and inorganic phosphate levels increased, while the ATP concentration remained unchanged. A progressive decrease and increase in creatine phosphate and inorganic phosphate levels respectively were then observed over the remaining periods of anoxia (Fig. 7.10C,D). Once creatine phosphate levels were depleted the intracellular concentration of ATP fell (Fig. 7.10D). Over the periods of anoxia studied the inorganic phosphate resonance shifted downfield relative to the creatine phosphate resonance. The precise chemical shift of the inorganic phosphate resonance was often difficult to determine due to peak splitting. While intracellular pH could not be measured accurately under these conditions the chemical shift in the inorganic phosphate resonance was taken to represent tissue acidosis.

Over similar periods of anoxia the changes in the relative concentrations of creatine phosphate, ATP and inorganic phosphate in control tissue were less dramatic. During the first 50 minutes of anoxia the levels of all
FIGURE 7.10 $^{31}$P-NMR Spectra of MH Susceptible Muscle

Before and After Periods of Anoxia

A. Conditions of normal oxygenation
B. After 50 minutes of anoxia
C. After a 100 minutes of anoxia
D. After 150 minutes of anoxia

B - A. The difference spectrum
C - A. The difference spectrum
D - A. The difference spectrum
resonances remained essentially stable (Fig. 7.11B and 7.12B). After 100 minutes of anoxia the relative concentration of metabolites continued to remain stable (Fig. 7.11C) or a small decrease in the levels of creatine phosphate and an increase in inorganic phosphate levels were observed. A progressive decrease in the levels of creatine phosphate and a rise in intracellular inorganic phosphate were then observed over 150 minutes of anoxia, however ATP depletion did not occur (Fig. 7.12C).

During anoxic experiments the Ringer buffer contained 1mM Tris. pH 7.4 buffer. The effect of 1mM Tris. on MH susceptible and control muscle was also investigated by pharmacological techniques (Methods described in Chapter 3). Tris (1mM) failed to contract MH (n = 6) or control (n = 6) isolated muscle preparations.

7.4 DISCUSSION

There were no differences in the phosphate metabolite profiles of control and MH susceptible muscle preparations (Fig. 7.2). Both muscle preparations displayed resonances from creatine phosphate, ATP and inorganic phosphate. Sugar phosphates were only observed after long periods of spectral accumulation. Resonances from creatine phosphate and ATP were easily identified and were present in high concentrations indicating healthy tissue. Similar metabolite profiles have been reported in excised skeletal
FIGURE 7.11 $^{31}$P-NMR Spectra of Control Muscle Before and After Periods of Anoxia

A. Conditions of normal oxygenation
B. After 50 minutes of anoxia
C. After 100 minutes of anoxia
B - A. The difference spectrum
C - A. The difference spectrum
FIGURE 7.12 $^{31}$P-NMR Spectra of Control Muscle Before and after Periods of Anoxia

A. Conditions of normal oxygenation
B. After 50 minutes of anoxia
C. After 150 minutes of anoxia

B - A. The difference spectrum
C - A. The difference spectrum
muscle from a number of species (Eakin, Morgan, Gregg and Matwityoff, 1972; Hoult et al., 1974; Barany, Barany, Burt, Glonek and Myers, 1975; Burt, Glonek and Barany, 1976; Dawson et al., 1977). However the resonance of inorganic phosphate was larger from pig skeletal muscle in comparison to other species. This may represent a species difference or increased energy consumption of the tissue preparation immediately after excision. After immersion into the Ringer solution the relative concentrations of all muscle metabolites were maintained for long periods using the recirculating superfusion method (Fig. 7.3), indicating an adequate supply of substrates and oxygen to muscle cells. The stability of the profile was sufficient to render any changes in metabolite levels under various conditions unequivocal.

On exposure to 3% halothane and 2mM caffeine changes in the metabolite levels occurred in MH susceptible (Fig. 7.6 and 7.7) but not control (Fig. 7.4 and 7.5) muscle. Creatine phosphate levels fell while inorganic phosphate levels increased. Dantrolene (6μM) reversed the effects of 2mM caffeine (Fig. 7.7). These results are consistent with the observed hypercontractility of isolated MH susceptible muscle in response to these agents, and with dantrolene acting as a skeletal muscle relaxant (Ellis et al., 1973) reversing contractility and promoting the synthesis of creatine phosphate. These observations also support the suggestion that the continued synthesis and
hydrolysis of ATP plays an important role in the hyperpyrexic response which occurs during fulminant MH. Once the demand for ATP is greater than the ability of oxidative phosphorylation and glycolysis to replenish its stores the concentration of creatine phosphate falls to maintain the level of ATP. The continued hydrolysis of ATP and creatine phosphate liberates heat. Similar changes in metabolite profiles have been observed using whole body $^{31}P$-NMR in MH swine after MH was induced by the administration of halothane (Roberts, Burt, Gouylai, Chance, Screter and Ryan, 1983). Creatine phosphate levels fell rapidly while ATP levels were maintained until glycogen and creatine phosphate were depleted.

The ATP concentration in MH muscle slices has been shown to decrease when exposed to halothane and ATP depletion has been proposed as an indicator of MH susceptibility (Harrison et al., 1969). However, results in this study indicate that in well oxygenated muscle strips exposed to halothane ATP levels remain constant as in control preparations. The ATP depletion observed by Harrison et al. (1969) is probably due to inadequate oxygenation, as large preparations (200 - 500mg) of muscle were used. Decreased oxidative metabolism in the presence of contractile activity induced by halothane would lead to a rapid depletion of creatine phosphate and ATP. This study supports previous observations (Britt et al., 1976) that the ATP depletion test is inadequate in identifying
susceptibility to MH.

Higher concentrations of caffeine (10mM) were required to produce similar changes in the metabolite profiles of control muscle. In the presence of 10mM caffeine ATP levels remained constant while creatine phosphate levels decreased and inorganic phosphate levels rose in control muscle (Fig. 7.8). Similar observations have been reported in caffeine-stimulated frog gastrocnemius muscles (Block and Maxwell, 1974). After MH susceptible muscle had undergone 10mM caffeine-stimulated isometric contraction, near total exhaustion of the high energy phosphates and a large increase in inorganic phosphate levels was observed (Fig. 7.9). This rapid utilization of the high energy phosphates is consistent with the increased contractile response obtained from MH susceptible muscle in response to higher concentrations of caffeine, in comparison to control muscle (Fig. 3.2, Chapter 3).

After the cessation of the flow of oxygenated buffer to MH susceptible muscle, a rapid lowering of creatine phosphate levels, acidosis and finally diminution of the ATP pool was observed after 150 minutes (Fig. 7.10). Over a similar period of anoxia creatine phosphate levels declined while ATP levels remained relatively constant in control tissue (Fig. 7.11 and 7.12). The onset of metabolic change was also slower in control
tissue, with the concentration of all metabolites remaining relatively constant over the first 50 minutes of anoxia. Thus a clear distinction can be made between MH susceptible and control metabolite profiles after various periods of anoxia.

Changes in metabolite levels occur as oxidative metabolism runs down. ATP and creatine phosphate levels fall as the level of inorganic phosphate rises. Intracellular pH also falls due to lactate accumulation. Analogous observations have been reported after similar periods of anoxia or ischaemia in skeletal and cardiac muscles (Hoult et al., 1974; Hollis, Nunnally, Taylor, Weisfeldt and Jacobun, 1978; Grove, Ackerman, Radda and Bore, 1980; Salhany, Stohs, Reinke, Pieper and Hassings, 1979; Garlick, Radda and Seeley, 1977).

Roberts, Ryan, Ali and Allen (1982) have also observed differences in skeletal muscle function during periods of ischaemia between MH susceptible and control individuals. After similar periods of ischaemia contractile response is significantly diminished in MH muscle in comparison to control muscle, suggesting an abnormally high regional oxygen consumption in MH muscle. Increased oxygen consumption may occur in ischaemic or anoxic MH muscle which leads to a rapid fall in the intracellular high energy phosphates. The excess heat produced during MH is considered to be derived from accelerated glycolysis.
and the breakdown of the high energy phosphates (Berman et al., 1970; Berman and Kench, 1973). In MH muscle an abnormality may exist in carbohydrate metabolism linked with poor resynthesis of the high energy phosphates, which may be precipitated under anaerobic conditions. Sybesma and Eikelenboom (1978) have reported accelerated muscle metabolism under anaerobic conditions in pigs susceptible to PSS which leads to the development of PSE meat.

Anoxic or ischaemic conditions may therefore provide a non-anaesthetic means of inducing accelerated regional metabolism in MH patients, which could be identified non-invasively in vivo using $^{31}$P-NMR.

7.5 SUMMARY

$^{31}$P-NMR spectroscopy may have the potential to aid in the non-invasive diagnosis of MH. Changes in the phosphate metabolite profile of MH susceptible skeletal muscle occurs more readily under conditions of anoxia in comparison to control muscle. Increased oxygen consumption may occur in anoxic MH muscle, which leads to accelerated glycolysis, a rapid fall in intracellular high energy phosphates and acidosis. Accelerated muscle metabolism is also observed in the presence of 2mM caffeine and 3% halothane in MH muscle. Changes in the concentrations of metabolites and intracellular pH
could be mapped non-invasively under anoxic conditions using topical $^{31}$P-NMR.
Malignant hyperpyrexia is a dangerous complication of anaesthesia occurring in individuals with an inherited muscular abnormality. Clinical features of MH and the observed hypercontractility of isolated MH susceptible skeletal muscle indicate a dysfunction in the processes which regulate myoplasmic Ca$^{2+}$ concentration. While the precise site of the lesion which predisposes to the abnormally high Ca$^{2+}$ level is unknown, processes which primarily control myoplasmic free Ca$^{2+}$ levels, such as the events of EC coupling and Ca$^{2+}$ transport by the SR have been implicated.

In excitable cells, VOCC mediate Ca$^{2+}$-dependent depolarization that initiates a number of specific cellular functions including EC coupling (Tsien, 1983). CCA block Ca$^{2+}$ influx through these channels. In skeletal muscle, voltage-dependent Ca$^{2+}$ currents flow almost exclusively across the T-tubular membrane system and are blocked by CCA (Almers et al., 1981; Chiarandini and Stefani, 1983). CCA receptors are preferentially localized to the T-tubular membrane, consistent with their association with calcium channels (Fosset et al., 1983; Glossmann and Ferry, 1983).
In the present investigation the effects of CCA were examined on Ca\(^{2+}\)-dependent functions which are primarily involved in the regulation of myoplasmic Ca\(^{2+}\) in skeletal muscle, with a view to defining whether CCA were effective in inhibiting the abnormal Ca\(^{2+}\) fluxes associated with MH muscle. The CCA diltiazem, verapamil and nifedipine induced changes in the contractile characteristics of both MH susceptible and control skeletal muscle fibres. Like the skeletal muscle relaxant dantrolene, diltiazem was found to be effective in inhibiting and reversing the drug-induced hypercontractility of MH susceptible human and porcine skeletal muscle fibres. Observations on muscle contractility in the presence of verapamil and nifedipine indicated that these agents would not be effective in inhibiting the abnormal Ca\(^{2+}\) fluxes associated with MH muscle. Dantrolene is known to primarily act by lowering myoplasmic Ca\(^{2+}\) concentrations (Ellis and Carpenter, 1972, 1974; Desmedt and Hainant, 1977, 1979; Lopez et al., 1979). Results with diltiazem were also consistent with the CCA acting to lower myoplasmic Ca\(^{2+}\) levels. Observations on detubulated porcine skeletal muscle suggested that diltiazem modifies contractile responses at the level of the T-tubule membrane, as responses to caffeine were not modified by diltiazem in these preparations. This result was consistent with studies that indicate that CCA primarily act at the level of the T-tubular membrane (Almers et al., 1971, 1981; Fosset et
al., 1983) and suggests that Ca\(^{2+}\) influx through T-tubules may play an important role in the aetiology of MH.

While CCA binding sites are primarily localized at the T-tubule membranes of skeletal muscle CCA have also been shown to interfere with Ca\(^{2+}\) fluxes associated with the SR in cardiac muscle (Naylor and Horowitz, 1983). In view of the high concentrations of CCA used in contractile studies and the possibility of inhibition of Ca\(^{2+}\) fluxes associated with the SR, the effects of diltiazem and verapamil were investigated on Ca\(^{2+}\) uptake and release by the SR. Both diltiazem and verapamil failed to affect the sequestration of Ca\(^{2+}\) by the longitudinal tubules, Ca\(^{2+}\) release from the terminal cisternae membrane or the activity of Ca\(^{2+}\)-dependent ATPase. These results are consistent with observations that CCA do not act on the SR in skeletal muscle and that Ca\(^{2+}\)-dependent ATPase does not form a specific Ca\(^{2+}\) channel (Jilka et al., 1975; Jilka and Martonosi, 1977). Dantrolene reversed caffeine-induced contractures in isolated muscle fibres in the presence of maximally effective concentrations of diltiazem and the converse was true. Furthermore, dantrolene inhibited Ca\(^{2+}\) fluxes associated with the terminal cisternae of the SR but failed to affect Ca\(^{2+}\)-uptake or the activity of Ca\(^{2+}\)-dependent ATPase. The muscle relaxant also inhibited contractile responses to caffeine in detubulated muscle fibres. These results suggest that dantrolene has a different site of action
to that of diltiazem and support the view that dantrolene primarily acts to lower myoplasmic Ca\(^{2+}\) levels by inhibiting Ca\(^{2+}\) release from the SR (Desmedt and Hainaut, 1977, 1979; Lopez et al., 1979), while diltiazem acts to modify contractile responses by inhibiting Ca\(^{2+}\) fluxes associated with T-tubule membranes.

One of the rationales for the use of dantrolene in the treatment of MH was based on the demonstration that the skeletal muscle relaxant could prevent and reverse contracture responses to the diagnostic agents in isolated muscle fibres. The effect of diltiazem was therefore investigated on the porcine MH syndrome. Diltiazem was shown to prevent the onset and further expression of an initiated MH syndrome. Higher concentrations of diltiazem were required to inhibit and reverse the drug-induced hypercontractility of isolated MH susceptible porcine muscle in comparison with dantrolene. However, similar concentrations produced these effects in isolated MH susceptible human muscle. In view of this and the observation that diltiazem could inhibit the expression of the porcine MH syndrome, diltiazem may be an effective therapeutic agent in the treatment of human MH if administered early in the onset of a crisis.

The precise site of the muscle cell membrane abnormality which predisposes to MH is not yet known. In vitro studies, using agents which act specifically
on different parts of the muscle contractile mechanism, suggest that the abnormality in MH susceptible swine muscle is distal to the postjunctional membrane. Two possible sites are involved, one being the sarcolemma and T-system, the other the SR. Previous studies on Ca²⁺ uptake and release by the SR isolated from MH susceptible human and porcine muscle have led to no firm conclusions so a detailed biochemical characterisation of the SR membrane from MH susceptible porcine muscle was conducted. The SR membrane from control and MH susceptible porcine skeletal muscle was separated into two morphologically distinct regions, the terminal cisternae and the longitudinal tubules. These fractions exhibited the functional differences that are related to their fundamental roles in Ca²⁺ transport in vivo (Campbell and Shamoo, 1980). Essentially, fractions differed in protein composition and Ca²⁺-transport activities. A comparative study of the same membrane fractions derived from MH susceptible and control muscle did not reveal any morphological differences or abnormalities in Ca²⁺ transport or Ca²⁺-dependent ATPase activities.

As no distinction could be made between Ca²⁺ fluxes associated with these distinct regions of SR from MH susceptible and control muscle the dysfunction may be at another site, possibly the T-tubule membrane. An extensive morphological characterisation and measurement of Ca²⁺ fluxes associated with T-tubular membranes from MH
susceptible and control muscle may answer this question. T-tubules can be isolated and are characterised by a unique protein and phospholipid composition (Campbell et al., 1980; Lau et al., 1977, 1979a), the presence of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and active $\text{Na}^+$ transport (Lau et al., 1977, 1979b), high cholesterol content (Lau et al., 1979a) and $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ (Brandt, Caswell and Brunswig, 1980).

ATP-dependent $\text{Ca}^{2+}$ transport activity has been demonstrated with vesicles derived from T-tubular membranes and differs from that of SR in several respects. The affinity of the T-tubular $\text{Ca}^{2+}$ transport ATPase is significantly lower than that of the SR $\text{Ca}^{2+}$ pump. Also, $\text{Ca}^{2+}$ transport by isolated T-tubular vesicles is not increased by calcium precipitating ions or enhanced by treatments that render the vesicle membranes leaky (Brandt et al., 1980; Hidalgo et al., 1983; Hidalgo, Gonzalez and Garcia, 1986). The physiological role of this $\text{Ca}^{2+}$ transport system is yet to be defined. However, it may participate in the removal of activating $\text{Ca}^{2+}$ from the junctional region of triads (Bianchi and Narayan, 1982). This ATPase system would therefore act in a similar way as the sarcolemmal systems which remove $\text{Ca}^{2+}$ from other excitable cells, such as the $\text{Na}^+\text{-Ca}^{2+}$ exchange system and $\text{Ca}^{2+}$ pumping ATPase of cardiac cells (Naylor and Horowitz, 1983). Recently, a $\text{Na}^+\text{-Ca}^{2+}$ exchange system as well as a calmodulin stimulated $\text{Ca}^{2+}$ pump were found in sarcolemmal preparations isolated from pig and rabbit skeletal muscle.
(Michalak, Famulski and Carafoli, 1984; Mickelson, Beaudry and Louis, 1985). Calmodulin stimulated Ca\(^{2+}\) transport by isolated T-tubule membranes has also been reported (Hidalgo et al., 1986). It was suggested that the physiological role of this high affinity Ca\(^{2+}\) pump is in maintaining a low intracellular Ca\(^{2+}\) concentration in resting muscle cells. An abnormality in one of these transport systems may lead to an elevation of myoplasmic Ca\(^{2+}\) levels. A comparison of Ca\(^{2+}\)-transport activities between isolated MH susceptible and control T-tubular membranes is therefore warranted.

Currently, the in vitro hypercontractility of MH susceptible skeletal muscle in response to various stimuli is the only consistent method of assessing susceptibility to MH. The disadvantage of this test is its cost, risk and invasive nature. Studies on muscle metabolism using \(^{31}\)P-NMR spectroscopy indicated the potential for this technique to be used for the non invasive diagnosis of MH. \(^{31}\)P-NMR was used to map intracellular changes in the group of key phosphate intermediates involved in energy metabolism in MH susceptible and control skeletal muscle. While there were no differences in the metabolite profiles of these muscle preparations under normal conditions, after the cessation of the flow of oxygenated buffer to MH susceptible tissue a rapid decrease in the levels of creatine phosphate and finally diminution of the ATP pool were observed. Over a similar period of anoxia
creatine phosphate levels declined while ATP levels remained relatively constant in control tissue. A clear distinction could be made between MH susceptible and control metabolite profiles after various periods of anoxia. Anoxic or ischaemic conditions may therefore provide a non-anaesthetic means of inducing accelerated regional metabolism in MH muscle. Thus decreased oxygen and nutrient supply in association with exercise (induced contractile activity) to specific muscle bundles may lead to a rapid depletion of creatine phosphate levels and a fall in intracellular pH in comparison to controls. This would allow the development of a simple non invasive diagnostic test for MH susceptibility using $^{31}$p-NMR.
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