IMPAIRMENT OF SARCOPLASMIC RETICULUM FUNCTIONS :
METABOLIC PERTURBATIONS DURING MUSCLE FATIGUE

by

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ABSTRACT

The purpose of these three studies was to examine the role of altered metabolic perturbations as a mechanism modulating sarcoplasmic reticulum (SR) function during the development of muscle fatigue. The first study examined the role of SR Ca^{2+} release during and following fatigue and attempted to define the role of specific intracellular metabolites in Ca^{2+} release and recovery of mechanical function. Following a 3 min stimulation protocol (15 Hz; 250 ms; 50 % duty cycle) designed to produce fatigue and metabolic perturbations (decreases in ATP and PCr and increases in Pi and H^+), *in vitro* rat diaphragms were subjected to one of the following three conditions: (1) two min of additional stimulation (2MS), (2) five min of passive recovery (5MR) and (3) twenty min of anoxic recovery (20MR). Similarly, a second fatigued diaphragm was exposed to one of these three conditions but in the presence of caffeine.

In the 2 MS group, fatigue at low frequencies (LFF) was only partially reversed by caffeine ($P < 0.05$), suggesting that part of the reduction in tension was due to an impaired cross-bridge activation. Caffeine exaggerated the reduction in tension ($P < 0.05$), with high-frequency fatigue (HFF), compared to the no caffeine group suggesting that the $[Ca^{2+}]_i$ was reduced beyond the level induced by the stimulation protocol only. For the 5MR, which was designed to allow for the normalization of ATP and PCr, the recovery period was not sufficient to reverse the effect of LFF in the no-caffeine diaphragm. However, caffeine mediated recovery produced increases in tension that were higher than the non-stimulated control ($P < 0.05$). Such an effect was notfound for HFF. This finding suggested that with LFF, there was no inhibition at the

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cross-bridges, that caffeine was capable of neutralizing the inhibition placed on the Ca^{2+} release channels by H^+ and that differences in the sensitivity of the myofibrillar apparatus to Ca^{2+} occurred. The lack of tension recovery for the 20MR condition, which would have allowed sufficient time for the dissipation of H^+, suggests that a suppressed phosphorylation potential and increased metabolic by-products are the main mechanism responsible for the reduced tension.

In the second study, the sensitivity Ca^{2+}-Mg^{2+}-ATPase activity to Ca^{2+}, H^+ and ATP was examined. This was done using homogenate preparations of muscles (white gastrocnemius medialis, WGM; red gastrocnemius medialis, RGM; soleus, S) containing different proportions of slow- and fast-twitch fibres known to reflect differences in SERCA 2a and 1 isoform content (Lytton et al., 1992). It was hypothesised that differences in sensitivity of Ca^{2+}-Mg^{2+}-ATPase activity in the homogenate to the above metabolic factors would be dependent on the fibre type composition, reflecting different contents of the two isoforms. Although, the results showed that the dependency to Ca^{2+} and ATP by the three muscle fibre types were similar, the dependency to H^+ between homogenates of WGM and RGM compared to S was different ($P < 0.05$). These results suggested that the sensitivity of SERCA 1a isoform to pH was greater than the sensitivity to pH of the SERCA 2a isoform.

The final study was designed to determine if the selectivity of the isolation protocol could be a contributing factor in explaining the discrepancy in results that exist between laboratories (Byrd et al., 1989a; Chin & Green, 1996) concerning the effect of prolonged exhaustive exercise on Ca^{2+}-Mg^{2+}-ATPase activity. The general procedures

of Byrd et al. (1989a) were carefully reproduced to minimise the experimental variability between the two studies. Rats were run to exhaustion on a slope treadmill. The red muscles (gastrocnemius and vastus) of two animals were pooled together for the control and run groups. Ca^{2+}-Mg^{2+}-ATPase activity and activities of marker enzyme's for mitochondrial and sarcolemmal contamination were measured in homogenate, various sub-fractions and final vesicle fractions. The results showed no differences in total, basal and maximal Ca^{2+}-stimulated activity between the control and run animals for the homogenate, sub-fractions and vesicle fractions. The results also confirmed previous published results (Dossett-Mercer et al., 1994; Chin & Green, 1996) from this laboratory that found no depression in Ca^{2+}-Mg^{2+}-ATPase activity following running exercise. The discrepancy that exists regarding the on the effect of prolonged exercise on Ca^{2+}-Mg^{2+}-ATPase activity cannot be explained on the basis of differences in species of rats, exercise protocol or isolation procedure, since these variables were similar in the two studies.

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"I can only tell you what I warned you: there is something about experimental science that makes strong men weak. It's the great vulnerability of having to live in close contact with yourself hour after hour looking for how you feel about experiments you once thought you understood and now discover in the act of doing are more complex and elusive than one supposed, and the ego begins to dissolve. It all adds up to feeling sick, liverish, dejected, scared, about the way one would feel in the middle of a hundred-mile run if one had trained for a five-mile run"

Adapted from a letter sent by Norman Mailer to an aspiring writer.

DEDICATION

Je dédie cet ouvrage aux trois femmes dans ma vie: ma fille Marion, ma compagne Louise et ma mère Louise.

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CHAPTER I

INTRODUCTION

1.0   INTRODUCTION

The characterization of prolonged activity on muscle function is not a new phenomenon. Muscle fatigue was observed experimentally as early as 1868 by Marey (see Asmussen, 1979). Thirty years later, Mosso (see Asmussen, 1979) observed that a colleague fatigued by a strength task could produce more work after the delivery of an oral presentation to an audience. This was attributed to an increased nervous arousal which led to the conclusion that central influences could affect physical performance.

More recently, studies (Merton, 1954; Ikai, 1967; Stephen & Taylor, 1972) presented divergent views on the mechanisms responsible for the development of neuromuscular fatigue thereby establishing the basis of a controversy that has yet to be resolved (Fitts & Balog, 1996). At present, investigations of neuromuscular fatigue include considerations of whether the mechanism(s) responsible for the inability to generate the required tension (Edwards, 1981) are located in the central nervous system or in the contractile apparatus (Bigland-Ritchie et al., 1984; Green, 1990; Enoka & Stuart, 1992; Fitts, 1994).

Although, considerable advances have been made, the emerging view is that the aetiology of neuromuscular fatigue is complex and depends on a number of factors acting either alone or in combination at various sites (Fitts, 1994). However, the work, generated for the most part by three laboratories (J. Lännergren and H. Westerblad; D.

G. Allen; and G. D. Lamb and D. G. Stephenson), has implicated both the T-tubular membrane and the sarcoplasmic reticulum (SR) as sites likely to play a major role in the development of muscle fatigue.

The SR in regulating Ca^{2+} homeostasis in the myoplasm displays four basic functions. During inactivation, it acts as a storage site for Ca^{2+}. During excitation, it releases Ca^{2+} so that the latter can act on a series of contractile regulatory proteins and trigger cross-bridge dynamics. It also has a mechanism for Ca^{2+} resequestration and for returning Ca^{2+} to resting levels during relaxation. Finally, if the site of release and uptake are separated, the SR must have a mechanism by which Ca^{2+} can be translocated from one site to the other. Any disturbances at one or more of these sites could disrupt normal Ca^{2+} kinetics and impair muscle function.

1.1 STRUCTURES INVOLVED IN CA^{2+} RELEASE AND CA^{2+} UPTAKE FUNCTIONS OF THE SARCOPLASMIC RETICULUM

Particular interest has been focused on the critical question of the coupling mechanism between excitation and contraction and this question has been addressed relentlessly by muscle physiologists for over 35 years (Ebashi, 1991; Dulhunty et al., 1996). In the last decade, considerable progress has been made in advancing knowledge in this intriguing area. Molecular tools and refined technology have revealed pertinent new information on the structures and processes involved in excitation-contraction coupling (ECC). The current concepts offer the possibility for establishing a mechanistic link between ECC and skeletal muscle fatigue. In a similar fashion, knowledge of the structure of the Ca^{2+} release channels, Ca^{2+}-Mg^{2+}-ATPase as well as other Ca^{2+} binding proteins of the SR has greatly advanced. This section reviews the structures of the

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Ca^{2+}-release channels and Ca^{2+}-Mg^{2+}-ATPase which are directly implicated in the experiments performed in this thesis.

1.1.1 Structures responsible for Ca^{2+} release by the sarcoplasmic reticulum

Close examination of the gap (10-20 nm) between the SR and the t-tubule reveals the existence of bridging structures (Somlyo, 1979; Saito et al., 1984; Dulhunty, 1987; Block et al., 1988). These protein structures, known as junctional feet, are located in the terminal cisternae membranes of the SR (Franzini-Armstrong, 1983). Two terminal cisternae border a t-tubule to form a structure known as a triad.

Although these feet structures were suggested to be responsible for Ca^{2+} release (Somlyo, 1979; Lai et al., 1987), a major step towards understanding the mechanism involved in Ca^{2+} release by the SR came with the use of pharmacological stimulators and inhibitors of Ca^{2+} release in skinned fibres, isolated heavy SR microsomes and purified membrane channels (Fleischer & Inui, 1989).

Among the plethora of drugs tested, ryanodine, a neutral plant alkaloid (Jenden & Fairhurst, 1969) was shown to demonstrate a high affinity for heavy (Fleischer et al., 1985) but not for light SR microsome preparations. This was the first evidence to suggest that the sites bound by the radiolabelled ryanodine were Ca^{2+} release channels. In addition, the action of pharmacological agents such as ruthenium red and adenine nucleotides on the ryanodine sites paralleled the effects on the native Ca^{2+} release channels. The effect of ryanodine is inhibited by the presence of micromolar concentrations of ruthenium red (Fleischer et al., 1985; Meissner, 1986; Hymel et al., 1988b) and facilitated by preincubation with micromolar levels of Ca^{2+} or millimolar

levels of adenine nucleotides (Meissner et al., 1986; Imagawa et al., 1987b).

The concentration of ryanodine has a differential effect on Ca^{2+} permeability of the SR (Fleischer et al., 1985; Meissner, 1986; Smith et al., 1988). In nM (20-200 nM) concentrations, it becomes a positive effector by locking the "feet" open (Fleischer et al., 1985; Smith et al., 1988; Bull et al., 1989). In concentrations in excess of 10 μM, it acts as a negative effector by blocking or partially closing the feet (Sutko et al., 1985; Meissner, 1986). An initial release followed by a deactivation of the channel has also been observed with high concentrations of ryanodine (Chu et al., 1990). The differential effect of concentration has been linked to the presence of low and high affinity sites for ryanodine that would favour different states of conductance (Bull et al., 1989; McGrew et al., 1989). Ryanodine binding at the high affinity sites activates the Ca^{2+} channel whereas binding to the low affinity sites causes inhibition of release (McGrew et al., 1989; Chu et al., 1990). Furthermore, dissociation of ryanodine from the high affinity sites appears to be hindered by binding of ryanodine to the low affinity sites, suggesting an allosteric association between the two sites (Lai et al., 1989; Chu et al., 1990). A companion effect of high (60 nM) concentrations of ryanodine is to inhibit the Ca^{2+} channel of the dihydropyridine (DHP) receptor of the t-tubular membrane, a structure also involved in ECC (see below) (Valdivia & Coronado, 1989).

The use of radiolabelled ryanodine led to the isolation of a ryanodine receptor (RyR) protein (Imagawa et al., 1987b; Pessah et al., 1986; Campbell et al., 1987; Hymel et al., 1988a; Lai et al., 1988). Evidence from single channel studies (Rousseau et al., 1987; Hymel et al., 1988a; Lai et al., 1988; Smith et al., 1988) supported the

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conclusions from vesicle studies and established that the native Ca^{2+} release channel was identical to the ryanodine receptor.

Three dimensional reconstruction of the RyR through negative staining electron microscopy revealed a large cytoplasmic tetragonal structure with overall dimensions of 29 x 29 x 12 nm, a baseplate of 14 x 14 x 7 nm and a protruding transmembrane assembly of 7 nm (Wagenknecht et al., 1989; Radermacher et al., 1994). These four polypeptides are symmetrically arranged around and connected to a central channel of 1-2 nm in diameter (Imagawa et al., 1987b; Lai et al., 1988; Wagenknecht et al., 1989). Four cavities on the side of the transmembrane assembly have been suggested to be the path by which Ca^{2+} is released from the lumen of the SR into the junctional gap (Radermacher et al., 1994).

Sequencing analysis using complementary DNA (cDNA) has revealed a primary structure made of 5,037 amino-acids with a M_r of 565 kDa for each peptide (Takeshima et al., 1989). Four to ten hydrophobic transmembrane segments residing in the COOH-terminal (Takeshima et al., 1989; Zorzato et al., 1990; Chen et al., 1993a; Grunwald & Meissner, 1995) and two more central segments (Zorzato et al., 1990) form a basal platform (14x14x4 nm) (Wagenknecht et al., 1989; Rademacher et al., 1995) which anchors the structure into the junctional membrane face.

Possible modulator regions such as calmodulin and nucleotide binding sites have been identified between the first tetramer (M1) and the foot region (Takeshima et al.,1989) and on the surface of the foot region (Zorzato et al., 1990). Reconstitution studies of the purified RyR have suggested that the Ca^{2+} channel contains two groups of

sites that either inhibit or activate the Ca^{2+} channels when phosphorylated (Hymel et al., 1989b). More recently, a phosphorylation site (Suko et al., 1993; Coronado et al., 1994) and a region that binds Ca^{2+} during Ca^{2+} induced Ca^{2+} release has been identified (Chen et al., 1992; 1993b). A binding protein (FK506-binding protein) that interacts with the folding and assembly of other proteins has been shown to activate the RyR and reduce the inhibitory effects of Mg^{2+} and H^+ (Ahern et al., 1994; Timerman et al., 1995).

At about the same time another receptor having an L-type voltage-dependent calcium channel (Rios & Brum, 1987; Smith et al., 1987; Campbell et al., 1988; Hymel et al., 1988b) was found in t-tubule (Ferguson et al., 1984). In contrast to T- or N-type channels, the L-type channel voltage dependent receptor was sensitive to dihydropyridines (nifedipine, nitrendipine; PN 200-110) (Lamb, 1986; Rios & Brum, 1987; Fill et al., 1989), D-600 (Hui et al., 1984; Melzer & Pohl, 1987) and benzothiazepine (Walsh et al., 1987; Striessnig et al., 1990). As with the Ca^{2+} channels of the SR, the use of a radiolabelled drug, in this case DHP, led to the isolation and purification of the receptor, providing information on its structure and function (Borsotto et al., 1984; Curtis & Catterall, 1984; Leung et al., 1987; Smith et al., 1987; Hymel et al., 1988b).

The DHP receptor is comprised of 1873 amino-acids (Tanabe et al., 1987), and is an ovoidal particle of 16x22 nm (Leung et al., 1988). The quaternary structure consists of five sub-units: α_1 (185 kDa), α_2 (143 kDa), β (54 kDa), γ (30 kDa) and δ (26 kDa) (Leung et al., 1987; Takahashi et al., 1987; Leung et al., 1988; De Jongh et

al., 1989; Lai et al., 1990; Striessnig et al., 1990). The α_1 is the key sub-unit because of its pharmacological properties, its many kinase phosphorylatable sites, and the arrangement of its structure which contains the voltage-sensing elements and ion pore structure (Campbell et al., 1988; Catterall, 1991).

It has been suggested that the DHP receptor may serve the dual role of Ca^{2+} channel and voltage sensor (Rios & Brum, 1987; Tanabe et al., 1987; Cognard et al., 1990). Reconstitution studies with phospholipid vesicles and lipid bilayers have shown that some of the receptors have voltage dependent gating Ca^{2+} channels that are blocked by DHPs (Smith et al., 1987). Initially, a range of conductance levels, matching more or less the native t-tubule channel, were reported for the purified channel (Smith et al., 1987). However, this unexpected finding was later explained by showing that with increased experimental time, the purified monochannels became oligochannels with conductance values that were integer multiples of the conductance recorded for the monochannel (Hymel et al., 1988b). In addition, pharmacological selectivity and phosphorylation by cAMP-dependent protein kinase (Tanabe et al., 1987; Hymel et al., 1988b, Nunoki et al., 1989) suggested that the reconstituted channel was an L-type Ca^{2+} channel.

The association between the t-tubular membrane and the Ca^{2+} release channels was first demonstrated in experiments using vesicle fractions of aggregates of heavy SR and t-tubule (Kim et al., 1983; Ikemoto et al., 1984). Ca^{2+} release was induced by depolarisation of the t-tubular membrane through ionic substitution and by the addition of caffeine. Results revealed that the junctional association between the t-tubule and the

SR was critical for the ionic depolarisation induced release. In vesicle fractions where the SR was separated from the t-tubule, only caffeine stimulated release was observed. (Ikemoto et al., 1984).

However, it was only from later work with dysgenic mouse muscle lacking the DHP receptor and hence ECC, that the role of the DHP receptor as a voltage sensor was confirmed (Tanabe et al., 1988). When plasmids containing cDNA of rabbit skeletal muscle DHP were microinjected into cultured myotubes of the dysgenic mouse, contractility and slow calcium current were restored (Tanabe et al., 1988). In contrast, cDNA of the DHP receptors from mouse cardiac muscle injected into the dysgenic skeletal muscle demonstrated that the receptor recovers its function as an L-type Ca^{2+} channel, but not as a voltage sensor (Tanabe et al., 1990). The presence of a specific loop between domain II and III in skeletal α_1 sub-units is a critical functional sequence that is responsible for signal transmission (Adam et al., 1990) and is structurally linked to the Ca^{2+} release channels of the SR (Lu, et al., 1994; Anderson & Meissner, 1995).

However, the role of the DHP receptor as a Ca^{2+} channel was initially challenged with evidence obtained from experiments involving phospholipid vesicles (Curtis & Catterall, 1986) and intact skeletal fibres (Schwartz et al., 1985). These experiments demonstrated that less than 5% of the DHP receptors had calcium channels. The identification of two forms of the α_1 sub-unit in skeletal muscle t-tubules may solve this issue (De Jongh et al., 1989).

One line of evidence which suggested that two forms of the α_1 existed was the different calculated M_r determined from sequence analysis (212 kDa) (Tanabe et al.,

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1987) and from SDS-PAGE (170-175 kDa) (Leung et al.,1988; Takahashi et al, 1987). Proteolytic modification *in vivo* or during the purification and/or inaccuracy of electrophoresis were first assumed to be responsible for the divergence in results (Tanabe et al., 1987; Vaghy et al., 1988). However, both forms of α_1 sub-units have been identified in t-tubule membranes of skeletal muscle (De Jong et al., 1989).

The existence of two α_1 sub-units of different mass and the high proportion of 175 kDa sub-units compared to 212 kDa sub-units (Lai et al., 1990) suggested a specific functional role for each sub-unit (De Jongh et al., 1989). It has been hypothesised that the 175 kDa sub-unit may act as the voltage sensor, whereas the 212 kDa sub-unit could represent the Ca^{2+} channel (De Jongh et al., 1989; Catterall, 1991). However, this hypothesis has been contested onthe basis of a study showing that the expression of a truncated form of the sub-unit restored both the Ca^{2+} current and skeletal ECC (Beam et al., 1992).

As for the other sub-units, the β sub-unit, which contains phosphorylation sites (De Jongh et al., 1989) appears to assist the α_1 sub-unit in regulating the Ca^{2+} channel. Coexpression of the β sub-unit with the α_1 sub-unit normalises the activation kinetics of the latter (Kuwajima, et al., 1992). The α_2 sub-unit is encoded by the same gene and forms an integral part of the DHP-receptor Ca^{2+} channel (De Jongh et al., 1990). Further studies using coexpression experiments will be necessary before definite functional roles are established for each of these sub-units (Melzer et al., 1994).

1.1.2 Structure of the Ca^{2+}-Mg^{2+}-ATPase of the sarcoplasmic reticulum

The Ca^{2+}-Mg^{2+}-ATPase, also called SR ATPase, is best described as an ion-

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transport enzyme. Sequencing of the protein has revealed a single polypeptide chain of 110 kDa and 997 residues (MacLennan et al., 1985; Brandl et al., 1986; Keresztes et al., 1989). It constitutes 30-90% of the protein comprising the SR membrane (Fleischer & Inui, 1989). The extramembranous portion of the enzyme, about two-thirds of the total mass, lies in the cytoplasm (MacLennan, 1990; Molnar et al., 1990). The remaining one third is surrounded in the membrane by 90 lipid molecules (Gillis, 1985) and only a few peptides are exposed to the luminal side (Molnar et al., 1990).

The template fitting procedure (Taylor & Thornton, 1984), which determines the position of super-secondary structures, a combination of three secondary structures (e.g. beta sheet-alpha helix- beta sheet) in the amino-acid sequence was used to predict the structure of the Ca^{2+}-ATPase (MacLennan et al.,1985). This prediction method is based on the assumption that there is a limited number of these secondary structures and that new protein structures can be predicted from known protein structures. By comparing the repeatable patterns of these super-secondary structures for the Ca^{2+}-ATPase with the repeatable patterns obtained from known proteins, the structure of the ATPase can be predicted.

From these types of predictions and information from electron micrographs of the ATPase, a structural model has been suggested consisting of basepiece, stalk and headpiece components (MacLennan et al., 1985; Brandl et al., 1986). Identification with monoclonal antibodies, of segments located on the luminal face (residues 870-890) and cytoplasmic side (residues 330-505 and 657-672) of the SR lend support to this model (Clarke et al., 1990c).

The basepiece is made of four and six transmembrane helices in the NH_2 and COOH terminals, respectively (MacLennan et al, 1985; MacLennan, 1990). These helices being buried in the phospholipid membrane are made up of a large number of hydrophobic residues compared to the cytoplasmic domains. These helices also form the transmembrane channel for the passage of the Ca^{2+}-Mg^{2+}-ATPase (Martonosi et al., 1988).

Initially, the stalk region, made of 5 alpha helices, was thought to contain the high affinity calcium binding sites on the cytoplasmic side (MacLennan et al., 1985). However, this hypothesis was revised with the introduction by MacLennan and colleagues (see MacLennan, 1990), of site-directed mutagenesis to elucidate structural and functional relationships in the Ca^{2+}-ATPase. Briefly, the technique consists of implanting mutants into vectors to form microsomes (COS-1 microsomes) (Muruyama & MacLennan, 1988). These microsomes can then be used to measure the Ca^{2+} dependency of Ca^{2+} transport, representing an estimate of Ca^{2+} affinity. Autophosphorylation with ATP or Pi with or without Ca^{2+} provides information on the effect of the mutation on phosphorylation and nucleotide binding sites.

Altering residues in the stalk region by site-directed mutagenesis of the Ca^{2+}-ATPase, did not affect Ca^{2+} transport suggesting that the Ca^{2+} binding sites were located elsewhere (Clarke et al., 1989a). Subsequent studies revealed that a number of residues in other regions affected Ca^{2+} transport to a different degree (Clarke et al., 1989b, Vilsen et al., 1989; Clarke et al., 1990a). In contrast to the wild-type enzyme, phosphorylation by Pi for some of these residues was not inhibited by Ca^{2+}, suggesting

that the mutation had affected the high affinity Ca^{2+} sites (Clarke et al., 1989b). Six residues, located in the middle of transmembrane stalks 4 (Glu^{309}), 5 (Glu^{771}), 6 (Asp^{796}, Thr^{799}, Asp^{800}) and 8 (Glu^{908}), have been suggested to act as the high Ca^{2+} affinity binding sites (Clarke et al., 1990a).

The headpiece contains a phosphorylation domain, a nucleotide domain for the binding of Mg . ATP and a transduction or ß strand domain (MacLennan et al., 1985; MacLennan, 1990). Site-directed mutagenesis has been conducted in these domains to establish the location of functional residues or sequences.

In the phosphorylation domain, mutation of two residues (Asp^{351} & Lys^{352}), part of a highly conserved sequence, altered phosphorylation and Ca^{2+} transport function of the enzyme (Muruyama & MacLennan, 1988). Mutation of a third residue (Thr^{353}) affected Ca^{2+} transport without altering phosphoenzyme formation (Maruyama et al., 1989).

Mutagenesis has also been conducted in four conserved segments (Lys-Gly-Ala-Pro-Glu^{519}, Asp-Pro-Pro-Arg^{604}, Thr-Gly-Asp^{627} and Thr-Gly-Asp-Gly-Val-Asn-Asp^{707}) that have been proposed to form an ATP binding cleft (Taylor & Green, 1989). Close-knit interactions have made it more difficult to assess the precise effect of site-directed mutagenesis on these residue sequences. Mutagenesis of residues involved in nucleotide binding may prevent phosphoenzyme formation not by affecting ATP binding, but rather by preventing Ca^{2+} binding to the high affinity sites (Clarke et al., 1990d). Although definite proof of the involvement of these sequences in nucleotide binding is difficult to obtain, site directed mutagenesis of these residues has revealed that a high interaction

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exists between the domains (Clarke et al., 1990d). Ligand formation with many of these residues appears to induce conformational changes that link the phosphorylation and Ca^{2+} binding sites (Clarke et al., 1990d).

Lys^{515}, suggested to be important for phosphorylation and ATP binding (Taylor & Green, 1989), was found to reduce Ca^{2+} transport, but not phosphoenzyme formation (Maruyama et al., 1989). However, it appears to be an important residue, involved in energy transduction following phosphorylation (Clarke et al., 1990d). Mutagenesis of Thr^{625}, Gly^{626} and Asp^{627} caused a reduction in Ca^{2+} transport and prevented the formation of the phosphoenzyme in the presence of both ATP and inorganic phosphate (Pi) (Maruyama et al., 1989; Clarke et al., 1990d). These results suggested that these residues were important for the conformation of the catalytic site and in linking the phophorylation and Ca^{2+} binding sites through ligand-induced conformational changes (Clarke et al., 1990d).

Results of mutagenesis of residues in the other two sequences (Asp-Pro-Pro-Arg^{604} and Thr-Gly-Asp-Gly-Val-Asn-Asp^{707}) have suggested that these residues are important in the conformation of the phosphorylation site (Asp^{601}, Pro^{603} and Asp^{707}) and/or for the transition of the enzyme from the E1P to the E2P state (Asp^{601}, Pro^{603} and Asp^{703}) (Clarke et al., 1990d). Reduced Ca^{2+} transport due to blocking of the transitional state from E1P to E2P has also been observed with mutagenesis of residues in the ß strand domain such as Thr^{181}, Gly^{182} Glu^{183} and Gly^{233} or of Pro^{312} in the transmembrane portion (Andersen et al., 1989; Vilsen et al., 1989; Clarke, 1990b).

1.2 IMPAIRMENT OF CA^{2+} RELEASE AND CA^{2+} UPTAKE FUNCTIONS OF THE SARCOPLASMIC THROUGH METABOLIC PERTURBATIONS: A MECHANISM IN THE DEVELOPMENT OF MUSCLE FATIGUE

At the periphery, on the post-synaptic side of the neuromuscular junction, the mechanism responsible for the initiation of muscle contraction is the conduction of action potentials by the sarcolemma (Fuglevand, 1995). These action potentials activate voltage sensors (dihydropyridine receptors) on the T-tubular membrane, leading to a release of Ca^{2+} by RyR receptors on the junctional face membrane of the SR. The mechanism that has been retained as most plausible to explain this electro-mechanical coupling is the intramembrane charge movement which suggests that charged particles are responsible for the transduction of the intracellular signal between the DHP and ryanodine receptors (Schneider & Chandler, 1973; Melzer et al., 1995). The released Ca^{2+} binds to specific sites on the troponin C (TnC) to regulate tension production by the contractile apparatus. Ten to 20 ms after the increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) from 10^{-7} to 10^{-5} M, the Ca^{2+} is re-sequestered by the Ca^{2+}-Mg^{2+}-ATPase pump of the longitudinal SR, bringing $[Ca^{2+}]_i$ back to pre-contraction levels and causing dissociation of the actin-myosin cross-bridges to occur.

Potential metabolic correlates of muscle fatigue have included adenosine diphosphate (ADP), adenosine monophosphate (AMP), monoprotonated phosphate (HPO_4^{2-}), diprotonated phosphate ($H_2PO_4^-$), hydrogen ion (H^+), ammonium ion (NH_4^+), potassium ion (K^+), magnesium ion (Mg^{2+}), lactate ion (Lac^-) or any of a number of glycolytic intermediates (Cooke & Pate, 1990). Evidence linking metabolites with

impaired performance has accumulated over the years through the use of the needle biopsy technique (Hultman et al. 1985), Magnetic Resonance Spectroscopy (Miller et al., 1987); whole muscle *in vitro* preparations (Renaud & Mainwood, 1985), intact (Westerblad & Lännergren, 1988) and single skinned (Godt & Nosek, 1989) fibre preparations, vesicle fraction preparations (Favero et al., 1995) and incorporation of vesicles into lipid bilayers (Rousseau & Pinkos, 1990), and homogenate preparation (Dossett-Mercer et al., 1994).

The majority of investigators interested in understanding the role of metabolic mediated perturbations on SR function, have conducted experiments using the above-mentioned preparations (single intact fibres, skinned fibres, vesicle fractions, lipid bilayers and homogenate). The following review examines the evidence linking metabolic disturbances to impairment in SR function by focusing on research done with these experimental preparations.

1.2.1 Evidence of metabolic mediated disturbances of SR functions from experiments with single intact fibre preparation

Since the t-tubular membrane and the SR are responsible for the propagation of the electrical impulse and the regulation of cytosolic Ca^{2+}, monitoring the Ca^{2+} transient during the contractile event using intact fibres is a pertinent technique for assessing the effect of repetitive contractions on muscle tension. The preparation of intact amphibian and mammalian muscle fibre has proven to be well suited for this kind of experiment since it offers the possibility of measuring $[Ca^{2+}]_i$ with Ca^{2+}-fluorescent dyes while monitoring the change in tension (Westerblad & Allen, 1996). The basic postulate on which this work relies is that $[Ca^{2+}]_i$ has a sigmoidal relationship with

tension and that it is determined by three processes: the release of Ca^{2+} by the RyR, the rate of uptake by the Ca^{2+}-Mg^{2+}-ATPase and myoplasmic buffering (Westerblad & Allen, 1996).

Pronounced changes in the intracellular environment follow sustained activation of the muscle fibre and these changes can produce a reduction in mechanical performance (Stephenson et al., 1995). The experimental approach chosen by the laboratories of Westerblad, Lännergren and Allen with the intact single fibre preparation consists first in identifying the main perturbations (e.g. reductions in pH) that are produced by the chosen contractile challenge (e.g. intermittent stimulation) and second examining how these perturbations alter the above three processes.

In the single intact preparation, evidence of the effect of metabolic disturbances on tension generation can be observed in both *Xenopus* (Allen et al., 1991; Lee et al., 1991) and in mouse single intact fibre (Westerblad & Allen, 1993b) preparations in the early stages of fatigue following intermittent stimulation. This review focuses more on the mouse fibre preparation, since rats were used for the experimental work of this thesis. In mouse fibre, tetanic tension remains depressed despite the addition of caffeine (Westerblad & Allen, 1993b). Caffeine is a well known potentiator of contractile tension (Weber & Herz, 1968; MacIntosh & Gardiner, 1987), resulting in an increase in $[Ca^{2+}]_i$ through three mechanisms:(1) a potentiation of Ca^{2+} release by the RyR (Rousseau et al., 1988a), (2) an increased sensitivity of the myofilament to Ca^{2+} (Wendt & Stephenson, 1983), and (3) an inhibition of Ca^{2+}-uptake (Koshita & Oba, 1989).

The lack of change in tension in response to the increased $[Ca^{2+}]_i$ is interpreted as a reduction in myofilament sensitivity (Lännergren & Westerblad, 1991; Westerblad & Allen, 1991). In the single intact mouse fibre, since intracellular pH is not significantly reduced, elevation in the by-products of ATP hydrolysis, especially Pi through the breakdown of creatine phosphate (CPr), are suggested to be the main inhibitor of contractility during this early fatigue phase (Westerblad & Allen, 1992a). The possibility that pH plays a more important role is nevertheless underlined in experiments where a reduction in pH is induced through CO_2 bubbling of the buffer solution (Mainwood & Renaud, 1985). The reduction in pH in this case produces an increase in $[Ca^{2+}]_i$ that is suggested to be caused by a reduced myoplasmic buffering and slower uptake kinetics (Westerblad & Allen, 1993a). The accompanying reduction in tension in the face of the increase in $[Ca^{2+}]_i$ is again explained by a decrease in myofibrillar sensitivity to Ca^{2+}, due to a reduced troponin binding constant for Ca^{2+} (Allen et al., 1995).

In the later stages of fatigue, the fall in $[Ca^{2+}]_i$, despite slower rate of uptake and reduced myoplasmic buffering, suggests an impairment in Ca^{2+} release (Westerblad & Allen, 1993b). Experiments with skinned fibre (Lamb & Stephenson, 1991) and vesicle preparations (Meissner et al., 1986) indicate that Mg^{2+} inhibits the release of Ca^{2+}. The concentration of diffusible Mg^{2+} in the myoplasm is 6 mM, and 4-5 mM is bound to ATP to form the physiological substrate Mg . ATP (Lamb & Stephenson, 1992). Tetanic tension was reduced by 50 % when Mg^{2+} was injected in mouse fibres (Westerblad & Allen, 1992b). However, this reduction in tension took place when

myoplasmic $[Mg^{2+}]_i$ was raised from 0.8 to 2.9 mM (Westerblad & Allen, 1992b). Lamb and Stephenson (1992) estimated that the $[Mg^{2+}]_i$ would not reach more than 0.4 mM during a tetanic contraction, unless PCr, which acts as a buffer, was depleted. Despite this finding, Westerblad and Allen (1992b) concluded that the increase in $[Mg^{2+}]_i$ was too small to explain the reduction in tension. Instead of $[Mg^{2+}]_i$, it was suggested that the reduction in tension was related to a 30 % decrease in [ATP] that could affect the Ca^{2+}-Mg^{2+}-ATPase pump. A reduction of Ca^{2+} in the lumen of the SR through binding with Pi (Fryer et al., 1995) has also been suggested to be an inhibitory factor.

Recently, the single intact fibre model has been used to study low frequency fatigue (LFF) (Westerblad et al., 1993; Lännergren et al., 1996). LFF was first identified by Edwards et al. (1977) when it was observed that tension elicited at low frequencies of stimulation (10-20 Hz) were relatively more depressed than tension elicited at high frequencies (50-100 Hz) of stimulation following repetitive contractions. Low- and high-frequency fatigue (HFF) are also characterised by a different recovery schedule. In single mouse fibre, LFF is very slow and has only recovered to 1/3 of initial tension after 60 min, whereas the recovery of tension elicited by high-frequency is much faster (Lännergren et al., 1996). LFF was explained by a reduction in Ca^{2+} release per stimulation pulse (Edwards et al., 1977). HFF appears to be due to a transmission block at the neuromuscular junction or an impairment in conduction along the sarcolemma and t-tubule (Bigland-Ritchie et al., 1979), although this conclusion has been challenged (Metzger & Fitts, 1986).

The results of single intact fibre experiments, confirm the initial hypothesis by showing that $[Ca^{2+}]_i$ was reduced, but that maximal Ca^{2+}-activated tension was unchanged with LFF (Westerblad et al., 1993). Sensitivity of the myofilament was unchanged and the uniform spatial gradient of $[Ca^{2+}]_i$ (Westerblad et al., 1990) suggests that the conduction of the signal by the t-tubular membrane is not impaired (Westerblad et al., 1993). SR pump function determined through the tail of elevated Ca^{2+} decline left by the $[Ca^{2+}]_i$ signal (Klein et al., 1991) suggests a diminished rate of uptake.

The sigmoidal relationship between tension and stimulation frequency explains why a small decrease in $[Ca^{2+}]_i$ causes a large reduction in tension at low frequencies of stimulation (Lännergren et al., 1996). Stimulation at 100 Hz produces a $[Ca^{2+}]_i$ that lies on the horizontal plateau of the frequency-tension relationship. At low frequencies of stimulation, the $[Ca^{2+}]_i$ lies on the steepest part of the curve and a small change results in a pronounced drop in tension (Lännergren et al., 1996).

The long time period required for the recovery of tension suggests that accumulation of metabolites does not play a critical role in this reduced uptake function. The resynthesis of PCr which takes place within 5-10 min of the cessation of activity (Baker et al., 1993) and suggests that Pi, if any, plays only a minimal role as an inhibitor of the Ca^{2+}-Mg^{2+}-ATPase.

However, longer lasting structural alterations in the Ca^{2+}-Mg^{2+}-ATPase pump protein induced by acute elevation of metabolites during contraction cannot be ruled out (William & Klug, 1995). The presence of a glycogenolytic complex (aldolase and glyceraldehyde-3-phosphate dehydrogenase) in the gap junction between the DHP and

RyR receptors (Brandt et al., 1990) suggests a high enzymatic activity in proximity of proteins that could be affected by the high turnover rate of the metabolic by-products generated by these enzymes (see Lännergren et al., 1996).

However, non-metabolic mechanisms cannot be ignored to explain the loss of tension with LFF. Mechanical stress imposed by stretching the fibre to 120 % of resting length abolished the capacity to generate pre-stretching tension for 10 h (Bruton et al., 1995). It is interesting to note that LFF is observed in human subjects that have performed a regimen of eccentric contractions, where the muscle is stretched against a resistance (Jones et al., 1996). Damage to protein structure could also occur through a temporary increase in $[Ca^{2+}]_i$. In an interesting study, a stimulation protocol that did not induce fatigue by itself resulted in the development of LFF when caffeine was present in the medium (Chin & Allen, 1996). Damage by the elevated $[Ca^{2+}]_i$ could be mediated by the activation of Ca^{2+} proteases, phospholipases or calmodulin (see Lännergren et al., 1996) but more studies are needed to confirm the relative importance of these mechanisms.

1.2.2 Evidence of metabolic mediated disturbances of SR functions from experiment with skinned fibre preparation

The effect of metabolic perturbations on ECC have been studied using skinned muscle fibre preparations where the composition of the intracellular environment can be controlled (Stephenson, 1981; 1989). The release of Ca^{2+} with this preparation is accomplished through depolarisation of the t-tubular membrane by ionic substitution (Stephenson, 1981; 1989). Ionic substitution of Na^+ for K^+, instead of choline chloride substitution has been demonstrated to stimulate Ca^{2+} release more rapidly and has very

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little electrical or osmotic effects on the Ca^{2+} release channels (Lamb & Stephenson, 1990).

The effect of $[Mg^{2+}]$ investigated with this preparation using either skinned fibres of toad (Lamb & Stephenson, 1991) or rat (Lamb & Stephenson, 1994) was shown to inhibit Ca^{2+} release induced by the ionic depolarisation. Reducing $[Mg^{2+}]$ from 1 to 0.05 mM induced a strong release of $[Ca^{2+}]_i$ in the presence of ATP. The effect was suggested to be dependent upon the RyR, since no release was observed when the Ca^{2+}-release channel blocker ruthenium red was used. In addition, involvement of the DHP receptor was rejected on the basis that a similar Ca^{2+} release was obtained with inactivated DHP receptors (Lamb & Stephenson, 1991). Complete inhibition of Ca^{2+} release was observed with 10 mM $[Mg^{2+}]$ (Lamb & Stephenson, 1991; Lamb & Stephenson, 1994).

Calcium release through ionic depolarisation was unaffected at a pH of 6.2 in both types of muscle fibres (Lamb et al., 1992; Lamb & Stephenson, 1994). A single 2-3 s depolarisation was sufficient to release all the stored Ca^{2+} (Lamb & Stephenson, 1994). This result contradicts the findings of vesicle fraction and bilayer experiments (Ma et al., 1988; Rousseau & Pinkos, 1990) (see below).

Skinned fibre experiments conducted with cardiac muscle have also shown an effect of metabolites on Ca^{2+}-Mg^{2+}-ATPase function (Zhu & Nosek, 1991). In an ATP-free (rigor) solution mimicking the intracellular milieu found during long-term hypoxia, a 44-48 % reduction in Ca^{2+} was found. The reduction was suggested to come from the reversal of the Ca^{2+}-Mg^{2+}-ATPase pump (Zhu & Nosek, 1991).

1.2.3 Evidence of metabolic mediated disturbances of SR functions from experiment with vesicle fractions, vesicle in lipid bilayers and homogenate preparation

Vesicle fractions isolated by zonal and differential centrifugation can be used to study the kinetics of Ca^{2+} release (Meissner et al, 1986), $Ca^{2+}-Mg^{2+}-ATPase$ activity (Meissner, 1973) and Ca^{2+} uptake (Palade et al., 1989) in the presence of various compounds. Calcium release can also be studied through reconstitution of isolated channels in lipid bilayers (Smith et al., 1985).

Calcium release was found to be inhibited in vesicles (Meissner & Henderson, 1987) and isolated channels (Ma et al., 1988; Rousseau & Pinkos, 1990; Favero et al., 1995) at pH values around 6.5. Lowering pH reduced the release rate of $^{45}Ca^{2+}$ efflux from heavy SR vesicles (Meissner & Henderson, 1987). The reduction was attributed to a decrease in the probability that the Ca^{2+} channels be open. Similar conclusions were found when the *cis*-chamber (cytoplasmic side) of a planar lipid bilayer preparation was acidified from a pH of 7.4 to 6.6 (Rousseau & Pinkos, 1990; Favero et al., 1995). [3][H]-ryanodine-binding to the release channels was reduced when pH was decreased from 7.1 to 6.5 (Favero et al., 1995). This suggests that the H^+ acts by decreasing the binding of Ca^{2+} to modulating sites (Rousseau et al., 1988a) on the cytoplasmic domains of the channels. The modulating effect of pH may be more refined if the changes are localised in the triadic gap (Dettbarn & Palade, 1991). Countertransport of H^+ into the SR during Ca^{2+} release may create a local alkalinization that inactivates the release while the increased H^+ concentration in the lumen may interact directly with the release pore (Rousseau & Pinkos, 1990). The unitary conductance of the channel was reduced when

the concentration of H^- in the *trans*-chamber (luminal side) was increased. Favero et al., (1995) also found that lactate inhibited Ca^{2+} release and $^3[H]$-ryanodine binding.

Calcium on its own is not considered to be a potent activator of release, but following the release by flash photolysis of caged Ca^{2+}, in the absence of ATP or Mg^{2+}, the release channels were shown to be quickly activated (Györke et al., 1994). However, the increase in $[Ca^{2+}]$ could be an artefact of the photolysis procedure (Stephenson et al., 1995).

Adenine nucleotides increase, whereas Mg^{2+} inhibit, the release of Ca^{2+} from the channels in reconstituted systems (Meissner et al., 1986, Rousseau et al., 1986; Smith et al., 1986; Meissner & Henderson, 1987; Favero et al., 1995). The inhibition due to H^+ and Mg^{2+} appeared to be additive (Favero et al., 1995). In the presence of Mg-ATP the Ca^{2+} sensitivity of the channel gating mechanism is increased (Rousseau et al., 1992). Ca^{2+} release appears to be activated without discrimination by any components of adenine nucleotide family (Westerblad et al., 1991). The ATP does not have to phosphorylate the RyR to affect the channel, since the non-hydrolysable analog AMP-PCP stimulates Ca^{2+} release (Rousseau et al., 1992). Physiological concentration of inorganic phosphate (Pi) increased Ca^{2+} release from vesicles by 50 % and $^3[H]$-ryanodine binding by 84 % (Fruen et al., 1994). The open probability of channels reconstituted in planar lipid bilayers increased by 91 %.

Calmodulin can activate multi-functional protein kinase and has been found to be an inhibitor of Ca^{2+} release in vesicle fractions (Meissner, 1986; Smith et al., 1989). It has been suggested that during repetitive activity, it could play a role similar to Mg^{2+},

slowing the release of Ca^{2+} to compensate for the increased intracellular Ca^{2+} concentration (Meissner, 1986; Smith et al., 1989). Finally, it could activate a Ca^{2+} dependent-phosphatase that would dephosphorylate a regulatory site on the channel.

The kinetics of the Ca^{2+}-Mg^{2+}-ATPase has also been extensively studied in vesicle fractions (De Meis & Vianna, 1977; Henderson et al., 1994a). The depression in Ca^{2+}-Mg^{2+}-ATPase activity at low pH is explained by a decrease in the affinity for Ca^{2+} of both the low- and high-affinity Ca^{2+} binding sites (Meissner, 1973; Verjovski-Almeida & de Meis, 1977) as well as a reduced cooperativity of Ca^{2+} binding (Hill & Inesi, 1982; Henderson et al., 1994a). The reduced affinity is explained by a competition between H^+ and Ca^{2+} for the Ca^{2+} binding sites (Pick & Karlish, 1982; Henderson et al., 1994). Magnesium in high concentrations (Guillain et al., 1982; Forge et al., 1993a) has been shown to have an inhibitory effect on Ca^{2+}-Mg^{2+}-ATPase activity by competing for the high affinity Ca^{2+} binding sites (Guillain et al., 1982; Forge et al., 1993a) and lowering cooperativity between the two sites (Forge et al., 1993a).

The concentration of Pi inhibits Ca^{2+}-Mg^{2+}-ATPase activity (Stienen, 1993) and Ca^{2+} uptake (Perlitz et al., 1990). Pi can also accumulate inside the lumen and form a phosphate complex ($CaHPO_4$) (Palade et al., 1989; Fryer et al., 1995) that reduces product back inhibition and favours Ca^{2+}-Mg^{2+}-ATPase activity and Ca^{2+} uptake (Korge & Campbell, unpublished). Therefore, whether Pi inhibits or activates the Ca^{2+}-Mg^{2+}-ATPase and Ca^{2+} uptake depends on the loading time of Pi by the lumen (Zhu & Nosek, 1991) or in vesicle preparation, the presence of precipitating anions like oxalate (Korge & Campbell, unpublished).

In vitro preparations of vesicle fractions and homogenates have also been used to assess Ca^{2+}-Mg^{2+}-ATPase activity and Ca^{2+} uptake (Byrd et al., 1989; Luckin et al., 1991; Belcastro et al., 1993; Chin & Green, 1996) as well as Ca^{2+} release (Favero et al., 1993) following exercise. A homogenate preparation (Simonides & van Hardeveld, 1990) has also been used to examine the effect of short-term high intensity exercise on SR pump function (Dossett-Mercer et al., 1994). In these experiments, the assay is performed under optimal conditions and it is assumed that exercise induces longer lasting structural changes in Ca^{2+}-Mg^{2+}-ATPase structure (Luckin et al., 1991; McCutcheon et al., 1992) that can be detected later.

The results for the effects of exercise on SR Ca^{2+} sequestration properties and particularly on Ca^{2+}-Mg^{2+}-ATPase activity remain controversial. A depression in Ca^{2+}-Mg^{2+}-ATPase activity has been reported following single bouts of exhaustive exercise by some investigators (Belcastro et al., 1981; Byrd et al., 1989a; Byrd et al., 1989b, Luckin et al., 1991; Belcastro et al., 1993), whereas others have been unable to show any alterations in activity (Fitts et al., 1982; Dossett-Mercer et al., 1994; Chin & Green, 1996).

1.3   STATEMENT OF THE PROBLEM

The purpose of the first two investigations was to examine the effects of contraction induced metabolic mediated disturbances on the structures responsible for the regulation of cytoplasmic Ca^{2+} concentration, namely the Ca^{2+} release channels and the Ca^{2+}-Mg^{2+}-ATPase of the SR. The purpose of the third investigation was to examine

the controversy surrounding the effects of prolonged exercise on the Ca^{2+}-Mg^{2+}-ATPase activity, focusing, particularly on the role of the isolation procedure.

1.3.1 Sub-problems

(i) Study 1

1. To examine the effect of caffeine mediated release of Ca^{2+} during electrical intermittent stimulation by monitoring contractile properties.

2. To examine the effect of caffeine mediated release of Ca^{2+} during the recovery of mechanical function.

3. To determine the role played by acidosis and specific by- products of high phosphate metabolism on caffeine Ca^{2+}-mediated release and recovery of mechanical function.

(ii) Study II

1. To determine if there is a difference in the dependency of H^+, ATP and Ca^{2+} on the Ca^{2+}-Mg^{2+}-ATPase between homogenates from three muscle fibre types with different proportions of SERCA 1 a and SERCA 2a isoforms.

(iii) Study III

1. To determine if the selectivity of the isolation protocol used to obtain an enriched vesicle fraction of sarcoplasmic reticulum could be a contributing factor to explain the discrepancy in results that exists in the literature

concerning the effect of prolonged exhaustive exercise on Ca^{2+}-Mg^{2+}-ATPase activity.

2. To determine if there is a difference in Ca^{2+}-Mg^{2+}-ATPase activity of muscle tissue between homogenates and sub-fractions of control and run animals.

3. To determine if there is a difference in purity between the homogenates and sub-fractions of control and run animals by analysing marker enzymes for mitochondrial and sarcolemmal contamination.

1.4 HYPOTHESES

The general hypothesis is that metabolic perturbations modulate the regulation of $[Ca^{2+}]_i$ by acting on the Ca^{2+} release channels and the Ca^{2+}-Mg^{2+}-ATPase of the SR. The sub-hypotheses are as follows:

(i) Study 1

1. The inhibition of the Ca^{2+} release is an important mechanism in the development of LFF as a consequence, caffeine should reverse in part the effects of fatigue. The recovery will be only partial because of the presence of Pi and its inhibitive effect on cross-bridge kinetics. For HFF, tension will be less depressed than for LFF. The depression will depend on the by-products of high-energy phosphate metabolism. Caffeine will have no further effect on tension since $[Ca^{2+}]_i$ is already high with HFF..

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2. LFF will not recover following a 5 min passive recovery period without the presence of caffeine, suggesting that factors other than the by-products of high energy phosphate metabolism, such as H^+, are responsible for the reduction in tension. The recovery in the presence of caffeine will be complete since the by-products of high energy phosphate metabolism will not impede cross-bridge kinetics. HFF will recover, at least in part, during the 5 min recovery period and caffeine will have no effect for the same reasons mentioned in (1).

3. LFF will partly recover following the 20 min period of anoxia because of the dissipation of H^+, but like the first condition, tension will be partly depressed because of the inhibition of cross-bridge kinetics by the by-products of the high energy phosphate metabolism maintained by the anoxia. The effect of caffeine will be less pronounced than in the first condition because of the recovery of the pH. HFF will recover and caffeine will have no effect for the reasons mentioned above.

(ii) Study II

1. The Ca^{2+}-Mg^{2+}-ATPase activity will be reduced under conditions of low pH, low ATP and high Ca^{2+} concentrations.

2. The depression in Ca^{2+}-Mg^{2+}-ATPase will be less pronounced in muscle fibre types containing a higher proportion of type I fibres than in muscles

predominantly composed of type IIb fibres, reflecting a lower sensitivity by SERCA 2a isoform to *in vitro* metabolic perturbations.

(iii) Study III

1. Exercise will predispose the homogenate to a differential effect by the isolation procedure. The Ca^{2+}-Mg^{2+}-ATPase activity will be lower in selected sub-fractions isolated from the exercised animals as a result of the effect of the homogenisation procedure.

2. Contamination profiles will reveal no difference between the control and exercised groups, suggesting that altered purity is not the reason for the difference in Ca^{2+}-Mg^{2+}-ATPase activity observed between the control and exercised group.

1.5 DELIMITATIONS

(i) Study I

1. The animals used in the first study were Wistar rats.

2. The rats were kept on a reverse 12:12 light/dark cycle.

3. Fatigue was induced by 15 Hz stimulation to mimic the average physiological firing frequency of motor units of the diaphragm.

4. Measurements of mechanical function were limited for twitch contractile properties: peak twitch tension, contraction time, half-relaxation time, rate of tension development and rate of tension decline. For tetanic contractile properties: tetanic tension, rate of tension development and rate of tension decline at 10, 20, 50 and 100 Hz were assessed.

5. Assessment of the effect of caffeine on Ca^{2+} release was limited to measurements of contractile function.

6. The diffusion of metabolites out of the muscle and of oxygen and caffeine to the muscle were specific to the *in vitro* diaphragm preparation.

(ii) Study II

1. The animals used in the study were male Sprague-Dawley rats.

2. Measurements of SR function were limited to Ca^{2+}-Mg^{2+}-ATPase activity in homogenates.

(iii) Study III

1. The animals used in the study were male Sprague-Dawley rats.

2. Fatigue was induced by treadmill running to exhaustion. The criteria for exhaustion was determined by the inability of the animal to avoid the shock grid.

3. Measurements of SR function were limited to Ca^{2+}-Mg^{2+}-ATPase activity.

4. Contamination of homogenates and sub-fractions was limited to measurements of p-nitrophenyl phosphatase (sarcolemmal) and citrate synthase (mitochondria).

1.6   LIMITATIONS

(i) study I

1. In the diaphragm *in vitro* preparation, the development of neuromuscular fatigue was limited to peripheral sites on the post-synaptic side of the neuromuscular junction.

(ii) Study II

1. The tissue separation of the superficial and deep compartment of the gastrocnemius medialis was done by visual inspection. The use of portions of muscle compared to single fibres reduces the purity of the sample.

2. Homogenates of the muscles were frozen and assayed later for Ca^{2+}-Mg^{2+}-ATPase activity. The Ca^{2+} activated activity in homogenates has been shown to be unaffected by freezing (Simonides & van Hardeveld, 1990; Chin et al., 1994). Although basal activity was found to be reduced (Chin et al., 1994) this reduction was only a negligible part of the total activity (Chin et al., 1994)

3. Free calcium concentrations were determined through the use of a calcium software package (Chelator, Schoenmakers, 1992). Numerous factors such as the contamination, the purity of the chelator, and the pH limit the accuracy of Ca^{2+} concentrations determined through a software program (For a detailed discussion on the determination of free calcium please see Appendix IIA).

(ii) Study III

1. A 5-10 min delay was necessary for sampling tissue following the point of exhaustion.

2. After homogenisation of the tissue sampled from the run animals, a delay of approximately 1 h was necessary for sampling and homogenisation of the control animal tissue.

3. Some delays were also encountered between steps of the isolation procedure to enable the suspension and freezing of intermediary sub-fractions. However, the supernatant was maintained in the ultracentrifuge below 4° C during these delays and the delays were similar for sub-fractions from both the control and run animals.

4. Animals were anaesthetised using sodium pentobarbital which has been shown to affect Ca^{2+}-Mg^{2+}-ATPase activity of isolated SR vesicles (Fernandez-Salguero et al., 1990) at high drug concentrations. Similar

dosages were used for control and run animals. (For more details please see Chin, 1993)

1. 7   LIST OF ABBREVIATIONS

ADP - adenosine diphosphate

AMP - adenosine monophosphate

ANOVA - Analysis of Variance

Arg - arginine

Asp - aspartate

ATP - adenosine triphosphate

Ca^{2+} - calcium ion

Cl^- - chloride ion

CSA - cross-sectional area

CT - contraction time

DHP -dihydropyridine

DNA -desoxyribonucleique acid

$+dP_t/dt_{max}$ - maximal rate of tension development

$-dP_t/dt_{max}$ - maximal rate of tension decline

EGTA - ethylene glycol-bis(ß-amino-ethyly ether)-N,N,N',N'-tetraacetic acid

ECC - excitation contraction-coupling

Glu - glutamine

Gly - glycine

g - gram

H^+ - hydrogen ion

Hom - homogenate

HPO_4^{2-} - monoprotonated phosphate

$H_2PO_4^-$ - diprotonated phosphate

Hz - Herz

K^+ - potassium ion

kDA - kilo Dalton

Lac^- - lactate ion

Lys - lysine

M - mole

mm - millimeter

mM - millimole

mg - mg

Mg^{2+} - magnesium

mV - millivolt

NaN_3 - sodium azide

Na^+ - sodium ion

NH_4^+ - ammonium ion

nm - nanometer

nM - nanomole

P_t - twitch tension

pCa - negative logarithm for base 10 ($\log_{10}$) of $[Ca^{2+}]$

PCr - phosphocreatine

pH - negative logarithm for base 10 ($\log_{10}$) of $[H^+]$

Pi - inorganic phosphate

Pro - proline

Q_{10} - relative increase in enzyme activity for a 10° C increase in temperature

RGM - red gastrocnemius medialis

$RT_{1/2}$ - half-relaxation time

RyR - ryanodine receptor

S - soleus

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SR - sarcoplasmic reticulum

TCSA - tension per cross-sectional area

Thr: threonine

Troponin C - TnC

V_{max} - maximal enzyme activity

Val - valine

WGM - white gastrocnemius medialis

μM: micromole

2MS - 2 min stimulation

5MR - 5 minute recovery

20MR - 20 minute recovery

CHAPTER II

ROLE OF CAFFEINE IN MEDIATING RECOVERY IN SHORT TERM STIMULATION OF THE *IN VITRO* DIAPHRAGM.

2.0 ABSTRACT

The role of sarcoplasmic reticulum Ca^{2+} release in fatigue was examined during and following fatigue induced by repetitive stimulation. Rat diaphragms were subjected initially to a 3 min stimulation protocol (15 Hz; 250 ms; 50 % duty cycle) designed to produce fatigue and metabolic perturbations (decreases in ATP and PCr and increases in Pi and H^+. The repetitive stimulation protocol (15 Hz; 250 ms; 50 % duty cycle) reduced twitch tension (P_t) by 44 % whereas tension at 100 Hz (P_{100Hz}) by % ($P <$ 0.05). Subsequently, caffeine was used to induce Ca^{2+} release under 3 different conditions in order to examine the role of specific intracellular metabolites. The three conditions were: (1) 2 min of additional stimulation (2MS); (2) 5 min of passive recovery (5MR) and (3) 20 min of anoxic recovery (20MR). Each animal provided two diaphragm muscle strips. One diaphragm strip was exposed to caffeine and the other served as a control. In all six different groups were studied (caffeine-2MS, caffeine-5MR, caffeine-20MR, no caffeine-2MS, no caffeine-5MR and no caffeine-20MR).

Results from the 2MS group indicated that the fatigue observed at low frequencies (LFF) was partially reversed by caffeine (35 %; $P < 0.05$). However, the less than full recovery (74 % compared to the resting conditions) suggested that part of the reduction in tension ($P < 0.05$) was due to an impaired cross-bridge activation. In contrast, with high-frequency fatigue (HFF), caffeine exaggerated the reduction in

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tension ($P < 0.05$) compared to no caffeine suggesting that the $[Ca^{2+}]_i$ was reduced beyond that induced by the stimulation only.

For the 5MR, the recovery period was not sufficient to reverse the effect of LFF in the control (no caffeine) diaphragm suggesting that H^+ may be an important factor responsible for the fatigue. Caffeine mediated recovery produced increases in tension ($P > 0.05$) that were higher than the non-stimulated control, suggesting that no inhibition at the cross-bridges was present and that the 5 min period was sufficient to allow resynthesis of PCr and [Pi] to return to resting levels. This finding also suggested that with LFF, caffeine was capable of neutralizing the inhibition placed on the Ca^{2+} release channels by H^+, and that differences in Ca^{2+} sensitivity of the myofibrillar apparatus occurred. The fast recovery in P_{100} during the 5 MR ($P > 0.05$) confirmed the presence of HFF. Caffeine had no effect on tension, suggesting that the $[Ca^{2+}]_i$ was already sufficient to fully saturate the regulatory proteins

No recovery in tension was observed for the 20MR for either P_t or P_{100Hz}. The 20 min time period would have allowed sufficient time for the dissipation of most of the H^+ leaving a suppressed phosphorylation potential (and increased by-products), as the most probable mechanism for the reduced tension. The lack of an effect of caffeine on recovery could be the result of a depletion of the SR Ca^{2+} stores due to high [Pi] and [ADP] (Zhu & Nosek, 1991). In conclusion, although transient increases in H^+ and Pi may impair Ca^{2+} release and tension recovery, other factors also appear to be implicated.

2.1 INTRODUCTION

At the muscle level, the development of tension is the result of two physiological processes. First, an activation process referred to as excitation-contraction coupling (ECC) acts to convert an action potential delivered by the t-tubule membrane to a Ca^{2+} flux generated by the sarcoplasmic reticulum (SR). Second, a mechanical process referred to as the cross-bridge cycle occurs whereby the myofilament transforms the excitation into a mechanical parameter that represents the generated tension. Muscular fatigue, defined as the inability to generate a required or expected tension (Edwards, 1981) has been attributed to a disruption in the mechanisms of either one or both of these two processes.

A contractile parameter that has been shown to involve the SR in tension failure is low-frequency fatigue (LFF) (Edwards et al., 1977). LFF is characterized by a more pronounced and longer lasting tension depression at low rather than at high frequencies of stimulation following repetitive contractions (Edwards et al., 1977; Wiles et al., 1983; Westerblad et al., 1993). LFF does not appear to be the result of a problem with the contractile elements since tension produced by high frequency stimulation is only slightly depressed, suggesting at this level of activation that the Ca^{2+} transient is maintained and that cross-bridge kinetics are not impaired. A reduction in the amount of Ca^{2+} released per stimulation pulse has been suggested to be the mechanism responsible for the depression (Edwards et al., 1977). Further evidence that tends to suggest that Ca^{2+} release is the problem behind the depression is the total or partial recovery in tension that is observed with the use of methylxanthines such as caffeine (Moxham et al., 1980; MacIntosh & Gardiner, 1987). The possible mechanisms by which tension is

reestablished with caffeine is through either (i) an increased sensitivity of the myofilament to Ca^{2+} (Wendt & Stephenson, 1983; Allen & Westerblad, 1995), (ii) an inhibition of Ca^{2+} uptake (Koshita & Oba, 1989; Fryer & Neering, 1989; Allen & Westerblad, 1995), and (iii) through a potentiation of Ca^{2+} release from the SR and an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Jones et al., 1982; Nassar-Gentina et al., 1981). More recent experiments using fluorescent Ca^{2+} dyes in single intact fibre preparations which enable both tension and the $[Ca^{2+}]_i$ to be simultaneously monitored, have confirmed that the reduction in tension is directly coupled to alterations in $[Ca^{2+}]_i$ and that these alterations are probably due to a reduced Ca^{2+} release from the sarcoplasmic reticulum (Westerblad et al., 1993).

The caffeine experiments and the single intact fibre experiments suggest that the loss of tension is related to low cytosolic level of Ca^{2+} and that the lower Ca^{2+} concentration does not depend on the depletion of luminal Ca^{2+} stores. However, these experiments do not reveal the cause of the reduced release or which step of the release mechanism is disrupted by the repeated activity.

The opening of the Ca^{2+} release channels is inhibited by changes in selected metabolites that occur during muscle fatigue such as a reduction in adenine nucleotides (Meissner et al., 1986), an increase in Mg^{2+} concentration (Favero et al., 1995) and a reduced pH (Rousseau & Pinkos, 1990). However, a metabolite mediated disturbance of Ca^{2+} release function in LFF is questionable since metabolic changes appear to recover more quickly than tension (Westerblad et al., 1993).

The overall aims of this study were to examine the role of sarcoplasmic reticulum Ca^{2+} release in both fatigue induced by repetitive stimulation and during

recovery from fatigue and to isolate the role of specific extracellular metabolites in Ca^{2+} release and recovery of mechanical function. Three sets of experiments were designed to address these purposes.

The aim of the first set of experiments was to induce a reduction in tension and a perturbed metabolic environment, and then to examine the significance of impaired Ca^{2+} release, using caffeine, on the disturbance in mechanical function that occurred. It was hypothesized that inhibition of the Ca^{2+} release channels is an important mechanism in this type of fatigue.

A first objective of the second set of experiments was to isolate which of the metabolic by-products, namely those resulting from high-energy phosphate metabolism (Pi, ADP, AMP) or from glycolysis (H^+) inhibit Ca^{2+} release. A second objective was to observe to what extent caffeine is capable of reversing these effects. To examine the role of acidosis, a 5 min recovery period was used to provide for the recovery of the phosphorylation potential without dissipation of the acidosis (Hood & Parent, 1991). It was hypothesized that tension would not recover in the acidic condition, but that it would be restored with caffeine.

To examine the role of the high-energy phosphate metabolites, a 20 min passive anoxic recovery period was provided to prevent recovery of the high-energy phosphate potential while at the same time enable lactate to diffuse out of the cell and permit pH to substantially recover. It was hypothesized that the fatigue induced by the stimulation would persist during the anoxic period and that the addition of caffeine would have minimal effect.

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2.2 METHODS

2.2.1 Animal care

The twenty-six adult male Wistar rats used for this study were housed in an environmentally controlled room (27° C) with a reversed 12 :12 light/dark cycle and fed on a diet of laboratory chow (Purina rat chow) and water *ad libitum*. Animal care was in accordance with the guidelines established by the Canadian Council on Animal Care and the study was approved by the University of Waterloo Animal Ethics Committee.

2.2.2 Removal and preparation of diaphragm

The rats were anesthetized with ether and the diaphragm was quickly excised. The excised diaphragm was placed in a petri dish containing cold (4-10° C) oxygenated (95% O_2- 5 % CO_2) Krebs solution : 137 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, and 11 mM glucose (Faulkner et al., 1982). Two costal strips of muscles (~ 3 mm wide) were cut parallel to the muscle fibre with a portion of rib left attached at one end and a piece of tendon at the other end. The inferior portion of one of the diaphragm strips was anchored by a small Plexiglass micro-clamp and its tendon end was attached to a force-displacement transducer (Cambridge Technologies, model 300H) with a length of silk thread (4-0). Two silk threads were tied at the tendon-thread and transducer thread junction to eliminate the influence of thread elasticity on force measurements (Kyle, 1988). The second diaphragm strip was prepared in a similar fashion but the silk thread was secured to a weight suspended around a pulley to maintain a passive tension of approximately 2 g,

which has been shown to enhance the viability of the muscle (Goldberg et al., 1975). Both strips were placed in jacketed baths (280 ml) containing oxygenated Krebs solution. The solution was maintained at 30° C (Mainwood & Cechetto, 1980) by circulating water coming from a heat exchanger around the jacketed organ bath. Field stimulation of the muscle was produced by two platinum electrodes to prevent electrolysis of the Ringer solution that were positioned at 2 to 3 mm along the entire length of the diaphragm strip.

2.2.3 Measurements of mechanical properties

The maximal voltage needed to produce peak twitch tension was determined by delivering single electrical pulses (square wave pulses of 0.2-ms duration) at 0.1 Hz (Grass Instrument S48 stimulator). Initially, 40 volts were used and the voltage was then increased in a stepwise fashion until twitch tension amplitude was maximal. To insure that supra-maximal voltage was obtained, the maximal voltage was multiplied by a factor of 1.5. Optimal length (Lo), representing the length at which tension is maximal, was determined by moving the tension transducer (Cambridge Technology Inc., Model 352), mounted on a micrometer clamp, while observing twitches at 0.1 Hz. After an equilibration period of 30 min, the tension was compared with the tension recorded initially and if it was found to vary, minor readjustments in length were made until a new Lo was established. The distance between the fibre-central tendon junction and the fibre rib-junction was used as the criterion length. Muscle cross-sectional area (CSA) was estimated by dividing muscle wet mass (g) by the product of muscle density $(1.056 \text{ g} . \text{cm}^3)$ and the length of the diaphragm strip (cm) (Close, 1972).

The time course for the dependent measurements is shown in Figure 2.1. Contractile properties were collected using the Watscope Data Acquisition Unit and Software (Northern Digital Inc., 1985-1987) at the beginning of the experiment, after the initial 3 min of stimulation and after imposing one of the three experimental conditions (see description below). Two sets of twitch and tension-frequency data were collected for pre-fatigue measurements. One set of the same data was collected following the initial 3 min stimulation and following the application of each of the three experimental conditions. For twitch data, peak twitch tension (P_t), twitch contraction time (CT), one-half relaxation time ($RT_{1/2}$), peak rate of tension development ($+dP_t/dt_{max}$) and peak rate of tension decline ($-dP_t/dt_{max}$) were determined (Figure 2.2). A delay of 10 s was provided between measurements to direct the results to a computer.

After a delay of 20 s, collection of the tension-frequency data, including $+dP_t/dt_{max}$ and $-dP_t/dt_{max}$ was started by stimulating the muscle strip at frequencies of 10 (P_{10Hz}), 20 (P_{20Hz}), 50 (P_{50Hz}), and 100 (P_{100Hz}) Hz for 1.5 s each and with 10 s between measurements. The force-frequency data were collected in a random order to minimize the possible variability due to potentiation produced by the previous measurement[LB1]. In addition, three different conditions were imposed on the diaphragm to isolate and distinguish between the effect of two specific perturbations, namely the effect of H^+ and the suspension of ATP recovery. Details of these three recovery conditions and an hypothesis related to each condition are given in the following three sub-sections.

FIGURE 1. TIME COURSE OF DEPENDENT MEASURES

Standard protocol   **Experimental protocol**

CONDITION# 1   3 min stimulation   2 min of additional stimulation

Con   ──────   NC   ──────

Trt   ╱╱   ──────   ╱╱   C   ──────   ╱╱

CONDITION# 2   3 min stimulation   5 min of normal recovery

Con   ──────   NC   ──────

Trt   ╱╱   ──────   ╱╱   C   ──────   ╱╱

CONDITION# 3   3 min stimulation   20 min of anoxic recovery

Con   ──────   NC   ──────

Trt   ╱╱   ──────   ╱╱   C   ──────   ╱╱
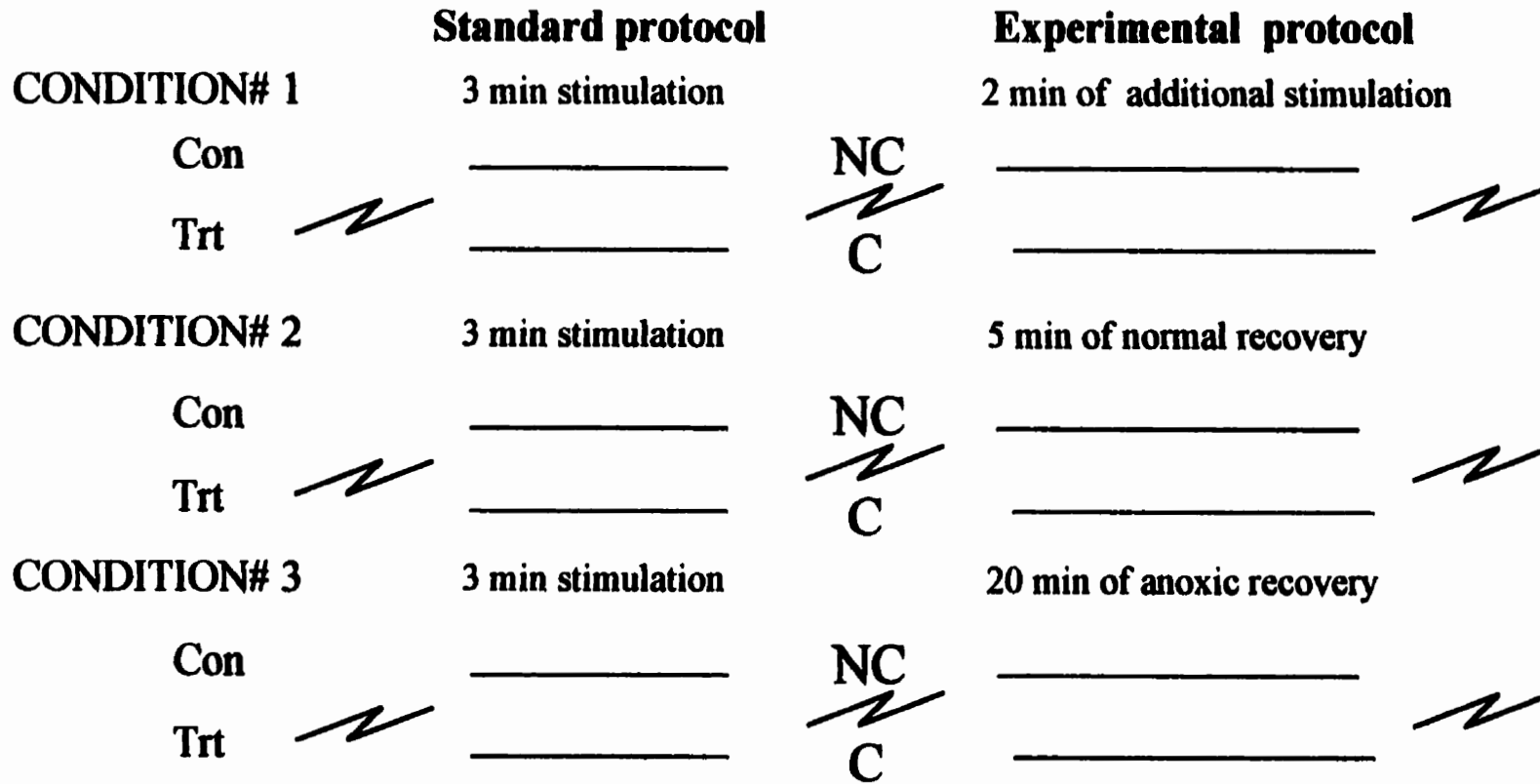
LEGEND: ╱╱   Evoked contractile properties

C: Caffeine   NC: No Caffeine

Con: Control   Trt :   Treatment

FIGURE 2. Twitch contractile properties
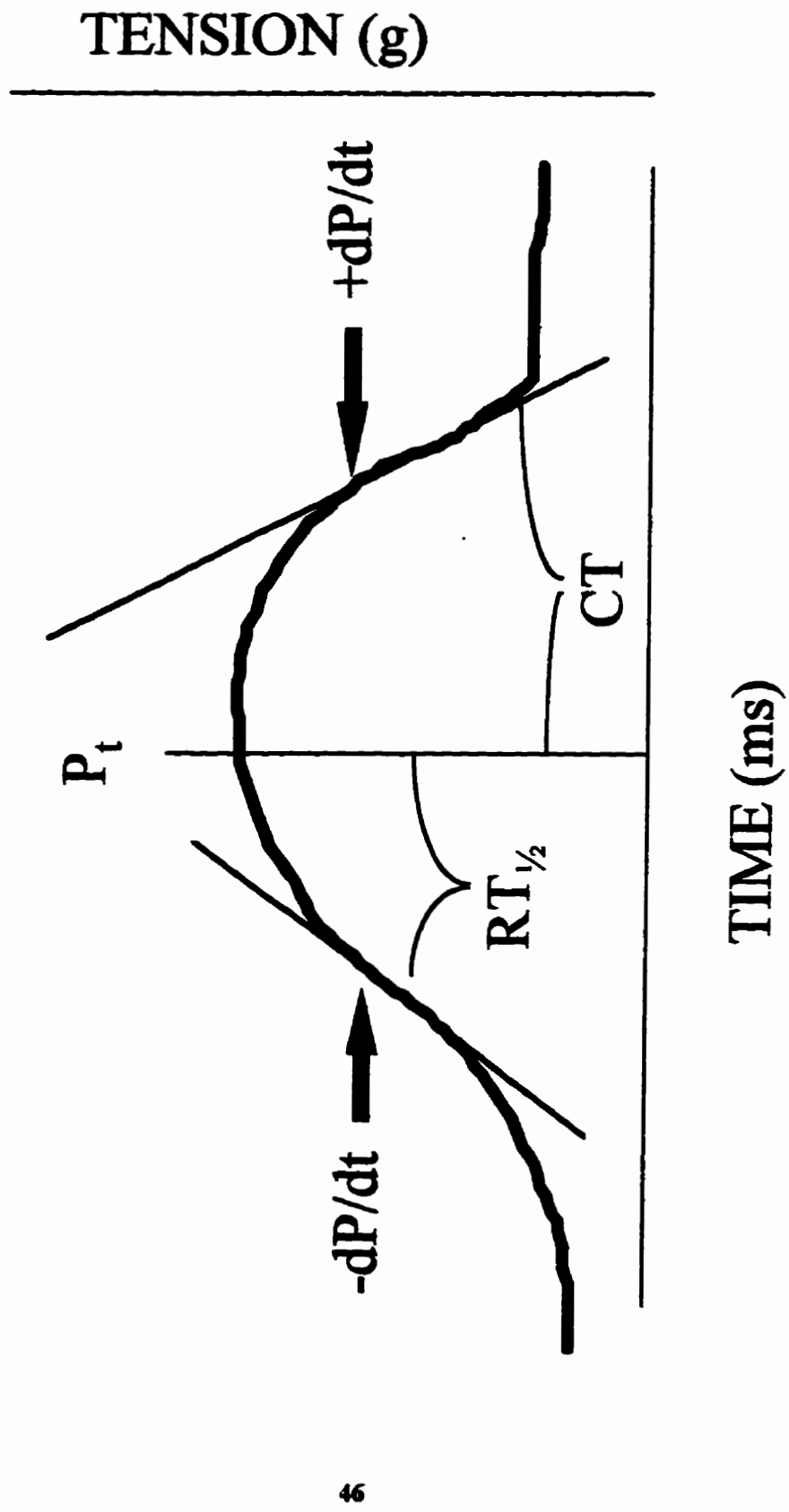
2.2.4 Experimental conditions

2.2.4.1 Three min stimulation protocol

Rats were divided into three groups based on the three experimental conditions used during the recovery period. The 3 conditions were: (1) 2 min of additional stimulation (2MS); (2) 5 min of passive recovery (5MR) and (3) 20 min of anoxic recovery (20MR) (Figure 2.1). These conditions are described in detail in the next section (2.4.2). Each animal provided two diaphragm muscle strips. One was exposed to caffeine, while the other served as a control. This arrangement produced a total of [K2]six different groups of diaphragm muscle strips (caffeine-2MS, caffeine-5MR, caffeine-20MR, no caffeine-2MS, no caffeine-5MR and no caffeine-20MR). Initially, all groups were subjected to a 3 min stimulation protocol (15 Hz; 250 ms; 50 % duty cycle) with [LB3]the purpose of producing a reduction in tension and large metabolic perturbations. The 15 Hz stimulation frequency was chosen because it approximates the firing frequency of phrenic motoneurons during resting breathing in the rat (Purves & Sakmann, 1974). A duration of 3 min using a protocol of continuous stimulation at 10 Hz has been shown to induce significant fatigue and severe metabolic perturbations in rat plantaris muscle after 3 min (Hood & Parent, 1991). The ATP content fell by 47 %, where 82 % of the decrease was due to an increase in IMP (Hood & Parent, 1991). After 1 min of stimulation, lactate increased 19-fold, pH fell from 7.02 to 6.36 and phosphocreatine (PCr) content felt to 11 % of resting control muscle. Although, repletion of PCr content had recovered after 3 min, lactate content was not

normalized until 15 min after stimulation (Hood & Parent, 1991). The plantaris is composed of a

high percentage of type IIb (53 %) and type IIa (40 %) fibres (Armstrong & Phelps, 1984), similar to the diaphragm. The diaphragm has also a high proportion of type IIb (35 %) and type IIa (35 %) fibres (Green et al., 1984).

The intermittent nature of the stimulation protocol provides a more physiological stimulus and its effect on metabolite production and energy demand is if anything more pronounced than continuous stimulation (Chasiotis et al., 1987; Bergström & Hultman, 1988). The choice of the 3 min intermittent stimulation protocol with a 50 % duty cycle was also validated by a study in our laboratory that showed that the most pronounced reduction in tension with the *in vitro* rat diaphragm preparation, during 30 min of stimulation, occurred by 3 min (Massarelli et al., 1989). Therefore, based on these observations, the 3 min stimulation protocol was assumed to produce a pronounced fatigue.

2.2.4.2 Recovery conditions

During the recovery from the 3 min stimulation, the effect of caffeine (1, 3, 7-methylxanthine) was monitored in a second parallel diaphragm through the contractile performance to determine the involvement of the Ca^{2+} release during the specific conditions of recovery.

The response to caffeine is dose-dependent, but the sensitivity of the muscle to caffeine is also dependent on fibre type composition (Fryer & Neering, 1989). Although a concentration of 5 mM has been demonstrated to have a maximal potentiating effect on twitch tension developed by the soleus, the potentiation of twitch tension in the

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extensor digitorum longus (EDL) was dose dependent over the range of 2-10 mM (Fryer & Neering, 1989). The concentration of 5 mM has been shown to elicit maximum Ca^{2+} release in skinned fast fibres of *Xenopus laevis* (Stienen et al., 1993). Higher concentrations produced similar Ca^{2+} release, but may have caused some deterioration of isometric tension development (Stienen et al., 1993). Since the diaphragm is composed of a mixture of fast and slow muscle fibre types (Green et al., 1984), it was decided that the concentration of 5 mM would be appropriate for these experiments.

2.2.4.2.1 Condition 1: additional 2 min of stimulation (2MS)

The rationale for the first experimental condition (2MS) was to maintain, if not exaggerate, the reduction in tension and the metabolic perturbations obtained following the initial 3 min of stimulation. This was accomplished by further stimulating the diaphragm strip for an additional 2 min using the same contractile schedule, following a brief interruption provided to collect contractile data. In addition, caffeine was administered in order to measure the extent to which increased Ca^{2+} release can reverse the effect of the induced muscle fatigue (Weber & Herz, 1968). Based on the well documented effect of caffeine, it was hypothesized that most of the reduction in tension produced by the above protocol would be reversed by caffeine, confirming the inhibition of the Ca^{2+} release channels and a reduced $[Ca^{2+}]_i$ as an important mechanism underlying muscle fatigue (Allen & Westerblad, 1995). This effect will be more pronounced in the case of twitch tension than tetanic tension at 100 Hz stimulation because the change in $[Ca^{2+}]_i$ takes place at a steeper part of the Ca^{2+}-tension relationship curve (Lännergren et al., 1996).

2.2.4.2.2 Condition 2: 5 min passive recovery (5MR)

During recovery under aerobic conditions, PCr is rapidly resynthesized while lactate remains high (Sahlin et al., 1979). This discrepancy in the recovery of PCr and lactate formed the basis of the rationale of the second condition, namely to distinguish between the importance of H^+ concentration versus phosphorylation potential on Ca^{2+} release. In order to achieve this, a 5 min recovery (5MR) without stimulation was chosen to allow enough time for the high-energy phosphate system and intracellular ADP and Pi to recover, while maintaining a high level of acidosis (Kushmerick & Meyer, 1985; Hood & Parent, 1991; Baker et al., 1993).

Evidence for the recovery of the phosphorylation potential was observed with continuous stimulation at 10 Hz of the rat gastrocnemius-plantaris (Hood & Parent, 1991). The level of PCr, which had decreased during the first min of stimulation, started to increase and was significantly higher after 60 min of stimulation (Hood & Parent, 1991). Rapid repletion took place during the initial 3 min of the recovery (Hood & Parent, 1991). Following 2 min of sustained maximal voluntary contractions of human tibialis anterior, substantial recovery in tension and in Pi concentrations were observed after 2 min, while pH required approximately 15 min to return to resting levels (Baker et al., 1993). In a study using the rat *in vitro* diaphragm preparation, the recovery of pH, following 1-2 min of either low- (5 Hz) or high-frequency (75 Hz) stimulation, required approximately 10 min to recover to resting levels (Metzger & Fitts, 1987a). This still represent still 5 min more than the length of the passive recovery period chosen for this study.

It was hypothesized that the 5 min recovery would allow no or a very slight recovery in tension, in the presence of the high acidosis. The presence of caffeine should reverse the inhibitory effect of the H^+ on the Ca^{2+} release channels (Favero et al., 1995) by increasing the $[Ca^{2+}]_i$. Furthermore, it is postulated that the recovery will be more pronounced for the twitch than for the 100 Hz stimulation, for the reasons mentioned above regarding the $[Ca^{2+}]_i$-tension relationship.

2.2.4.2.3 Condition 3: 20 min of anoxic recovery (20MR)

Finally, for the last condition, a 20 min anoxic recovery period without stimulation was utilized. This was done by replacing the O_2-CO_2 mixture (95 % O_2 - 5 % CO_2) with one containing 95 % N_2 and 5 % CO_2. Oxygen was returned to the organ bath just prior to the collection of the contractile data. The rationale for this anoxic recovery was to maintain the deficit in the phosphorylation potential achieved through the initial 3 min of stimulation, and at the same time, allowing sufficient time for lactate to diffuse out of the muscle and for pH to recover.

Recovery of pH following contractile activity in intact muscle appears to be around 10-15 min (Metzger & Fitts, 1987a; Baker et al., 1993). It has been suggested that based on the lactate content and the rate of resynthesis of PCr, that the intracellular concentration of H^+ continues to rise during the first 15 min of recovery (Mainwood & Lucier, 1972).

In rat extensor digitorum longus (EDL) muscle made ischemic, muscle lactate is increased 10-fold only after 2 h anoxia (Sahlin et al., 1990). In agreement with the rationale of the second condition, a 5 min anoxic period immediately following stimulation did not reduce the level of lactate (Sahlin et al., 1990).

The lactate concentration, following a 20 min anoxic recovery, the time period chosen for this third condition, was not measured in the study of Sahlin et al. (1990). However, even if no recovery took place and the lactate levels were to remain close to the levels found after 5 min, it would be difficult to extrapolate these results to the present study. It is probable that the rates of lactate diffusion are very different in an *in situ* EDL preparation than a *in vitro* diaphragm preparation.

Diaphragms of rats weighing between 100-200 g, like the ones used in this study, have been shown to be thin enough to enable the diffusion of 95 % O_2 used to oxygenate the organ bath (Creese et al., 1958). The thickness of a muscle which can enable diffusion depends on the geometry of the tissue, the distance of a specific cell from the surface , and the rate of utilization or release of substances from the cells (see Goldberg et al., 1975). For rat diaphragm incubated in 100 % O_2 and at 37° C, the muscle thickness at which O_2 tension falls to zero has been estimated at 0.54 mm (see Goldberg et al., 1975). The thickness of the diaphragm of rats weighing over 550 g is 1 mm (Reid et al. 1987) so that the diaphragms of rats weighing approximately 150 g, such as those used in this study, should be under 0.54 mm and should provide adequate diffusion of metabolites. Therefore, assuming that lactate will diffuse out of the muscle, the metabolic by-products of phosphorylation should be the major factor contributing to the reduction in tension. It is hypothesized that in this case, the main factor causing the reduced tension is the inhibition of phosphorylation due to the deprived O_2 environment. Since the depressed tension is due to the suspension of ATP recovery, the release of Ca^{2+} by caffeine should have only minor repercussions in reversing the effect of fatigue.

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2.2.5 Statistical analysis

A three way analysis of variance (ANOVA) with two grouping factors: pharmacological intervention (caffeine-no caffeine) and condition (2MS, 5MR and 20MR) and one repeated measure (Time1 and Time 2) was used to analyze the effect of the initial 3 min stimulation (fatigue). A second three way ANOVA with the same grouping factors and repeated measures was used to analyze the combined effect of the pharmacological intervention (caffeine) and the experimental condition (2MS, 5MR and 20 MR). Planned comparisons were performed to determine differences between pairs of the six group means from the three way interaction. A one way ANOVA using the Tukey post-hoc test was used to see if there were any differences among the values at rest and after the initial 3 min of stimulation. The use of "different" in the Results section indicates that a significant difference was found. The significance level was accepted at $P < 0.05$.

2.3 RESULTS

2.3.1 Muscle strip characteristics, and resting contractile measurements

There were no differences in body weight between the six experimental groups (Table 2.1). Similarly, no differences were found for the diaphragm muscle characteristics for weight, length and CSA (Table 2.1). The effect of the various combinations of treatment and experimental conditions polarized the results in two distinct tension responses according to the test stimulation frequency. Low frequency stimulation produced a similar response for P_t, P_{10Hz} and P_{20Hz} while high frequency stimulation resulted in a similar response for P_{50Hz} and P_{100Hz}. Since the effect of the experimental treatments were similar, within these categories, only the results for the twitch and the 100 Hz stimulation frequency are presented and discussed in this section. The results for the 10 Hz, 20 Hz and 50 Hz frequencies can be found in Appendix IC.

Resting contractile properties including twitch tension (P_t) normalized for CSA, contraction time (CT), half-relaxation-time ($RT_{1/2}$) and rate of tension development ($+dP_t/dt_{max}$) and decline ($-dP_t/dt_{max}$), also normalized for CSA, were similar for the six experimental groups (Table 2.2). Similarly, tension at 100 Hz (P_{100Hz}) representing maximal tension (P_o), ($+dP_t/dt_{max}$) and ($-dP_t/dt_{max}$), all expressed as a function of muscle CSA, were not different among the six groups (Table 2.3).

Table 2.1 Body weight and diaphragm muscle strip characteristics for the six groups of rats.

Group	n	Body weight (g)	Diaphragm weight (mg)	Diaphragm length (cm)	Diaphragm Cross-sectional Area (cm^2)
NC-2MS	8	149.0 ± 7.1	19.5 ± 3.3	1.29 ± 0.07	0.014 ± 0.003
NC.-5MR	8	157.1 ± 5.2	17.4 ± 1.1	1.40 ± 0.08	0.012 ± 0.001
NC.-20MR	9	136.5 ± 6.1	22.9 ± 2.3	1.34 ± 0.08	0.017 ± 0.002
C- 2MS	8	149.0 ± 7.1	18.0 ± 1.8	1.38 ± 0.05	0.013 ± 0.001
C-5MR	9	157.8 ± 4.2	20.9 ± 1.7	1.32 ± 0.09	0.015 ± 0.001
C-20MR	8	137.5 ± 6.9	18.4 ± 1.3	1.23 ± 0.06	0.014 ± 0.001

Values represent means ± SE. NC, no caffeine; C, caffeine; 2MS, 2 min stimulation; 5MR, 5 min recovery; 20MR, 20 min recovery. Weight of diaphragm strips determined on wet tissue.

Table 2.2 Isometric twitch contractile data of isolated rat diaphragm strips at rest and following 3 min of cyclical stimulation for six experimental groups.

Group	0 min	3 min
P_t, N. cm^{-2}		
NC-2MS	2.37 ± 0.30	1.35 ± 0.17 [a]
NC-5MR	3.42 ± 0.37	1.95 ± 0.22 [a]
NC-20MR	2.83 ± 0.66	1.35 ± 0.32 [a]
C - 2MS	3.13 ± 0.57	1.71 ± 0.26 [a]
C -5MR	2.19 ± 0.26	1.36 ± 0.14 [a]
C -20MR	2.55 ± 0.48	1.45 ± 0.19 [a]
CT, ms		
NC-2MS	35.2 ± 2.4	38.0 ± 3.5
NC-5MR	32.3 ± 1.3	35.1 ± 1.3
NC-20MR	32.9 ± 1.3	38.4 ± 1.4 [b]
C - 2MS	37.6 ± 4.4	39.4 ± 3.7
C -5MR	42.6 ± 6.2	45.5 ± 7.8
C -20MR	34.1 ± 0.9	36.6 ± 1.1
$RT_{1/2}$, ms		
NC-2MS	35.3 ± 2.2	45.4 ± 2.5 [a]
NC-5MR	29.3 ± 0.7	47.9 ± 1.8 [a]
NC-20MR	30.6 ± 1.3	50.2 ± 4.6 [a]
C - 2MS	30.9 ± 1.9	43.4 ± 1.6 [a]
C -5MR	32.6 ± 1.7	47.7 ± 1.9 [a]
C -20MR	33.8 ± 2.2	47.8 ± 2.5 [a]
+dP$_t$/d t, mN. cm^{-2}. s^{-1}		
NC-2MS	147 ± 22	72 ± 13 [a]
NC-5MR	210 ± 35	116 ± 17 [a]
NC-20MR	165 ± 34	73 ± 13 [a]
C - 2MS	173 ± 45	102 ± 24 [a]
C -5MR	115 ± 28	71 ± 12 [b]
C -20MR	157 ± 29	90 ± 11 [a]
-dP$_t$/d t, mN . cm^{-2} . s^{-1}		
NC-2MS	53 ± 6	23 ± 2.6 [b]
NC-5MR	81 ± 10	27 ± 3.3 [a]
NC-20MR	66 ± 15	21 ± 2.9 [a]
C - 2MS	74 ± 17	29 ± 4.5 [a]
C -5MR	52 ± 9	20 ± 2.4 [a]
C -20MR	55 ± 12	23 ± 3.4 [a]

Values represent means ± SE. NC, no caffeine; C, caffeine; 2MS, 2 min stimulation; 5MR, 5 min recovery; 20MR, 20 min recovery; P_t, peak twitch tension; CT, contraction time; $RT_{1/2}$, twitch one-half relaxation time; +dP$_t$/dt, peak rate of tension development; -dP$_t$/dt, peak rate of tension decline. [a] Significantly different from control ($P < 0.01$), [b] Significantly different from control ($P < 0.05$).

Table 2.3. Isometric tetanic contractile data at 100 Hz stimulation frequency at rest and following 3 min of stimulation for the six experimental groups.

Group	0 min	3 min
P_{100Hz}, N . cm^{-2}		
NC-2MS	9.2 ± 0.7	7.4 ± 1.0 [b]
NC-5MR	11.9 ± 1.3	9.6 ± 1.2 [a]
NC-20MR	9.7 ± 1.9	7.6 ± 1.3 [a]
C - 2MS	12.6 ± 1.6	9.0 ± 1.0 [a]
C -5MR	8.1 ± 0.9	7.0 ± 0.7
C -20MR	9.1 ± 1.5	7.7 ± 0.8
+dP_t/d t, mN . cm^{-2} . s^{-1}		
NC-2MS	171 ± 20	111 ± 16 [a]
NC-5MR	232 ± 32	141 ± 17 [a]
NC-20MR	182 ± 34	102 ± 17 [a]
C - 2MS	249 ± 39	135 ± 19 [a]
C -5MR	143 ± 21	102 ± 12 [b]
C -20MR	198 ± 36	124 ± 13 [a]
-dP_t/d t, mN . cm^{-2} . s^{-1}		
NC-2MS	181 ± 8	103 ± 20 [a]
NC-5MR	263 ± 35	107 ± 16 [a]
NC-20MR	219 ± 40	90 ± 14 [a]
C - 2MS	312 ± 47	112 ± 14 [a]
C -5MR	176 ± 26	93 ± 11 [a]
C -20MR	214 ± 36	104 ± 8 [a]

Values represent means ± SE. NC, no caffeine; C, caffeine; 2MS, 2 min stimulation; 5MR, 5 min recovery; 20MR, 20 min recovery; P_{100} , peak tetanic tension at 100 Hz; +dP_t/dt, peak rate of tension development; -dP_t/dt, peak rate of tension decline. [a] Significantly different from control (P < 0.01), [b] Significantly different from control (P < 0.05).

2.3.2 Effect of initial 3 min stimulation on contractility

Twitch contractile changes induced by the initial 3 min of stimulation are summarized in the second column of Table 2.2. The effect of the 3 min stimulation protocol on twitch tension (P_t) was similar for all groups and overall this translated into a 44 % reduction from the resting value. Contraction time (CT) was not affected by the 3 min fatigue protocol, with the exception of a slight but significant increase for the no caffeine-20MR group. Half-relaxation time ($RT_{1/2}$) was increased for all groups resulting in an overall increase of 47 %. The rate of tension development ($+dP_t/dt_{max}$) was decreased in the six groups for an overall reduction of 47 %. However, if the $+dP_t/dt_{max}$ was corrected for the fall in tension by dividing $+dP_t/dt_{max}$ by P_t (Thompson et al., 1992a), only two out of the six groups displayed lower values after the 3 min stimulation. The rate of tension decline ($-dP_t/dt_{max}$) was depressed for the six experimental groups, with the overall reduction averaging 63 %. The relationship was maintained for the six groups when the reduced twitch tension was taken into account. However, the overall reduction was attenuated, falling to 29 %.

Following the 3 min of stimulation, only four of the six groups showed decreases in tetanic tension at the test stimulation frequency of 100 Hz (Table 2.3). The reduction was 20 % of initial tension (Table 2.3). The reductions in P_{100Hz} were not significant for the caffeine-5MR (13 %) and the caffeine-20MR (14 %) groups. The tetanic $+dP_t/dt_{max}$ and $-d_t/dt_{max}$ were reduced in all six groups and the overall reduction came to 39 % and 55 %, respectively (Table 2.3). When these parameters where expressed relative to tetanic tension, the values, although still significant, were reduced to 22 % and 42 %, respectively.

2.3.3 Effect of experimental conditions on caffeine modulated contractile response

No differences were found between the six groups after the initial 3 min of stimulation and before the application of one of the three conditions for any of the contractile properties for either the twitch (Table 2.2) or the 100 Hz stimulation frequency (Table 2.3).

2.3.3.1 Condition 1: Additional 2 min of stimulation (2MS)

During the first experimental condition (2MS), consisting of an additional 2 min of stimulation at 15 Hz, twitch tension (P_t) (Figure 2.3A) and tetanic tension at 100 Hz (P_{100Hz}) (Figure 2.4A) did not recover from the reduction produced initially by the 3 min of stimulation protocol (Figure 2.3A & 2.4A). The addition of caffeine produced an increase in P_t of 35 % which represented 74 % of the tension measured at rest before the initial 3 min stimulation. For P_{100Hz}, the addition of caffeine to the organ bath had the reverse effect causing a further reduction in tension of 27 % (Figure 2.4A). The caffeine generated tension was different than the no-caffeine tension both before and after caffeine treatment. However, this difference was in the opposite direction, the caffeine group being higher for the pre-values and lower for the post-values than the control group.

For the 2MS condition, caffeine produced a strong and significant effect on three twitch time related contractile properties (Figure 2.3B, C, & D). Contraction time (CT) was increased by 26 % representing 132 % of the initial pre-fatigue value (Figure 2.3B). In the control diaphragm, the additional 2 min of stimulation further reduced CT by 11 %. Half-relaxation time ($RT_{1/2}$) was prolonged by 61 % with the addition of

Figure 2.3 Effect of caffeine after two min of additional
stimulation (2MS) on twitch tension (A), contraction time
(B), half-relaxation time (C), rate of tension development (D),
rate of tension decline (E). PRE and POST; before and after
addition of caffeine and 2 min of additional stimulation. Data
are means ± SE. ** Significantly different from pre-comparable
condition ($P < 0.01$). * ($P < 0.05$).‡ Significantly different from
no-caffeine ($P < 0.01$).
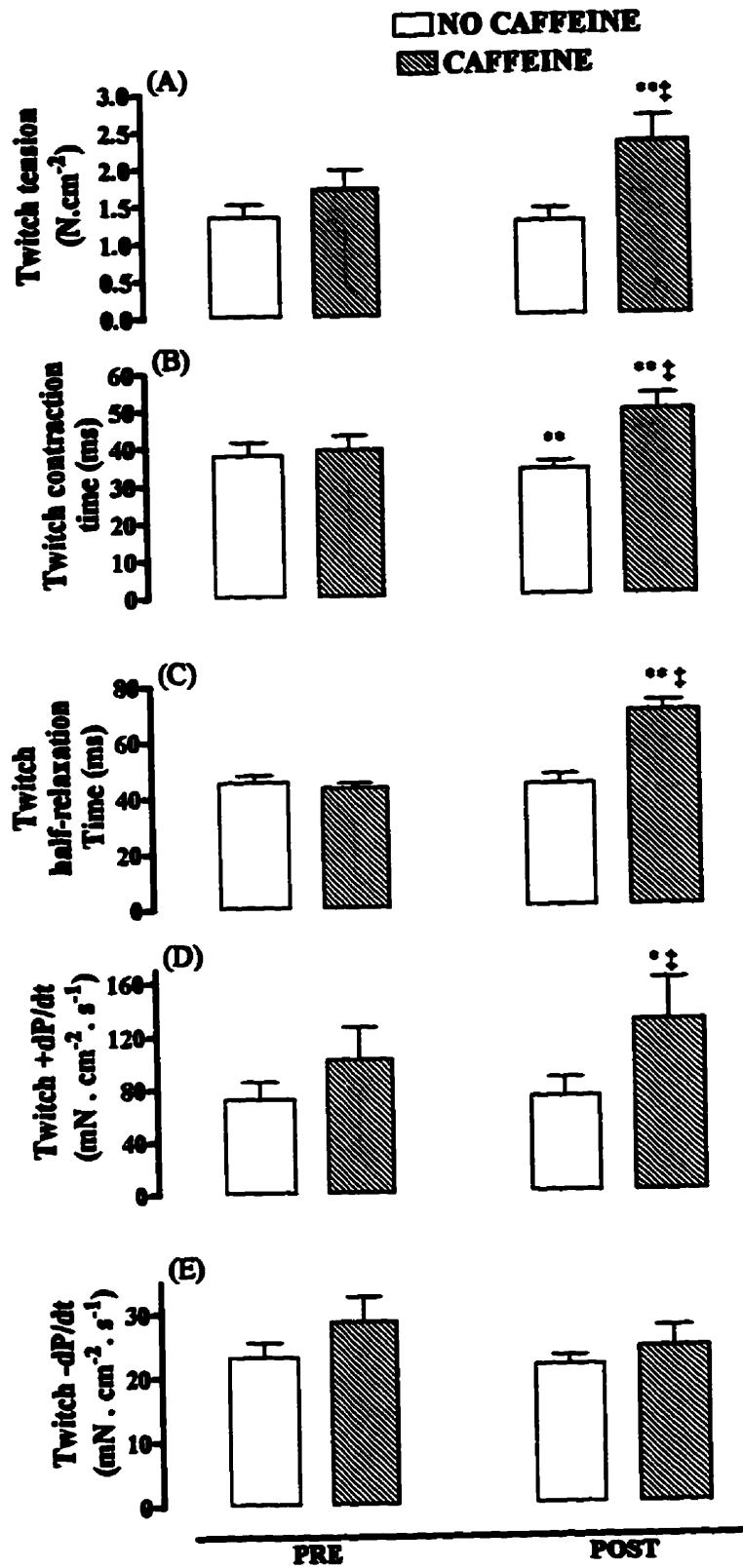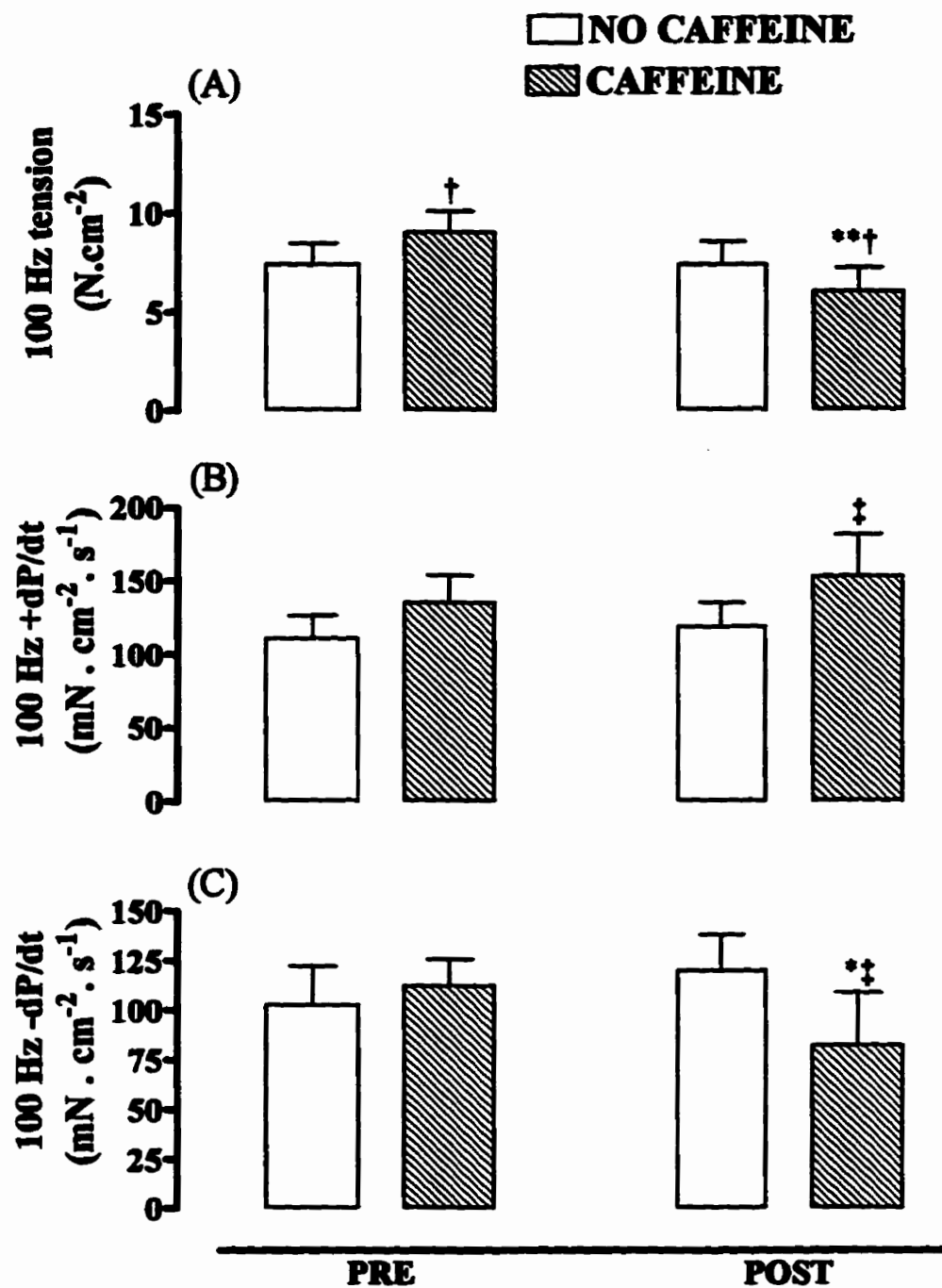
Figure 2.4 Effect of caffeine after two min of additional stimulation (2MS) on 100 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and additional 2 min of stimulation. Data are means ± SE. ** Significantly different from pre-comparable ($P < 0.01$).* ($P < 0.05$). ‡ Significantly different from no-caffeine ($P < 0.01$).†($P < 0.05$).

caffeine (226 % of pre-fatigue value) but was not significantly altered in the control strip (Figure 3C). Caffeine produced a 27 % increase in $+dP_t/dt_{max}$ (Figure 2.3D), representing 75 % of the pre-fatigue value. However, no significant difference was found when the reduction in twitch tension was normalized by dividing $+dP_t/dt_{max}$ by P_t ($+dP_t/dt_{max}$. P_t^{-1}). The additional stimulation had no effect on $-dP_t/dt_{max}$ in either the control or the caffeine treated strip (Figure 2.3E). When the correction for the loss of tension was calculated, a 34 % decrease was observed for the caffeine contracture. For the 100 Hz tension, $+dP_t/dt_{max}$ was not affected by caffeine, but $-dP_t/dt_{max}$ was reduced by 27 % (Figure 2.4B & 2.4C). However, when corrected for the change in tension, no effect of fatigue was found for $+dP_t/dt_{max}$. P_{100Hz}^{-1}.

2.3.3.2 Condition 2: five min of passive recovery (5MR)

For the second experimental condition, the 5 min passive recovery period was not sufficient to allow twitch tension (P_t) to recover (Figure 2.5A). However, at 100 Hz, a significant recovery of tension of 30 % was observed from post-fatigue (Figure 2.6A). The P_{100Hz} represented 105 % of the initial pre-fatigue value.

The addition of caffeine had a marked effect on P_t, producing a 148 % increase from the post-fatigue value (Figure 5A). This represented 119 % of the initial pre-

Figure 2.5 Effect of caffeine after five min of passive recovery
(5MR) on twitch tension (A), contraction time (B), half-relaxation
time (C), rate of tension development (D), rate of tension decline (E).
PRE and POST; before and after addition of caffeine and 5 min recovery
period. Data are means ⊕ SE. ** Significantly different from pre-comparable
condition ($P < 0.01$). ($P < 0.05$).‡ Significantly different from no-caffeine
($P < 0.01$). † ($P < 0.05$).
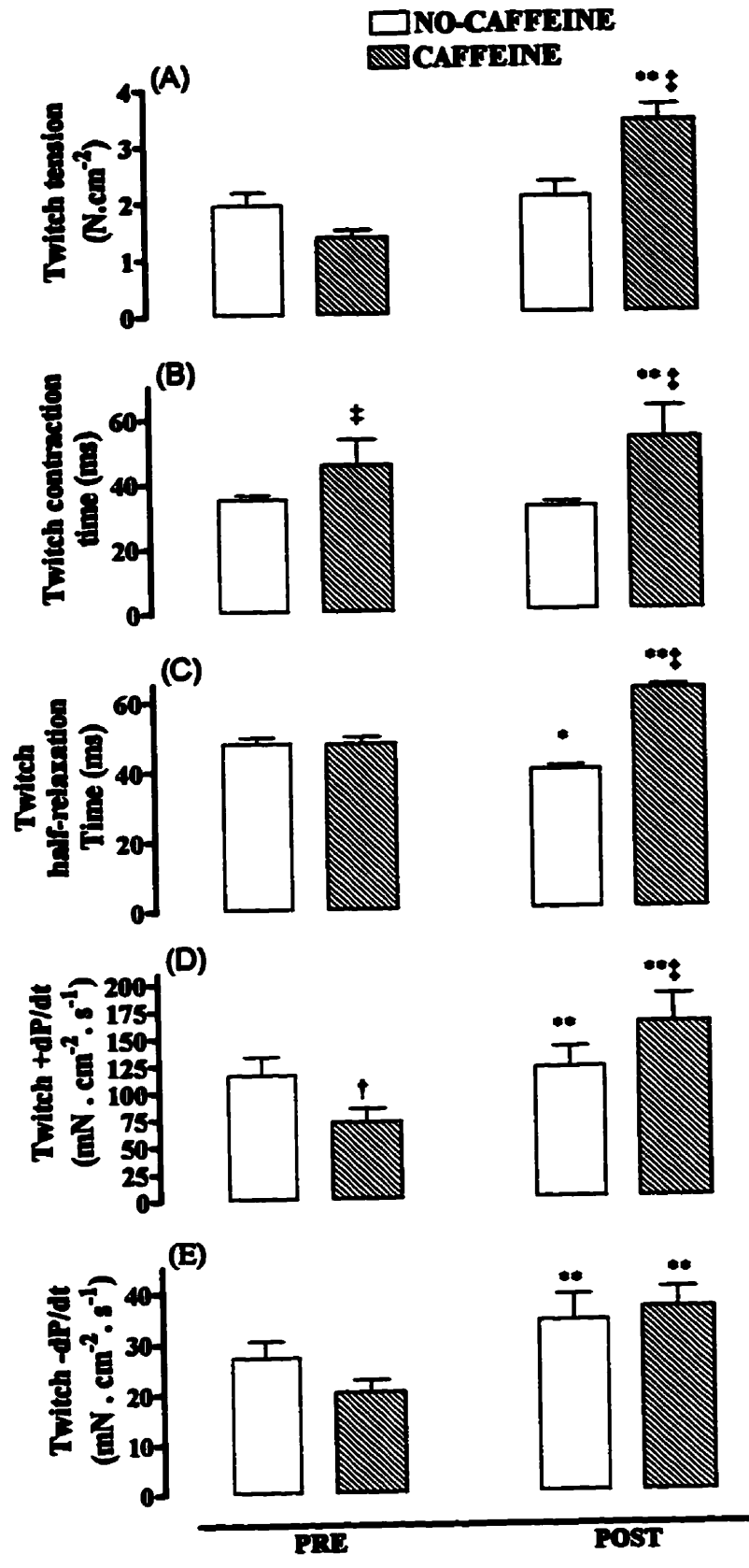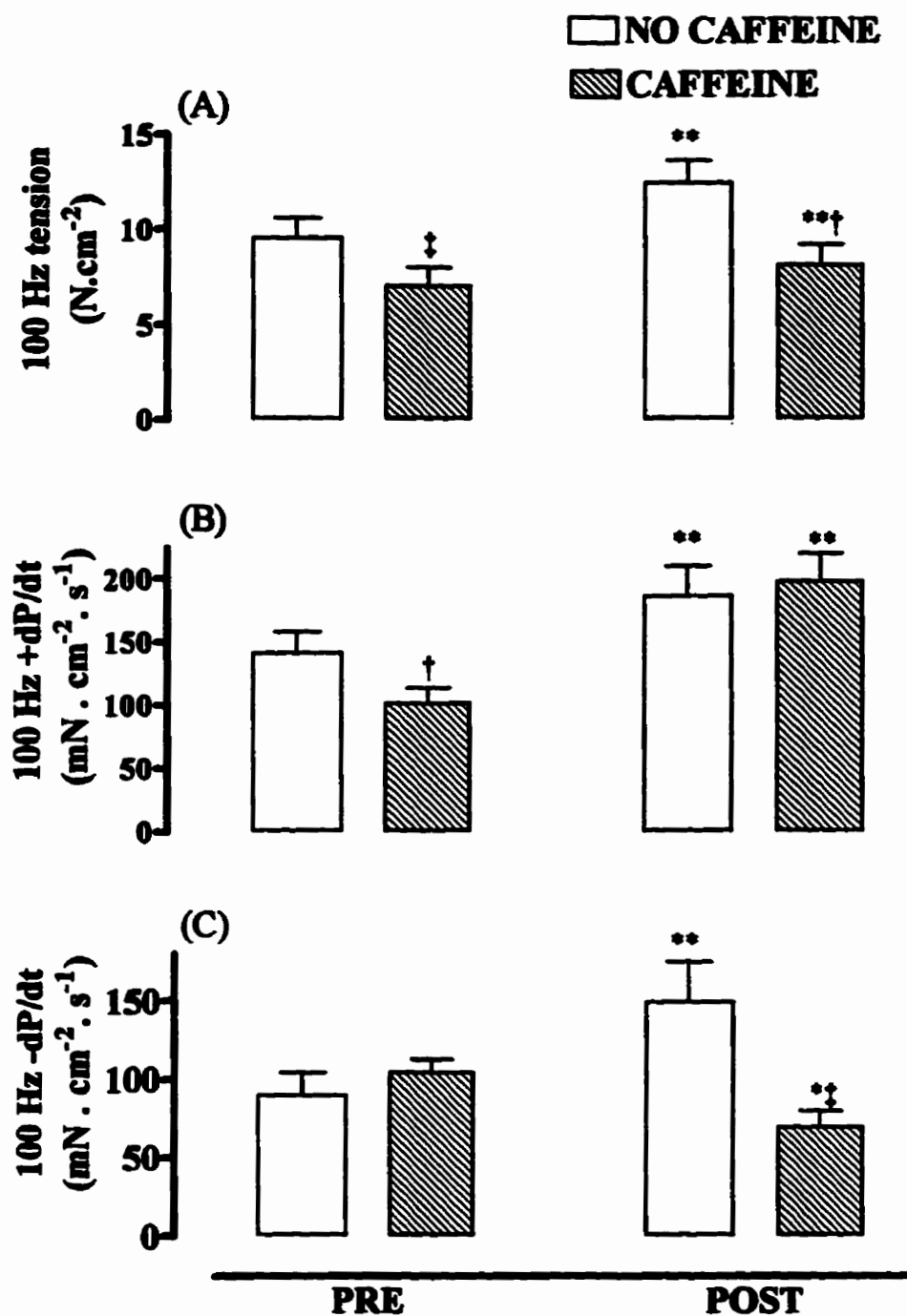
Figure. 2.6 Effect of caffeine after 5 min of recovery (5MR) on 100 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE ($P < 0.01$), * ($P < 0.05$) Significantly different from no-caffeine. ‡ ($P < 0.01$).† ($P < 0.05$).

fatigue value (Figure 2.5A). For P_{100Hz}, the increase of 15 % for the caffeine stimulated tissue was less than for the no caffeine tissue (Figure 2.6A). However, this can be explained by the reduction in P_{100Hz} for this particular group (Caffeine-5MR) (see above) following the initial 3 min of stimulation which was not significant and therefore left less room for improvement. The smaller increase, nevertheless, returned P_{100Hz} to its pre-fatigue value.

The effects of the 5MR condition on time related contractile properties were in general similar to those produced by the 2MS condition. The results, following the initial 3 min of stimulation, indicated that there was a difference between the control and caffeine treated muscle for CT, making it difficult to attribute the 17 % increase to the specific action of the drug (Figure 2.5B). Half-relaxation time ($RT_{1/2}$) was prolonged 32 % (193 % of pre-fatigue value) by caffeine and was shortened by 17 % in the control strip (Figure 2.5C). As well, $+dP_t/dt_{max}$ increased for the caffeine muscle by a value of 128 %, representing 140 % of its pre-fatigue value (Figure 2.5D). However, this increase was not different than the value found at the beginning of the 5 min recovery period when corrected for the change in P_t.

The $-dP_t/dt_{max}$ increased during the 5MR condition for both the caffeine (81 %) and the control (26 %) groups (Figure 2.5E), but were not different when corrected for P_t. Increases of 95 % for the caffeine and of 32 % for the control group with recovery were also observed for $+dP_t/dt_{max}$ at the 100 Hz stimulation frequency (Figure 2.6B), but only the increase for the caffeine group (57 %) remained significant after taking in consideration the change in P_{100}. This finding is consistent with a caffeine specific effect at this frequency of stimulation. The $-dP_t/dt_{max}$ of the control diaphragm

increased by 128 %, representing 93 % of the resting value before imposing the 3 min stimulation (Figure 2.6C). The increase fell to 74 % after taking in account the change in tension at the 100 Hz frequency of stimulation.

2.3.3.3 Condition 3 : twenty min of recovery (20MR)

For the third experimental condition (20MR), no change in twitch tension (P_t) was observed over the 20 min period for either the caffeine or the no caffeine group (Figure 2.7A). At 100 Hz, P_{100Hz} was reduced by 32 % after the addition of caffeine (Figure 2.8A).

In general, the 20MR condition produced similar changes on twitch dynamic parameters. Twitch contraction time (CT) was shortened by 39 % for the control strip and by 28 % for the treatment tissue (Figure 2.7B). Half-relaxation time ($RT_{1/2}$) decreased by 55 % and 41 % in the control and caffeine muscle, respectively (figure 7C). As with CT and RT $_{1/2}$, $-dP_t/dt_{max}$ in both control (88 %) and caffeine muscles (42 %) displayed increases (Figure 2.7E). These increases remained different for the control (86 %) and the caffeine diaphragms (94 %) even after correcting for the change in tension. Finally, for twitch $+dP_t/dt_{max}$, an increase (51 %) was observed only for the control tissue (Figure 2.7D). This was also the case at 100 Hz, where the increase in $+dP_t/dt_{max}$ (42 %) was only significant for the control muscle (Figure 2.8B). However,

Figure 2.7 Effect of caffeine after twenty min of anoxic recovery (20MR) on twitch tension (A), contraction time (B), half-relaxation time (C), rate of tension development (D),rate of tension decline (E). PRE and POST; before and after addition of caffeine and 20 min recovery period. Data are means ± SE. ** Significantly different from pre-comparable condition ($P < 0.01$). ($P < 0.05$). † Significantly different from no-caffeine ($P < 0.05$).
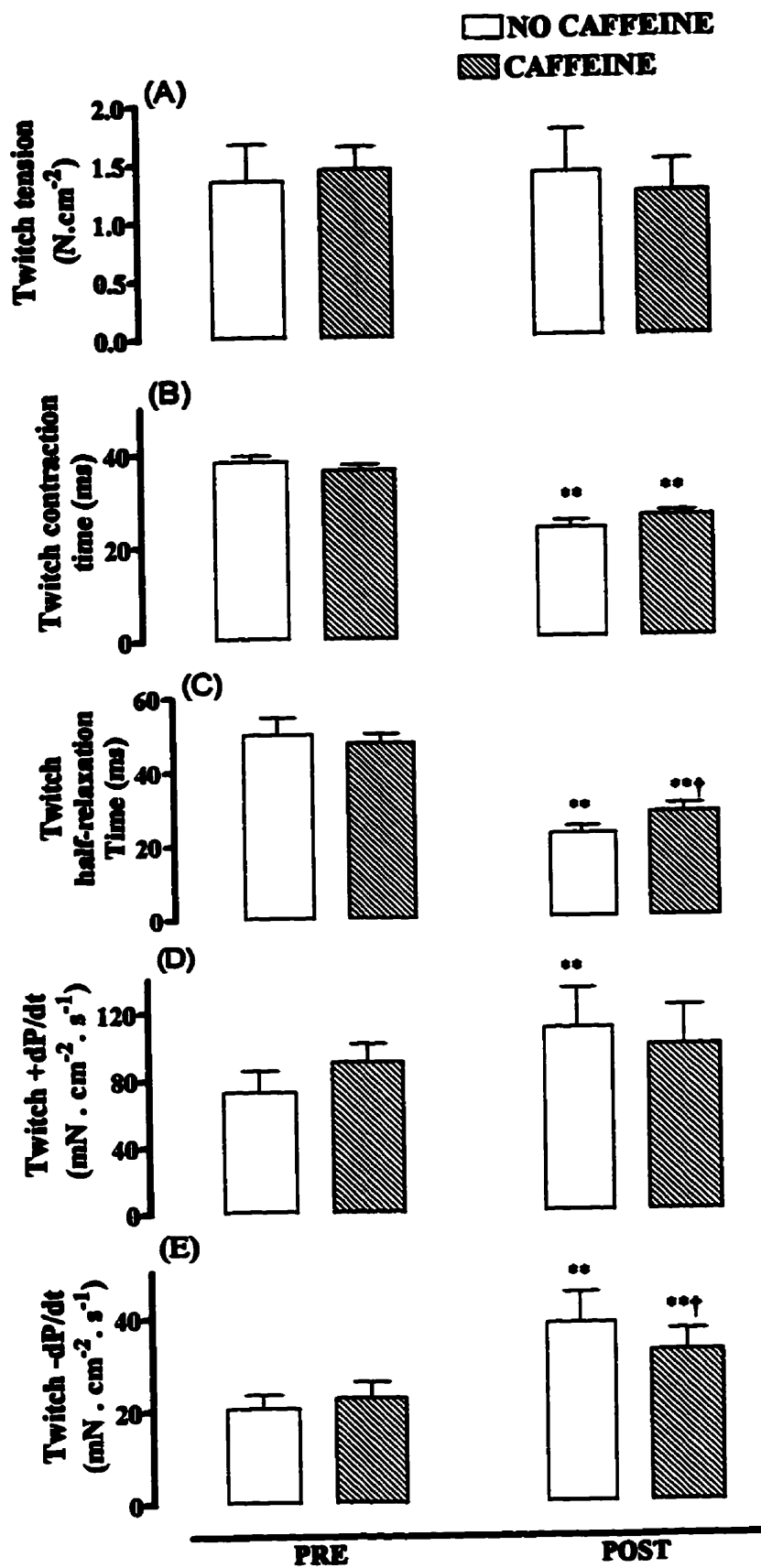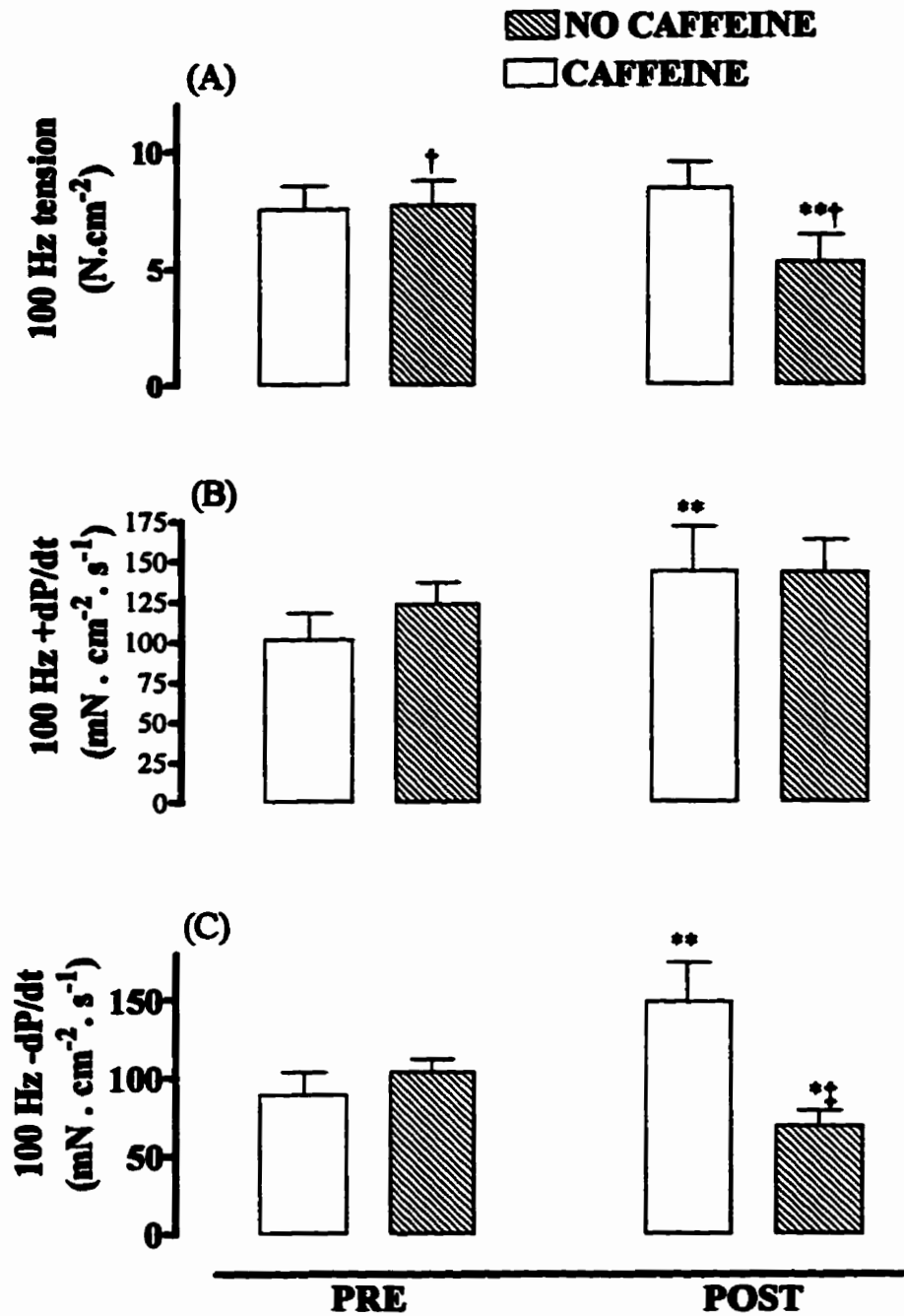
Figure 2.8 Effect of caffeine after 20 min anoxic recovery (20MR) on 100 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. * Significantly different from PRE ($P < 0.05$) † Significantly different from no-caffeine.

when the correction for the fall in tension was made, this increase was not significant. In contrast, an increase was found for the caffeine group after the correction was made (65 %). The $-dP_t/dt_{max}$ was increased at 100 Hz (67 %) for the control strip, but was reduced for the caffeine stimulated strip by 34 % (Figure 2.8C). The correction for the fall in tension at 100 Hz eliminated the effect for the caffeine contracture but a 42 % increase was still observed for the control muscle.

2.4   DISCUSSION

2.4.1 Resting contractile measurements

Resting contractile properties including twitch tension (P_t) normalized for CSA, contraction time (CT), half-relaxation-time ($RT_{1/2}$) and maximal rate of tension development ($+dP_t/dt_{max}$) and decline ($-dP_t/dt_{max}$), also normalized for CSA, were similar to values reported previously in this laboratory (Kile, 1988) for rat diaphragm muscle. Resting twitch tension could not be compared to the value obtained by another investigator in this laboratory (Massarelli et al., 1989) because it was not expressed as a function of muscle cross-sectional area. The average CT (25.6 ms) and $RT_{1/2}$ (25.7 ms) reported by Massarelli et al. (1989) were slightly lower than the values of CT (35.5 ms) and $RT_{1/2}$ (32 ms) obtained in the present study. For the $+dP_t/dt_{max}$ and $-dP_t/dt_{max}$, the results of Massarelli et al. (1989), like the tension data, were not expressed per unit of cross-sectional area and no comparisons could be made with the present values.

The average resting twitch tension for the six groups: 2.74 N . cm^{-2} or 279 g . cm^{-2} was lower than the value of 1231 g . cm^{-2} obtained in an experiment conducted under similar control conditions by another laboratory (Metzger et al., 1985). However, the value in the present study was close to the value (3.8 N . cm^{-2}) obtained in another study conducted at a temperature of 25° C instead of 30° C by the same group (Reid et al., 1987). The value obtained in this study was also lower than the value (6.2 N. cm^{-2}) obtained at 22° C by Hervé et al. (1988). The difference in temperature could explain in part the difference between these three studies, although a Q_{10} of only 1.06 was found for P_t of the ventral costal diaphragm (Metzger et al., 1985). The dependence of

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contractile properties to temperature is rather complex, and will vary according to muscle fibre composition (Buller et al., 1984). However, temperature does not appear to be the only factor causing variability between laboratories. Mainwood et al., (1982) obtained a value of 7.4 N . cm^{-2} at 30° C, the same temperature used for the present experiment. In summary, there appears to be wide fluctuations in P_t values from one laboratory to the next and although temperature may play a role, a number of factors including pH, oxygen diffusion, glucose diffusion in the incubation medium into the fibres and survivability of the isolated muscle also appear critical (Segal & Faulkner, 1985). Finally, the sensitivity of the tension transducer and the phosphorylation state of the muscle at low frequencies of stimulation will also have an effect on tension.

The CT obtained for this study was nearly identical to the values of 34 ms and 30 ms reported by Metzger et al. (1985) and Mainwood et al. (1982), respectively. However, the values were lower than the 56.3 ms reported by Reid et al. (1987). In the case of $RT_{1/2}$, the mean value was lower than the value of 43 ms of Metzger et al. (1985) and of 55.6 ms by Reid et al. (1987) but similar to the value of 29.8 ms found by Mainwood et al. (1982).

The comparison of the values of $+dP_t/dt_{max}$ and $-dP_t/dt_{max}$ between studies is not always obvious because of the various units used to express these parameters from one study to the other. Kile (1988) reported values of 12.8 g . cm^{-2}. s^{-1} for $+dP_t/dt_{max}$ and 3.7 for $-dP_t/dt_{max}$ and attributed the higher values reported by Metzger and Fitts (1986) to a difference in the estimation of the cross-sectional area. Kile (1988) suggested that Metzger and Fitts (1986) used only the resting length of the diaphragm to predict cross-sectional area instead of using resting length and the actual weight of the muscle.

However, the values of 49.2 for $+dP_t/dt_{max}$ and 16.2 for $-dP_t/dt_{max}$ are given in $g \cdot ms^{-1} \cdot cm^{-2}$ instead of $g \cdot s^{-1} \cdot cm^{-2}$. Assuming that the units provided by Metzger and Fitts (1986) are correct, the $+dP_t/dt_{max}$ and $-dP_t/dt_{max}$ would be considerably higher than the values reported both by Kile (1988) and the present study for the costal rat diaphragm at 30° C.

The value of 10 $N \cdot cm^{-2}$ reported in the present study for tetanic tension at 100 Hz agrees with the value of 11.2 and 12.8 $N \cdot cm^{-2}$ obtained by Kile (1988) and Reid et al. (1987), respectively. However, it is much lower than the values found by Mainwood et al. (1982) (34.3 $N \cdot cm^{-2}$) and Metzger et al. (1987b) (31.4 $N \cdot cm^{-2}$).

2.4.2 Effect of 3 min stimulation protocol on contractile performance

The 3 min stimulation protocol was sufficient to produce the significant changes in twitch contractile properties that are normally associated with muscle fatigue. Although, the protocol reduced tension at the 100 Hz frequency (P_{100Hz}) in all groups, significance was only obtained for 4 of the 6 groups. Overall, twitch tension was reduced by 44 % but P_{100Hz} by only 20 %.

This distinctive response at both low- and high-frequencies of stimulation following an intensive session of exercise is known as the well established phenomenon of LFF (Edwards et al., 1977). LFF has been documented using the *in vitro* rat diaphragm preparation (Kelsen et al., 1982; Metzger and Fitts, 1987a, b). Following various sessions of direct stimulation of the phrenic nerve at 15 Hz, Kelsen et al., (1982) observed a proportionally greater reduction (53 %) in the tension elicited at 20 Hz compared to the reduction elicited at 100 Hz (23 %). After 1.5 min of indirect stimulation at 5 Hz, the tension produced at that frequency was more depressed than

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the tension produced by a 75 Hz stimulus (Metzger & Fitts, 1987b). LFF has also been observed more recently in the intact mouse muscle fibre, where tension was reduced at 30 Hz and 50 Hz while tension at 100 Hz, the stimulation frequency used to induce the fatigue, was close to control values (Westerblad et al., 1993).

Another characteristic of LFF is the persistence of the effect which may last many hours after the end of the period of activity (Edwards et al., 1977). Although, the recovery period for the second condition lasted only 5 min, the distinctive response between low and high frequencies of stimulation indicated a selective fatigue specific to the low frequencies of stimulation in the present experiments. P_{100Hz} for the control group had completely recovered whereas P_t was still depressed after the 5 min period.

This discrepancy in response between the low- and high-frequencies of stimulation was originally attributed to a reduction in the amount of Ca^{2+} released by the sarcoplasmic reticulum (SR) per stimulation pulse (Edwards et al., 1977). More recently, similar conclusions were reached following experiments using the single mouse intact fibre preparation where the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored with a fluorescent Ca^{2+} dye (Westerblad et al., 1993). The $[Ca^{2+}]_i$ was found to decrease at both low and high frequencies of stimulation in the mouse intact fibre preparation (Westerblad et al., 1993). However, the differential effect of the decrease in $[Ca^{2+}]_i$ at low and high frequencies of stimulation on tension was explained by the position on the $[Ca^{2+}]_i$-tension relationship. At 30 Hz and 50 Hz, as opposed to 100 Hz, the Ca^{2+}-tension relationship fell on the steep part of the curve. Therefore a small decrease in $[Ca^{2+}]_i$ produced a pronounced reduction in tension. Other possibilities that could explain the greater attenuated tension reduction at low frequencies of stimulation,

such as a change in Ca^{2+} sensitivity or in maximal activation by Ca^{2+}, were rejected on the basis that neither were affected 30 min after recovery. The finding that Ca^{2+} is homogeneously distributed throughout the cytosol of the fibre argues against the reduction in $[Ca^{2+}]_i$ being caused by an impairment in the conduction of the action potential along the t-tubule membrane. In addition, by plotting the rate of $[Ca^{2+}]_i$ decline against the $[Ca^{2+}]_i$ during the tail of elevated $[Ca^{2+}]_i$ (Westerblad & Allen, 1993b), it was demonstrated that the rate of uptake by the Ca^{2+}-Mg^{2+}-ATPase pump was not altered during LFF.

The dynamic contractile properties following fatigue are suggested to reflect the time course of the Ca^{2+} transient (Westerblad et al., 1990) and offer additional information concerning the mechanisms involved in force depression with repeated stimulation. During the contraction phase, the decrease in P_t coupled to a contraction time (CT) that displayed a tendency to increase translated into an absolute reduction in the $+dP_t/dt_{max}$. CT has been shown to either be prolonged (Metzger & Fitts, 1987b) or reduced with fatigue (MacIntosh & Gardiner, 1987). In the present study, the low stimulation frequencies used to induce the fatigue (Metzger & Fitts, 1987b) in conjunction with the metabolic profile of the muscle (Fitts et al., 1982) are factors that could explain why CT showed significant changes in only one of the six groups. Surprisingly, the additional 2 min of stimulation during condition 2MS produced a reduction in CT in the control diaphragm instead of emphasizing the increase observed after the initial 3 min of stimulation.

The reduction in $+dP_t/dt_{max}$ is a commonly observed in muscle fatigue (Fitts et al., 1982; MacIntosh & Gardiner, 1987) and has been linked to the rate of cross-bridge

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binding from weakly bound, low tension state to the strongly bound, high tension state (Brenner, 1988). It has been suggested that both an increase in [H+] (Thompson et al., 1992b) or an increase [Pi] (Thompson et al., 1992c) could produce a reduction of the rate constant for cross-bridge cycling. However, part of this reduction is due to the reduced P_t which represents a smaller number of cross-bridges acting in parallel (Thompson et al., 1992a). If the $+dP_t/dt_{max}$ is corrected for the fall in tension ($+dP_t/dt_{max} \cdot P_t^{-1}$), the reduction is much smaller (Massarelli et al., 1989; Thompson et al., 1992a) and significant differences are found only for two out of the six groups. At 100 Hz , both the absolute ($+dP_t/dt_{max}$) and relative ($+dP_t/dt_{max} \cdot P_{100Hz}^{-1}$) rate of tension decay were significantly reduced.

For the relaxation phase, the reduced twitch tension was accompanied by a prolongation in twitch half-relaxation time ($RT_{1/2}$) of approximately 50 %. The slowed relaxation is a common feature of muscle fatigue and similar reductions in $RT_{1/2}$ have been reported with the *in vitro* rat diaphragm preparation following indirect stimulation (Metzger & Fitts, 1987b; Herve et al., 1988). Recently, increases in $RT_{1/2}$ have been attributed to reduced cross-bridge kinetics rather than an inhibition of the Ca^{2+} handling processes (Westerblad et al., 1993; Westerblad & Allen, 1993b). An increase in Pi concentration is suggested to be the factor responsible for the longer $RT_{1/2}$ (Westerblad et al., 1993).

The lower increase in $RT_{1/2}$ translated into a reduction in $-dP_t/dt_{max}$ (63 %) that was much smaller (29 %) when the correction for the reduced tension ($-dP_t/dt_{max} \cdot P_t^{-1}$) was applied. At 100 Hz, $-dP_t/dt_{max}$ was significantly reduced after fatigue. Since P_{100Hz} was not affected by the stimulation as much as P_t, this parameter remained

significantly reduced when corrected for the fall in tension. The rate of cross-bridge binding has been suggested to be the limiting factor for $+dP_t/dt_{max}$ and it could also be the main reason for the reduced $-dP_t/dt_{max} . P_t^{-1}$ (Lännergren & Westerblad, 1989; Kolbeck & Nosek, 1994). Other processes that could influence this parameter are a reduced rate in the detachment of the actin-myosin cross-bridges (Westerblad & Allen, 1993b), a slower dissociation of Ca^{2+} from TnC (Westerblad & Lännergren, 1989), an increase Ca^{2+} saturation of parvalbumin (Westerblad & Lännergren, 1990) and a slower rate of Ca^{2+} uptake by the $Ca^{2+}-Mg^{2+}$-ATPase (Allen et al., 1989).

2.4.3 Effect of experimental conditions on caffeine mediated contractile response

2.4.3.1 Condition 1: Additional 2 min of stimulation (2MS)

As anticipated, caffeine had a potentiating effect on twitch tension following the additional 2 min of stimulation (2MS). The reversal of the effect of fatigue on twitch and other low frequency stimulation's (see Appendix IC) by caffeine brought tension back to approximately 80 % of its initial pre-fatigue value. The less than full recovery in the presence of caffeine following the additional 2 min of stimulation suggests that some of the tension loss is due to an impaired activation of the contractile proteins (Lännergren & Westerblad, 1991).

The value obtained in the present study agrees closely with a similar increase of 82.5 % previously obtained with intact mouse muscle fibre, despite the fact that the increase was obtained with a much higher stimulation frequency of 70 Hz (Lännergren & Westerblad, 1991). It should be mentioned that the 70 Hz stimulation frequency did not produce a fused tetani in the mouse fibre (Lännergren & Westerblad, 1991),

whereas 50 Hz did for the diaphragm muscle strips used in this study. Therefore, the contractile behavior at 70 Hz for the mouse fibre may be more closely related to contractility at lower frequencies of stimulation in the diaphragm muscle preparation.

The inotropic effect of caffeine on twitch tension is the result of an increase release of Ca^{2+} by the Ca^{2+} release channels of the sarcoplasmic reticulum (Rousseau et al., 1988a) and to a lesser extent an increase in the sensitivity of the myofibrillar filament to Ca^{2+} (Wendt & Stephenson, 1983) and an inhibition of Ca^{2+} uptake (Koshita & Oba, 1989). The prolonged contraction time (CT) and half-relaxation time ($RT_{1/2}$) are features that normally accompany the increase in tension with caffeine contracture (MacIntosh & Gardiner, 1987; Fryer & Neering, 1989) and reflect a prolonged Ca^{2+} transient (Thompson et al., 1992a). The extended CT suggests a longer opening time for the Ca^{2+} channels (Rousseau et al., 1988a) which would be needed to release the extra Ca^{2+} (MacIntosh & Gardiner, 1987). The prolonged $RT_{1/2}$ is required to reduce the intracellular Ca^{2+} below the level of saturation needed for contraction. The prolongation of the $RT_{1/2}$ was sufficiently large that it caused a reduction in the normalized rate of tension decay ($-dP/dt_{max} \cdot P_t^{-1}$), despite the increase P_t.

Condition 2MS had no further detrimental effect on tension for the 100 Hz stimulation frequency. However, the addition of caffeine produced a further decrease in P_{100Hz} reducing tension from 77 % to 50 % of the initial pre-fatigue value. This suggests that the caffeine stimulated release of Ca^{2+} was not only incapable of reversing the effect of fatigue on P_{100Hz}, but that it actually enhanced the disturbance in tension.

A reduction of tension in the presence of caffeine has been reported by Wittman and Kelsen (1982). However, the decrease was only observed when high

frequencies of stimulation (> 50 Hz) where used to induce the fatigue through the phrenic nerve. A failure at the neuromuscular junction was suggested to be responsible for the depressed tension. In contrast, a loss of tension with caffeine was also observed with an "situ" preparation of rat gastrocnemius stimulated at 10 Hz , where an impairment at the neuromuscular junction is unlikely (MacIntosh & Kupsh, 1987).

In the latter study, caffeine was injected into the muscle following a 10 min rest period and two min into a 5 min stimulation protocol. The 10 Hz tension was immediately depressed in the group receiving caffeine. It was concluded that the depression in tension, following the injection of caffeine was caused by a depletion of the Ca^{2+} stores (MacIntosh & Kupsh, 1987).

In the present experiment, tension was increased in the presence of caffeine at the low frequencies of stimulation. At 10 Hz, a frequency close to the stimulation of 15 Hz used to fatigue the muscle, tension was increased by 67 % (see Appendix IC). Apart from the experimental model other differences between the present and MacIntosh and Kupsh (1987) 's experiment could explain the difference in results.[LB5] The intermittent nature of the stimulation in this study may have provided more time for Ca^{2+} uptake to replenish the SR which may have delayed the depletion of the Ca^{2+} stores.

It is of interest that the time of caffeine application had an effect on the results of MacIntosh and Kupsh (1987). No depression in tension was observed when caffeine was injected in the muscle at the mid-point of the 10 min rest period, instead of 2 min into the stimulation.[LB6] The single mouse muscle fibre has also been shown to be sensitive to the time of application (Westerblad & Allen, 1991). Caffeine increased

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$[Ca^{2+}]_i$ in the control condition, during a middle long phase of stable tension production (phase 2) and during a final phase of rapid tension decline (phase 3) but the added caffeine had an effect on tension only at the end of the final phase (Westerblad & Allen, 1991). Reduction in maximum tension was credited to be the main mechanism responsible for the tension depression observed during phase 1 and 2, while reduced Ca^{2+} sensitivity and Ca^{2+} release were quantitatively more important for tension depression during phase 3 (Westerblad & Allen, 1991).

Could a depletion of the Ca^{2+} luminal stores be responsible for the reduced tension observed at 100 Hz in the presence of caffeine ? Studies using clamped cut fibres have showed that Ca^{2+} release in the presence of caffeine is increased because the release takes place not only during depolarisation, but also during the repolarization phase (Simon et al., 1989; Klein et al., 1990). This increased release of Ca^{2+} with caffeine during stimulation was used with the intact mouse fibre preparation to demonstrate that long interval stimulation, which did not induced fatigue on its own, produced LFF when caffeine was present (Chin & Allen, 1996). Based on these findings, it is possible that the 1.5 s stimulation train at 100 Hz, in the presence of caffeine, is sufficient to produce a $[Ca^{2+}]_i$ that is high enough to accentuate the effect of the 3 min stimulation protocol.

2.4.3.2 Condition 2: five min of passive recovery (5MR)

The 5 min of passive recovery was not sufficient to allow tension to recover in the no caffeine control. diaphragm suggesting that H^+ may be an important factor responsible for the fatigue. The result from the present study disagrees with another study which used a similar model and found after a 5 min rest period, a full recovery

from 1.5 min of continuous indirect stimulation   (Metzger & Fitts, 1987b).

Nevertheless, the fatigue observed for the twitch and the other two lower frequencies of

stimulation (see Appendix 1C) agrees with the well documented pattern of LFF that is

characterized by a mechanical recovery that requires many hours (Edwards, 1977).

The addition of caffeine completely reversed fatigue for the low frequencies of

stimulation, with the increases surpassing the initial pre-fatigue values (120-160 %).

The higher reversal of tension loss observed for the 5MR condition compared to the

2MS condition, suggest that perhaps more intracellular Ca^{2+} may have been available for

activation of the contractile proteins and could explain why full tension was not

obtained following the 2MS condition. This could come from three sources: (1) an

increase amount of Ca^{2+} release by the SR or (2) Ca^{2+} being release from myoplasmic

Ca^{2+} buffers, such as parvalbumin and TnC or (3) a slower uptake.

The fact that little Ca^{2+} (10-20 %) was left in the SR after applying caffeine to

skinned frog fibre in rigor condition (no ATP) (Stienen et al., 1993) suggest that full

release of Ca^{2+} was obtained in both the 2MS and 5MR conditions. However, if CT and

$RT_{1/2}$ reflect the Ca^{2+} transient, than this would tend to suggest that less not more Ca^{2+}

was released, from the Ca^{2+} channels during the 5MR than during the 2MS. Both CT

and $RT_{1/2}$ were prolonged following the 5 min passive recovery, but not as much as

after the 2MS condition.

The increased intracellular Ca^{2+} appears not to be linked to a lower Ca^{2+}

saturation of parvalbumin, since the Ca^{2+} saturation of parvalbumin appears to remain

constant during fatigue (Westerblad & Lännergren, 1991). In addition, the

concentration of parvalbumin is very low in oxidative fibres (Heizmann et al., 1982) and

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therefore would not be expected to play a major role in a muscle like the diaphragm. However, low saturation of another Ca^{2+} buffer; TnC during regular tetani in single mouse fibre is suggested to be reason why higher tension can be reach in the presence of caffeine (Westerblad & Allen, 1996). If the same mechanism is used to explain the greater tension measured in the 5MS condition, this would suggest that the saturation of TnC during the 5 min recovery period is lower than after the additional 2 min of stimulation of condition 2MS. This lower saturation in the 5MR condition could be explained by Ca^{2+} leaving TnC and being stored into the SR by the $Ca^{2+}-Mg^{2+}-ATPase$ pump. In the 2Ms, the stimulation would maintained the $[Ca^{2+}]_i$ by releasing Ca^{2+} from the Ca^{2+} release channels. The higher twitch tension observed in the presence of caffeine could also be due to phosphorylation of myosin light chain kinase that is known to potentiate tension at low frequencies of stimulation (Sweeney & Stull, 1990).

The fact that caffeine mediated recovery produced increases in tension that were higher than the unstimulated control demonstrated that no inhibition at the cross-bridges was present. The fact that full tension recovery did not take place with the 2MS, suggest that the concentration of Pi, an ion that is known to inhibits cross-bridge dynamics , was back to resting level after the 5 min of passive recovery. The 5 min time period appeared to be long enough to favor the resynthesis of ATP from ADP and Pi by the creatine kinase reaction (Hood & Parent, 1991).

Although, the lack of tension recovery could be explained by the concentration of H^+, LFF is suggested not to be caused by increases in metabolites because it is long lasting and metabolic disturbances have a faster recovery time (Westerblad et al., 1993; Lännergren et al., 1996). Studies, showing that patients lacking

myophosphorylase or phosphofructokinase still develop LFF, suggesting that metabolites, at least H^+ production, is not the main culprit behind the compromised tension loss with this type of fatigue (Wiles et al., 1981; Allen et al., 1995). However, transient increases in H^+ could produce lasting alterations in RyR that could maintained Ca^{2+} release depressed for longer period of times.

The cell concentration of ATP does not fall below 1 mM even during severe fatigue (Nagesser et al., 1992). However, it has been suggested that ATP might be compartmentalized and that depletion of these compartments could lead to muscle fatigue (Korge & Campbell, 1995). If this is the case, the 5 min recovery period would provide enough time for the phosphagen supply to replenish localized pools of ATP that may have been depleted by the 3 min of repetitive stimulation. These local pools of ATP could have facilitated release of Ca^{2+} by acting directly on the Ca^{2+} channels (Meissner & Henderson, 1987). Ca^{2+} release has been shown to be affected by ATP concentrations ranging between 0.1 and 0.5 mM in frog skinned fibre preparations but since caffeine is very efficient in releasing Ca^{2+} in rigor conditions, the effect of ATP on the release channels is probably modest (Stienen et al., 1993). The fact that recovery did not take place in the control diaphragm reinforces the assumption that regeneration of localized ATP is not a critical factor in caffeine mediated tension recovery.

P_{100Hz} was increased by only 30 % for the control and by only 15 % for the caffeine contracture in the respective diaphragm strips. This could be explained by the 3 min stimulation protocol that did not produce a significant loss in tension for the caffeine group for that condition so that less room was available for recovery. The increase still represented 100 % of the initial pre-fatigue tension. In contrast, P_{50Hz} was

significantly affected by the initial fatigue protocol and recovered by over 32 % a value slightly superior to the increase registered for the control strip (26 %) and representing 106 % of the resting value (see Appendix IC). The cytosolic Ca^{2+} concentration produced by the high stimulation frequency has been suggested to provide Ca^{2+} in excess of what is needed for maximal activation of the contractile myofilament (Edwards et al., 1977). Thus the extra Ca^{2+} triggered by the application of caffeine would have no additional impact on tension elicited by high frequency stimulation, but would foster a recovery in tension for low frequency stimulation.

2.4.3.3 Condition 3 : twenty min recovery (20MR)

No recovery in tension was observed for either the caffeine or control group following the 20 min recovery period without oxygenation. CT and $RT_{1/2}$ were reduced considerably for both control and caffeine contractions, resulting in longer $+dP_t/dt_{max} \cdot P_t^{-1}$ and $-dP_t/dt_{max} \cdot P_t^{-1}$. The sustained depression could be the result of the metabolic environment generated during the 3 min that would have been in part sustained during the 20 min period of passive recovery. However, caffeine was able to reverse partially the effect of this intracellular milieu in the case of condition 2MS. This suggest that the presence of O_2 is essential for full tension recovery both with and without caffeine.

As previously mentioned, the thickness of the diaphragm strips used for these experiments was probably adequate for diffusion of metabolites, such as lactate, out of the muscle. The 20 min recovery period may have been sufficient to allow lactate diffusion and recovery of pH under the anoxic condition that prevailed for this treatment. When the solution is gassed with 5 % CO_2 and 95 % O_2, the pH is 7.4 and a

period of only 1-2 min is necessary to reach half-time recovery (Mainwood & Alward, 1982). In the present experiment, the anoxia was produced by substituting the O_2 by N_2. Given that N_2, an inert gas, contrary to CO_2, should not affect the pH, it is likely that the level of acidosis in the muscle decreased during the 20 min period.

A second possibility is that the high levels of by-products of the high-energy phosphate system increased $[Ca^{2+}]_i$. In a skinned heart fibre preparation, where an ATP-free (rigor) solution was used to mimic the effects of hypoxia and ischemia, the elevation of both Pi and ADP reduced the SR Ca^{2+} content by 44-48 % (Zhu & Nosek, 1991). The mechanism behind the reduction of the Ca^{2+} lumenal stores was through a reversal of the Ca^{2+}-Mg^{2+}-ATPase pump (Zhu & Nosek, 1991). Although, the anoxic period was only 20 min long, it was preceded by 3 min of stimulation. Therefore, assuming that Pi and ADP do not diffuse out of the cell, it is possible that the concentrations of these metabolites may have been comparable to the levels used in the skinned heart fibre study. The increase $[Ca^{2+}]_i$, if it is sufficiently high could again be the factor involved through an inhibition of Ca^{2+} release in fatigued muscles.

Finally, the anoxia would have prevented a metabolic recovery by inhibiting oxidative phosphorylation (Sahlin et al., 1990). Therefore, if the acidosis and the metabolic by-products of the high-energy phosphate system phosphorylation were dissipated during the 20 min period, the depressed tension following the 20 min of anoxia would be the result of the lower phosphorylation.

2.5 SUMMARY

The effect of LFF on tension were partially reversed by caffeine, suggesting some inhibition at the level of the cross-bridges. In the control diaphragm, the 5 min recovery was not sufficient to reverse the effect of LFF, suggesting a possible role by H^+. The caffeine mediated recovery produced increases in tension that were higher than the rested tensions. This suggested that caffeine was capable of neutralizing the inhibition placed on the Ca^{2+} release channels and that the inhibition on the cross-bridges observed with the 2MS condition was not present. The fact that no inhibition was observed on the cross-bridges with the 5MR condition suggest that Pi is responsible for the inhibition in the 2MS condition and that full resynthesis of PCr took place during the 5 min of passive recovery. It is probable that the 20 min anoxic recovery prevented resynthesis of phosphocreatine. The 20 min time period would have allowed sufficient time for the dissipation of most of the H^+ leaving a suppress phosphorylation has the most probable mechanism for the reduced tension. Results from this study suggest that the inhibition of the Ca^{2+} release channels could due to H^+. Despite the fact that the recovery of metabolites normally precedes the recovery in tension with low frequency stimulation, a transient increase in H^+ produce longer lasting alterations in the structures of the Ca^{2+} release channels.

CHAPTER III

EFFECTS OF CALCIUM, PROTONS AND ATP CONCENTRATION ON CA^{2+}-MG^{2+}-ATPASE ACTIVITY IN HOMOGENATES OF MUSCLES OF DIFFERENT FIBRE-TYPE COMPOSITION

3.0 ABSTRACT

The sensitivity of Ca^{2+}-Mg^{2+}-ATPase activity to Ca^{2+}, H^+ and ATP was examined using an homogenate (Hom) preparation. Homogenates of tissue from white gastrocnemius medialis (WGM), red gastrocnemius medialis (RGM) and soleus, (S) containing different proportions of slow- and fast-twitch fibres were chosen to reflect differences in SERCA 2a and 1a isoform contents (Lytton et al., 1992). It was hypothesised that in Hom preparations in which the native environment was preserved, the sensitivity of the Ca^{2+}-Mg^{2+}-ATPase to Ca^{2+}, H^+, and ATP would be dependent on the fibre type composition, reflecting different contents of the two isoforms. Kinetic characteristics for Ca^{2+} which included maximal velocity (V_{max}), cooperativity (n_H) and Ca^{2+} needed for half-saturation of the high affinity binding sites ($Ca_{1/2}$) indicated no differences ($P > 0.05$) in dependency to Ca^{2+} for the two isoforms. However, the WGM and RGM demonstrated a greater sensitivity to H^+ than the S, suggesting a greater sensitivity by the SERCA 1a isoform to H^+. For ATP, a lower enzymatic activity was found at 1 mM for the RGM compared to WGM and S. However, since no differences were observed for the WGM and the S, the lower activity found for the RGM cannot be explained by differences in sensitivity between the two isoforms. The difference in H^+ sensitivity found between the WGM and RGM, compared and S, suggests that the SERCA 1a isoform may provide a way to exert a

tighter control in slowing by allowing SR Ca^{2+} uptake kinetics to be slowed, thereby

preserving $[Ca^{2+}]_i$, extending the Ca^{2+} transient and prolonging mechanical tension.

3.1   INTRODUCTION

The Ca^{2+}-Mg^{2+}-ATPase of the SR is best described as an ion-transport enzyme responsible for the uptake of two Ca^{2+} from the cytoplasm at the expense of the hydrolysis of one ATP. The enzyme-pump exists in two conformational states (de Meis & Vienna, 1979). The E_1 conformation displays high affinity Ca^{2+} binding sites on the cytoplasmic side while the E_2 conformation displays low affinity Ca^{2+} binding sites on the lumenal side (de Meis & Vienna, 1979). Upon binding of Ca^{2+}, a carboxyl group of Asp^{351} is phosphorylated by Mg · ATP (Mg . ATP^{2-}) and Mg . ADP (Mg . ADP^-) is released into the cytoplasm (MacLennan, 1990). The phosphorylation changes the conformation of the Ca^{2+} binding sites and the enzyme undergoes a conformational change from a high energy state E_1 to a low energy state E_2. The enzyme is translocated through the membrane and into the lumen where the Ca^{2+} ions are released as a result of a three fold decrease in Ca^{2+} binding site affinity. Following the release of Ca^{2+} the enzyme is dephosphorylated, Pi is released on the cytoplasmic surface and the ATPase returns to the E_1 conformation with reformation of the high Ca^{2+} affinity sites (Martonosi, 1984; MacLennan, 1990).

During repetitive contractions the increase kinetics of intracellular Ca^{2+} impose a greater demand on the activity of the Ca^{2+}-Mg^{2+}-ATPase. Prolonged or short term intensive bouts of exercise also produces changes in the intracellular milieu through increases in the metabolic by-products of muscle contraction. As a result of increased glycolysis, lactate and H^+ are elevated despite the presence of HCO_3^- and other cell buffer systems. Reduction in pH from 7.1 to 6.1 have been observed with severe contractile activity (Metzger & Fitts, 1987a; Westerblad & Lannergren, 1988). Changes

in one or more of these metabolites are believed to depress the activity of the cellular ATPases and to limit the rate at which ATP can be hydrolyzed and consequently the rate at which energy can be produced. Although, overall cell ATP tends to stay above the K_m for ATP of any of the ATPases, differences in local rate of production versus rate of utilisation in the vicinity of the ATPases, could make ATP a potential regulator of the enzyme (Korge & Campbell, 1995).

Changes in intracellular environment during acute hypoxia in cardiac muscle have been shown to impair SR Ca^{2+} transport by altering Ca^{2+}-Mg^{2+}-ATPase activity (Zhu & Nosek, 1991). In skeletal muscles, most of the studies reporting a down regulation of Ca^{2+}-Mg^{2+}-ATPase activity following acute activity have adopted an *in vitro* model of isolated vesicle fractions where Ca^{2+}-Mg^{2+}-ATPase activity is measured under optimal conditions (Byrd et al., 1989a; Byrd et al., 1989b, Luckin et al., 1991, Belcastro et al., 1993) . Although, this does not allow conclusions to be made on the acute effects of the metabolites on Ca^{2+}-Mg^{2+}-ATPase activity, it does not preclude a transient role for these metabolites as initiators of longer lasting reductions in Ca^{2+}-Mg^{2+}-ATPase activity (Byrd et al., 1989a; 1989b) and of alterations in (Luckin et al., 1991) that have been previously reported to result with contractile activity.

The metabolic response in muscle to exercise is largely dependent upon the fibre type composition of the muscle. In slow- and fast-twitch oxidative fibres, elevated ATP hydrolysis is not accompanied by large elevation in metabolite concentration (Meyer et al., 1980; Dudley & Terjung, 1985b). In contrast, fast-twitch glycolytic fibres demonstrate a high glycolytic activity with a large imbalance between utilisation and production of ATP (Hochachka & Matheson, 1992) which leads to a large

accumulation of metabolic by-products (Meyer et al., 1980). The response of the cellular ATPases and in particular the Ca^{2+}-Mg^{2+}-ATPase may depend on the isoform type. Mainly, two different isoforms of the Ca^{2+}-Mg^{2+}-ATPase are expressed in skeletal muscle fibre types (MacLennan et al., 1985; Brandl et al., 1986). SERCA 1a encodes the Ca^{2+}-Mg^{2+}-ATPase isoform that is expressed in fast-twitch fibres, whereas SERCA 2a encodes the isoform found in slow-twitch muscle fibres (Lytton et al., 1992). Although some structural differences exist between these isoforms (MacLennan et al., 1985; Brandl et al., 1987), characterisation through COS expression system have revealed no differences in biochemical properties, suggesting that the two isoforms demonstrate similar affinity to Ca^{2+}, H^+ and ATP (Lytton et al., 1992; Pozzan et al., 1994). Although, differences in relaxation in the various muscle fibre types appear to be regulated in part by the number of Ca^{2+}-Mg^{2+}-ATPase pumps in the specific tissue (Wu & Lytton, 1993), the presence of two isoforms suggest the existence of some diversity in function. Modulation of the pumping activity by intracellular metabolic changes could be the result of simply different production levels in the two muscle fibre types or could rely on a distinct sensitivity to selected metabolic by-products by the two isoforms.

The Ca^{2+}-Mg^{2+}-ATPase reconstituted in COS expression system or in vesicle fractions obtained by differential centrifugation is removed from it's native environment. This environment, mainly the lipid membrane surrounding the enzyme, may play a specific role in the regulation of the enzyme and the affinity of the two isoforms to levels of metabolites sustained during contraction. An homogenate assay where the natural environment of the Ca^{2+}-Mg^{2+}-ATPase is maintained may offer a more

physiological way of studying the dependency of the two isoforms to Ca^{2+}, H^+ and ATP, as examples, and enable one to determine if these affinities vary among different muscle fibre types. It was therefore hypothesised that Ca^{2+}-Mg^{2+}-ATPase activity would be reduced under conditions of low pH, low ATP and high Ca^{2+} concentrations. It was also hypothesised that the depression in Ca^{2+}-Mg^{2+}-ATPase activity would be less pronounced in muscle fibre type containing a higher proportion of slow-twitch fibres than in muscle fibre type predominantly composed of fast-twitch fibres, reflecting a lower sensitivity by SERCA 2a isoform to *in vitro* metabolic perturbations.

3.2 METHODS

3.2.1 Experimental design

Ca^{2+}-Mg^{2+}-ATPase activity was examined under various conditions of Ca^{2+}, H^+ and ATP concentrations. The Ca^{2+} dependency was determined by measuring activity under 10 free Ca^{2+} concentrations ranging from under 0.095 μM to 160.9 μM. Three pH values: 6.4, 7.0 and 7.6, were chosen to assess the sensitivity to H^+ ion concentration. The three values of pH were representative of muscle cell under resting condition (pH 7.0), under acidosis following intense repetitive contraction (pH 6.4) and under alkalosis (pH 7.6). For ATP concentration, the physiological concentration of 5 mM was used as the starting point and Ca^{2+}-Mg^{2+}-ATPase activity was also examined under conditions of 3 mM and 1 mM.

Ca^{2+}-Mg^{2+}-ATPase activity was measured under the above conditions in three muscle fibre types for 8 animals. The soleus (S) consists of a majority of highly oxidative slow-twitch fibres, and some fast-twitch oxidative fibres (80 % type I & 20 % type IIa) (Close, 1972). The gastrocnemius medialis is divided into two compartments. The deeper red compartments (RGM) is made up of a majority of type IIa fibres and a white superficial compartment (WGM); composed of a majority of type IIa fibres (Gardiner et al., 1991; De Hann et al., 1993; De Ruiter et al., 1995). The isoform of the Ca^{2+}-Mg^{2+}-ATPase found in slow-twitch fibres is encoded by the gene SERCA 2a (Lytton et al., 1992; Pozzan et al., 1994). For fast-twitch fibres, both fast-twitch glycolytic-oxidative and fast-twitch glycolytic fibres are encoded by the same gene SERCA 1, despite the existence of a SERCA 1b that appears to be present only during the developmental stages (Lytton et al., 1992).

The homogenate assay of Simonides and van Hardeveld (1990) was used to measure Ca^{2+}-Mg^{2+}-ATPase activity. A definite practical advantage of the homogenate technique over a differential centrifugation method is the amount of tissue needed to obtain a sample. This is a critical factor when assaying activity in a small muscle like the soleus, where the sacrifice of many animals would be needed to obtain sufficient SR vesicle fractions for the various trials.

3.2.2 Animal and muscle preparation

. Male Sprague-Dawley rats (307 ± 4.5 g, mean $\pm$ SE) were maintained in an environmentally controlled room ($27°$ C) with a reverse 12 : 12 light/dark cycle and fed on a diet of laboratory chow (Purina rat chow) and water *ad libitum*. Animal care was in accordance with the guidelines established by the Canadian Council on Animal Care. This study approved by the University of Waterloo Animal Ethics Committee.

The animals were anaesthetised with an intraperitonial injection of sodium pentobarbital (0.07 ml . g^{-1} of tissue). Following anaesthetisation, the soleus (S) and the gastrocnemius medialis (GM) of both hindlimbs were quickly excised from the animal and placed into ice-cold homogenising buffer containing: 200 mM sucrose, 10 mM NaN_3, 1 mM EDTA, 40 mM L-Histidine and 5 mM 1,4-dithiothreitol. The GM was further divided into two sections; a deep red compartment (RGM) containing a high proportion of fast-glycolytic-oxidative fibres and a white superficial compartment (WGM); composed of a majority of type IIb fibres (De Hann et al., 1993).

3.2.3 Tissue homogenisation

Visible blood, fascia and fat were removed from the excised muscle samples. The muscles were then blotted dry, weighed, returned in the ice-cold buffer and minced into small pieces with scissors. The tissue was then homogenised (11:1 dilution) by hand with a glass to glass homogeniser. A portion of the pre-measured buffer volume was retained to rinse the homogeniser and recover homogenate sticking to the glass wall. The homogenate was spinned and then sonicated for 2 s with a 5 s interruption, five times at a power output of 60 (Sonics & Materials Inc., Danbury, Con.). All manipulations were done on ice. Aliquots of the homogenate were pipetted in Eppendorf tubes, dropped in liquid nitrogen and stored at -80° C. Freezing of homogenate has been shown not to affect Ca^{2+}-activated ATPase activity (Simonides and van Hardeveld, 1990; Chin et al., 1994; Dossett-Mercer et al., 1994). Protein determination was done with the method of Lowry et al. (1951) as modified by Schacterle & Pollock (1973).

3.2.4 Determination of free Ca^{2+} concentration

All solutions were made with water filtered through a Milli-Q Water Purification System (Millipore) and plastic ware was used to reduce Ca^{2+} leaching associated with glassware (Bers et al., 1994). The plastic ware was also soaked in a solution of EGTA (3 mM) and EDTA (1mM) (Meissner, 1973) and rinsed five times with ultra-pure water. A 100 mM $CaCl_2$ standard solution (Orion Research Incorporated, Boston, Ma.) was used as the Ca^{2+} source. The free Ca^{2+} concentrations ($[Ca^{2+}]_f$) were determined using a calcium software program (Chelator; Schoenmakers,

1992), taking into consideration the precise ionic strength, temperature (37° C) and pH of the buffer. Binding constants determined under standard conditions (0.1 M ionic strength, pH 7, 20° C) were adjusted to the specific conditions of the present experiments. A proton binding constant for phosphoenolpyruvate (Martell & Smith, 1974) was entered into the Ca^{2+} software program to control for the effect of this compound on $[Ca^{2+}]_f$.

The volumes of $CaCl_2$ for the Ca^{2+} dependency curve were chosen through pilot work to yield a well distributed activity curve. To counter the effect of proton release from the EGTA that occurs with the addition of Ca^{2+} (Tsien & Pozzan, 1989), precise volumes (1-3 µl) of KOH 100 mM were added to minimise fluctuation from the desired pH. The specific volumes of KOH were determined before hand under a set of similar conditions in a jacketed micro-vessel at 37° C using a glass pH electrode calibrated to 0.01 unit at that temperature. For the dependency curve, the Ca^{2+} binding data was analysed through non-linear regression with a computer software (GraphPad Software Inc.) using the following sigmoidal dose-response equation:

$$Y = Y_{bot} + (Y_{top} - Y_{bot}) / 1 + 10^{((\log Ca_{1/2} - X) \cdot n_H)}$$

where Y_{bot} is the Y value at the bottom of the plateau; Y_{top} is the value at the top of the plateau, $\log Ca_{1/2}$ is the logarithm of $Ca_{1/2}$, the concentration that gives a response halfway between Y_{bot} and Y_{top} and n_H, the Hill coefficient.

For the ATP and pH dependency, single low and high $[Ca^{2+}]_f$, were derived from the Ca^{2+} dependency curve, were used for the measurements. The total Ca^{2+} concentration was adjusted using the software program to insure that the $[Ca^{2+}]_f$ remains similar across all of the different conditions. As with the Ca^{2+} dependency

measurements, pre-determined volumes of KOH were added to preserve pH. The $[Ca^{2+}]_r$ were estimated, considering the accurate volume of buffer, enzymes, $CaCl_2$, KOH and homogenate added to the cuvette.

3.2.5   Assay for Ca^{2+}-Mg^{2+}-ATPase activity

Ca^{2+}-Mg^{2+}-ATPase activity was measured with the method of Simonides and van Hardeveld (1990) consisting of the coupled enzyme assay involving the pyruvate kinase and lactate dehydrogenase reactions in which the disappearance of NADH is monitored spectrophotometrically. The assay medium (Simonides & van Hardeveld, 1990) contained 20 mM Hepes, 200 mM KCl, 15 mM $MgCl_2$, 10 mM NaN_3, 1 mM EGTA, 10 mM phosphoenolpyruvate (PEP), 5 mM MgATP, 18 U ml^{-1} each of purified pyruvate kinase and lactate dehydrogenase and 2 μM Ca^{2+} ionophore A23187 and pH 7. The pH electrode was calibrated in pH buffers thermostatted at 37° C and the pH of the assay buffer was adjusted at the same temperature (Tsien & Pozzan, 1989). The ionophore, which make the vesicles leaky and prevents the accumulation of Ca^{2+} in the lumen, was added to the assay medium to insure that the activity was maximised. The concentration of 2 μM was chosen after pilot work demonstrated that similar activities were reached with 2 or 5 μM.

The frozen homogenate was allowed approximately 15 min to thaw on ice. One ml of buffer was added to a plastic cuvette and allowed at least 8 min to equilibrate to 37° C. The enzymes and volumes of homogenate (11 μl for the RGM and WGM, and 33 μl for the S) were added to the cuvette. The higher volume of homogenate for the S was used to increase sensitivity because of the low Ca^{2+}-Mg^{2+}-ATPase activity

demonstrated in this muscle compared to the red and white gastrocnemius (Dossett-Mercer et al., 1994). After another 2 min of incubation and before addition of Ca^{2+} from any external source, the reaction was followed spectrophotometrically (Shimadsu, UV-1601PC, Tek Science, Missisauga, Ont.) by measurement of NADH at 340 nm (ϵ_{340}). After monitoring the assay for approximately 2 min, the first volume of 100 mM $CaCl_2$ standard (Orion Research Incorporated, Boston, Ma) and KOH were added and activity monitored again for approximately 2 min. Basal or Mg^{2+} dependent activity was determined by two successive additions of 2 M Ca^{2+} to a final concentration of 20 mM to saturate the low affinity Ca^{2+} binding sites and inhibit the Ca^{2+}-dependent activity (Simonides & van Hardeveld, 1990).

Assay time, following the initial 10 min of incubation, was between 20-22 min for Ca^{2+} dependency and 8-10 minutes for each of the three pH and ATP conditions. Two trials were run for each condition and on average the assays of one tissue type for one animal were run during an analytical session. Ca^{2+}-activated ATPase activity was obtained by subtracting basal ATPase activity from total ATPase activity. The molar absorption coefficient used for NADH to calculate the rate of hydrolysis [LBI]was 6.27 x 10^{-3} . M^{-1} . cm^{-1}.

3.2.6   Data and statistical analysis

The data was collected with Personal Spectroscopy Software (Shimadzu Scientific Instruments Inc.) program. Regression analysis was used to determine the slope of the absorbance signal at the various conditions. One-way Analyses of Variance (ANOVA) with one repeated measure, condition (e.g. pCa 5.99 and ATP 3 mM) were used to determine the statistical analysis for each of the three muscle fibre types. One

way ANOVA's were also used to compare kinetic characteristics of the Ca^{2+}-activated activity among the three muscle fibre types. Planned comparisons were performed to determine differences between means from the one way interaction. The significance level was accepted at $P < 0.05$.

3.3 RESULTS

3.3.1 Ca^{2+} dependency of Ca^{2+}-Mg^{2+}-ATPase activity in homogenate.

Maximal Ca^{2+}-Mg^{2+}-ATPase activity (V_{max}) was reached at a pCa of 5.41 for the WGM. However, no significant differences were found between activities at pCa values ranging from 4.85 (85.7 %) to 5.74 (74 %) (Figure 3.1). For the RGM, the maximal activity was reached at a pCa of 5.74 with a plateau extending down to pCa 5.19 (88 % of maximal activity). Finally for the S, maximal activity was found at pCa 5.46 with a plateau extending over a wide range of pCa values from 3.85 (85.6 %) to 6.02 (81.3 %). The Hill coefficient (n_H), which evaluates the interaction between the two high affinity Ca^{2+} binding sites, confirmed a positive cooperativity that was similar for the three muscle fibre type with values of 2.0 for the WGM, 2.2 for the RGM and 1.8 for the S (Table 3.1). The $[Ca^{2+}]_f$ needed for half-saturation of the high affinity sites ($Ca_{1/2}$) was also similar among the three muscle fibre type with values of 1.1 μM for the WGM, 0.8 μM for the RGM and 0.9 μM for the S (Table 3.1). Basal or Mg^{2+}-dependent ATPase activity was 6.1 % for the WGM, 9.7 % for the RGM and 15.9 % for the S of the total ATPase activity.

3.3.2 Effect of pH on Ca^{2+}-Mg^{2+}-ATPase activity in homogenates.

The effects of the three pH conditions are summarised in Table 3.2. To make comparisons between the conditions, the values, for each muscle fibre types, are presented as a % of the activity measured at pH 7.0 and pCa 4.78. The enzymatic Ca^{2+}-stimulated activity was reduced at an acidosis comparable to that produced during intense repetitive muscular contraction (pH 6.4). In general, the extent of this reduction

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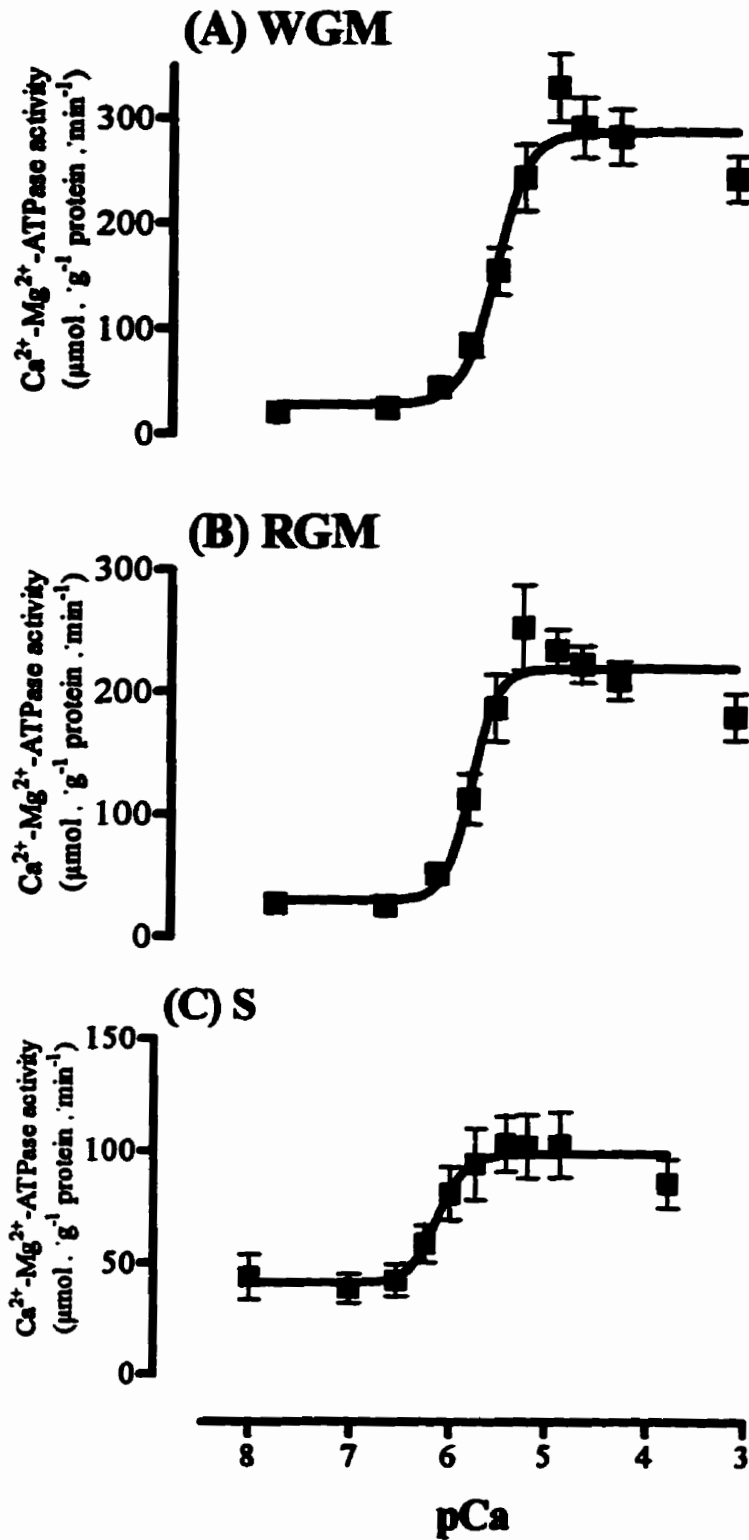
Figure 3.1. Ca^{2+}-Mg^{2+}-ATPase activity at 37°C, pH 7 and 5 mM ATP over the pCa range of 3.8-8.0 (n = 10) for white gastrocnemius medialis (WGM) (A); red gastrocnemius medialis (RGM) (B); soleus (C).

Table 3.1. Kinetic characteristics of Ca^{2+}- activated ATPase activity: (V_{max}), Hill coefficient (n_H) and Ca^{2+} concentration for half-saturation ($Ca_{1/2}$) of the high affintiy sites.

	WGM	RGM	S
V_{max} (μmol . g^{-1} protein . min^{-1})	291 ± 24	229 ± 13	100 ± 12 *
n_H	2.0 ± 0.4	2.2 ± 0.3	1.8 ± 0.6
$Ca_{1/2}$ (μM)	1.0 ± 0.2	0.7 ± 0.2	0.9 ± 0.2

Kinetic characteristics were determined from plot obtained from sigmoid fits of equation $y = Y_{bot} + (Y_{top} - Y_{bot}) / 1 + 10^{((\log Ca_{1/2} - x) \cdot n_H}$ (see methods) of Ca^{2+}-Mg^{2+}-ATPase activity data measured at $[Ca^{2+}]_f$ ranging from pCa 3.79 to 7.02 for White Gastrocnemius Medialis (WGM) and Red Gastrocnemius Medialis (RGM) and pCa 3.85 to 7.03 for Soleus (S). n_H was determined from Hill plots of the portion of the curve of Ca^{2+}-dependent activity as a function of $[Ca^{2+}]_f$ which correspond for the WGM and RGM to 20 to 90 % and for the S to 45 % to 95 % of maximal activity. Ca^{2+}-Mg^{2+}-ATPase activity measured at 37° C, pH 7.0, 5 mM ATP and 10 mM of free Mg^{2+}. Values: mean $\pm$ SE. * Significantly different than WGM and RGM ($P < 0.05$).

Table 3.2. Effect of pH on Ca^{2+}- activated ATPase activity for (A) White Gastrocnemius Medialis (WGM); (B) Red Gastrocnemius Medialis (RGM); (C) Soleus (S).

		pCa		
		8.0	5.99	4.78
(A) WGM				
	6.4	4.1 ± 1.8	20 ± 3.0 [*]	300 ± 28 [*†]
pH	7.0	9.5 ± 3.1 [‡]	137 ± 25 [*‡]	394 ± 27 [*†]
	7.6	31 ± 1.0 [‡]	269 ± 31 [*†§]	230 ± 28 [*†§]
(B) RGM				
	6.4	7.6 ± 3.8	20.7 ± 4.7 [*]	185 ± 23 [*†]
pH	7.0	19.5 ± 4.6	161 ± 23 [*‡]	371 ± 40 [*†‡]
	7.6	24.9 ± 4.3 [‡]	227 ± 18 [*‡]	212 ± 10 [*§]
(C) S				
	6.4	51 ± 11	57 ± 12	118 ± 15 [*†]
pH	7.0	20 ± 3.0	82 ± 17 [*]	108 ± 16 [*]
	7.6	40 ± 13	93 ± 9.0 [*‡]	79 ± 8.0 [*†§]

Values are means $\pm$ SE. [*] Significantly different from pCa 8 at comparable pH, ($P < 0.05$). [†] Significantly different from pCa 5.99 at comparable pH, ($P < 0.05$). [‡] Significantly different from pH 6.4 at comparable pCa, ($P < 0.05$). [§] Significantly different from pH 7.0 at comparable pCa, ($P < 0.05$).
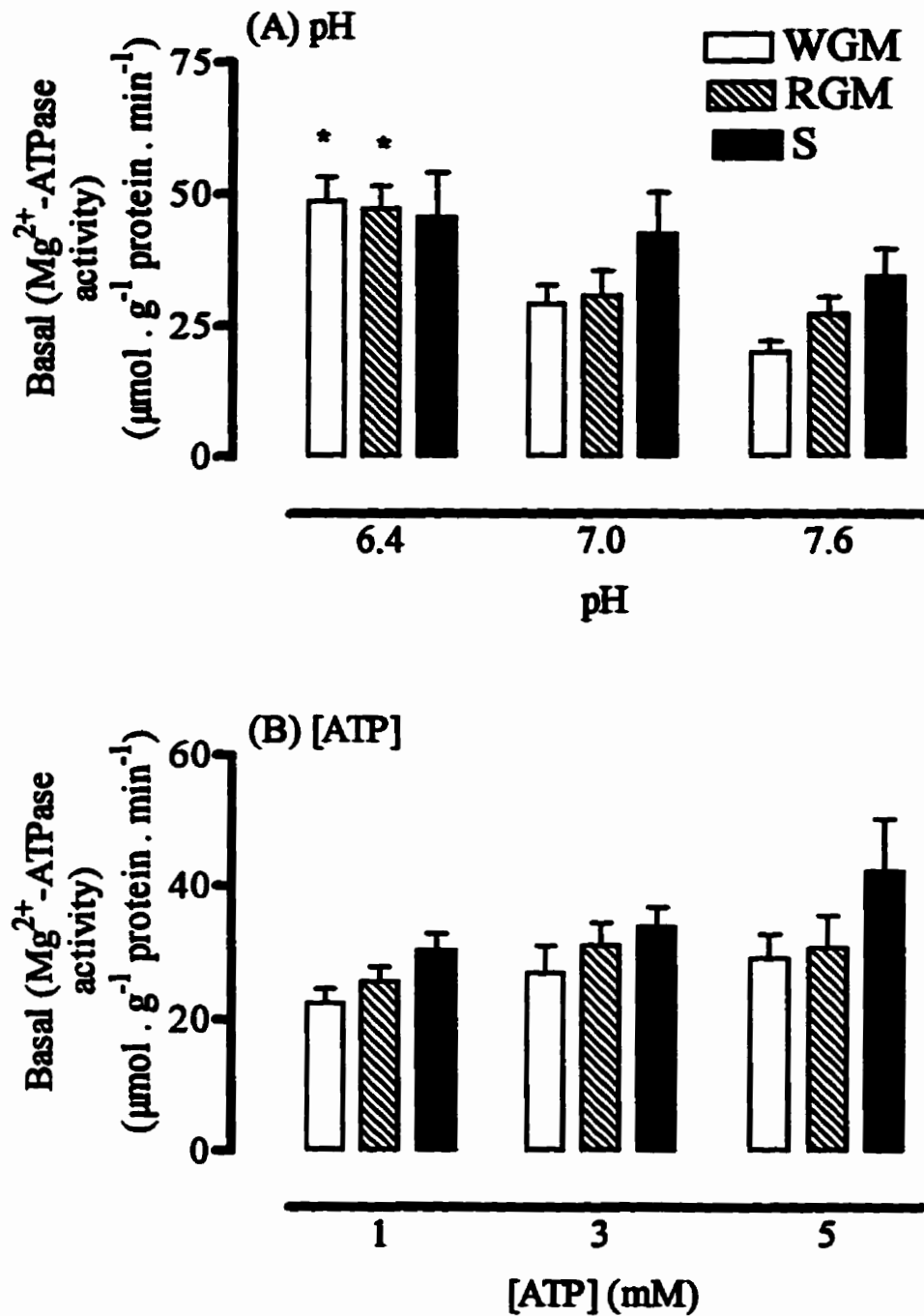
Figure 3.2. Effect of pH (A); and [ATP] (B); on
basal (Mg^{2+}-activated) activity. * Significantly
different than pH 7.0 and 7.6 ($P < 0.05$).

was dependent on the $[Ca^{2+}]_f$ levels. However, the WGM and RGM displayed a much more pronounced sensitivity to pH than the S. At pH 6.4, the activity measured before the addition of any external Ca^{2+} (pCa = 8.0) was only lower in the WGM, representing only 1 % of the maximal activity measured under pH 7 and pCa 4.78, considered as the optimal condition for these experiments. With the first addition of Ca^{2+} (pCa 5.99), the difference in activity became more pronounced in the WGM (5 % vs. 34. 7 %) and this difference extended to the RGM (5.6 % vs. 43.3 %). At pCa 4.78, a difference in activity was only found for the RGM (49.8 % vs. 100 %).

As with the acidosis, the effect of increasing pH to 7.6 was dependent upon the $[Ca^{2+}]_f$. At pCa 8, the activity at pH 7.6 was similar to the activity found at pH 7.0. Increasing $[Ca^{2+}]_f$ to pCa 5.99 produced an increase in activity only for the WGM (34.7 % vs. 68.3 %), although a trend was observed for the RGM. The second addition of Ca^{2+} (pCa 4.78) produced a significant decrease in activity for all three muscle fibre types. However, this increase appeared to be more pronounced for the WGM (58.3 %) and RGM (57.2 %) than for the S (73.8 %). Basal (Mg^{2+}-activated) activity was found to be higher at pH 6.4 than at pH 7.0 in the RGM and WGM (Figure 3.2A).

3.3.3 Effect of ATP concentration on Ca^{2+}-Mg^{2+}-ATPase activity in homogenates

The dependence of the Ca^{2+}-Mg^{2+}-ATPase activity in homogenates to ATP was examined at three concentrations: 1 mM, 3 mM and 5 mM. The results are presented in Table 3.3. To make comparisons between the conditions, the values, for each muscle fibre types, are presented as a % of the activity measured in the presence of 5 mM [ATP] and pCa 4.8. As with pH, the activities at the three ATP concentrations were

Table 3.3. Effect of ATP concentration on Ca^{2+}-activated activity (A) White Gastrocnemius Medialis (WGM); (B) Red Gastrocnemius Medialis (RGM); (C) Soleus (S).

		pCa		
		8.00	5.98	4.8
(A) WGM				
	1	17 ± 6	121 ± 13 [*]	355 ± 44 [*†]
[ATP] mM	3	15 ± 5	146 ± 25 [*]	418 ± 30 [*†]
	5	10 ± 3	137 ± 25 [*]	394 ± 26 [*†]
(B) RGM				
	1	15 ± 4	79 ± 16 [*]	271 ± 35 [*†]
[ATP] mM	3	16 ± 5	114 ± 20 [*‡]	323 ± 33 [*†]
	5	20 ± 5 [‡]	161 ± 23 [*‡]	371 ± 40 [*†]
(C) S				
	1	30 ± 5	70 ± 22	105 ± 14 [*†]
[ATP] mM	3	27 ± 4	56 ± 10 [*]	91 ± 9 [*†]
	5	18 ± 3	81 ± 17 [*]	108 ± 16 [*]

Values are means ± SE. [*] Significantly different from pCa 8 at comparable pH, ($P < 0.05$). [†] Significantly different from pCa 5.98 at comparable pH, ($P < 0.05$). [‡] Significantly different from ATP 1 mM at comparable pCa, ($P < 0.05$).

dependent upon the $[Ca^{2+}]$. As for pH, the activities were first measured without the addition of any external Ca^{2+}. No differences were observed between the three concentrations with the exception of the RGM where the activity at 1 mM (3.9 %) was lower than the activity measured at 5 mM (5.3 %).

At pCa 5.98, no differences in activities were observed for the WGM and S at the three ATP concentrations. However, in the RGM, the activity was higher not only at 5 mM ATP (43.3 %), but also at 3 mM ATP (30.7 %) when compared with the activity at 1 mM (21.2 %). The concentration of ATP had no differential effect on basal activity for any of the three muscles (Figure 3.2B).

3.4   DISCUSSION

In agreement with the findings of Simonides and van Hardeveld (1990), results from these experiments suggest that the homogenate preparation can be effectively used to study the dependency of Ca^{2+}-Mg^{2+}-ATPase activity to Ca^{2+}, H^+, and ATP. Although overall, the dependency to Ca^{2+} and ATP by the three muscle fibre types were similar, differences in pH dependency between WGM and RGM, and S support the hypothesis that the sensitivity to pH of SERCA 1a isoform is greater than the sensitivity to pH of SERCA 2a isoform.

The expression of isoforms of the Ca^{2+}-Mg^{2+}-ATPase has been suggested to play an important role in modulating the Ca^{2+} stores in tissues (Pozzan et al., 1994). Molecular cloning studies have identified three genes encoding the Ca^{2+}-Mg^{2+}-ATPase : SERCA 1, SERCA 2 and SERCA 3. In skeletal muscle, SERCA 1 encodes the pump enzyme that is exclusively expressed in fast-twitch fibres (Lytton et al., 1992). Two spliced species of the gene, termed SERCA 1a and 1b have been identified in adult and neonatal tissues, respectively. A second isoform, with a similar number of amino-acid residues and molecular weight but with some structural differences, is encoded by SERCA 2a in slow-twitch muscle fibre type (MacLennan et al., 1985; Brandl et al., 1987). Mapping of the amino-acid sequence has revealed a structure that varies by approximately 15 % (Brandl et al., 1986).

Biochemical characteristics of these isoforms such as Ca^{2+} and ATP affinities and pH dependency have been studied with COS cell expression system (Marayuma et al., 1989; Lytton et al., 1992). Although, some differences were observed for SERCA

2b and SERCA 3 when compared with other SERCAs, no differences were demonstrated between SERCA 1 and SERCA 2a with this type of preparation. This is surprising considering that the existence of two isoforms should confer some distinctive functional characteristics (Hawkins et al., 1994). One of these potential characteristics is the modulation of enzymatic activity through levels of metabolic by-products induced by muscle contraction. This modulation could occur through difference in levels of production in the two muscle fibre types or through a distinct difference in sensitivity of the isoforms to the levels of metabolites.

3.4.1 Homogenate preparation.

One disadvantage of the COS expression system or of vesicle fractions isolated through differential centrifugation is that the Ca^{2+}-Mg^{2+}-ATPase is translocated from its native environment. This translocation may affect the regulation of the enzyme and eliminate a distinctive affinity of the two isoforms to levels of metabolites sustained during increased contractile activity. In the case of vesicle fractions isolated by differential centrifugation, although a pure fraction is obtained, a large amount of the SR is discarded during the differential centrifugation process and what is left may not be representative of longitudinal SR. Another possibility is that the condition to which the muscle is exposed *in vivo* may predispose the tissue to a differential effect by the isolation process. In cardiac muscle, as the ischemic period increased, it was found that a greater fraction of SR that was able to accumulate Ca^{2+} was discarded during the isolation protocols (Rapundalo et al., 1986)

In this study, an homogenate assay (Simonides & van Hardeveld, 1990) was

used to examine the dependency of the Ca^{2+}-Mg^{2+}-ATPase to ATP and H^+ as modified by the $[Ca^{2+}]_f$ in three muscle tissue composed of different proportions of muscle fibre types and consequently of SERCA 1a and 2a. It is assumed that this homogenate assay would enable the measurement of Ca^{2+}-Mg^{2+}-ATPase activity in a more intact preparation where the enzyme remains embedded into the lipid bilayer and consequently be more representative of *in vivo* conditions.

3.4.2 Muscle fibre type and SERCA isoform distribution.

Ca^{2+}-Mg^{2+}-ATPase activity was measured in three tissue composed of a predominant percentage of the different fibre types. The WGM is made up of a majority of fast-twitch glycolytic fibres whereas the RGM is composed of a higher proportion of fast-twitch oxidative fibres (Gardiner et al., 1991; De Hann et al., 1993; Ruiter et al., 1995). The S is composed mostly of high oxidative slow-twitch fibres with a small proportion of fast-twitch glycolytic oxidative fibres (80 % type I & 20 % type IIa) (Ruiter et al., 1995). The mRNA and protein levels of the different isoforms of Ca^{2+}-Mg^{2+}-ATPase isoform has been determined in a number of muscles of the rat (Wu & Lytton, 1993). In the extensor digitorum longus (EDL) and white semitendinosus (WST), muscles that would compare favourably in terms of fibre composition with the WGM used in the present study, mRNA and protein quantities of SERCA 1 were 3.2-4 and 5-6 fold higher, respectively, than in soleus (mRNa : 5.3 ± 0.2 fmol . 100 mg^{-1} and proteins : 1.8 ± 0.2 nmol . 100 mg^{-1}). In the soleus, despite a high proportion of type I fibres, SERCA 2a mRNA was only 70 % (3.8 fmol . 100 mg) of SERCA 1 (Wu & Lytton, 1993). For the RGM, the value would be close to the WGM, since most of the

fibre composition is made of type IIa and the isoform of these fibres appears to be encoded by the same SERCA gene. The small proportion of type I fibres would suggest slightly higher levels of SERCA 2a in the RGM than in the WGM.

3.4.3 Ca^{2+} dependency of Ca^{2+}-Mg^{2+}-ATPase activity in homogenates

Maximal Ca^{2+}- activated activities (V_{max}) measured in this study were similar to the values reported in the original study that used the homogenate technique (Simonides and van Hardeveld, 1990). Ca^{2+}-activated Ca^{2+}-Mg^{2+}-ATPase activities, measured by these investigators, were between 85-107 $\mu M \cdot g^{-1}$ protein $\cdot min^{-1}$ for rat gastrocnemius and 57 $\mu M \cdot g^{-1}$ protein $\cdot min^{-1}$ for rat soleus. The values reported in the present study agree well with these values considering the Q_{10} effect since the activity in our assay medium was measured at 37° C instead of 25° C.

Despite the high variability in maximal activities, V_{max} was about 2.9 fold higher in WGM and 2.3 fold higher in RGM than in S (Table 1). Simonides and van Hardeveld (1990) found a 1.6-1.9 fold difference in activity between S and undivided white and red gastrocnemius. This difference has also been confirmed by previous studies examining Ca^{2+}-uptake properties in fast- and slow-fibre muscle types of the rat (Briggs et al., 1977; Kim et al., 1981). Maximum oxalate-supported Ca^{2+} uptake was 5-6 times higher in homogenate of fast- than slow-twitch muscle fibre type (Brigg et al., 1977). Also in rat, homogenates and vesicle fractions obtained from fibres of the deep (70 % IIa) and superficial (100 % IIb) vastus lateralis showed 2-3 times higher Ca^{2+} uptake than homogenate and vesicle fractions obtained from S (Kim et al., 1981).

These differences are explained by a greater volume of SR in fast-twitch muscle

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types (Gillis, 1985) and higher content of Ca^{2+}-Mg^{2+}-ATPase (Leberer & Pette, 1986). In guinea pig, 34.5 % and 19.7 % of the surface of the terminal cisternae and of the longitudinal SR, respectively were free of pumps, whereas only 1 % of the total surface in white vastus was not covered by Ca^{2+}-Mg^{2+}-ATPases (Ferguson & Franzini-Armstrong, 1988). As for maximal activity between tissues composed of a predominance of type IIa and type IIb fibres, although a tendency towards a higher content was observed for type IIb, the difference was not significant. (Leberer & Pette, 1986; Maier et al., 1986; Krenacs et al., 1989). Differences in density have also been reported in guinea pig, where the number of Ca^{2+}-Mg^{2+}-ATPase was found to be 32,000 . μm^{-2} in white vastus lateralis and 25,000 . μm^{-2} in soleus (Ferguson & Franzini-Armstrong, 1988). Finally, recent quantification of SERCA isoforms reveals that the fast-twitch muscle type contains about three times the total amount of Ca^{2+}-Mg^{2+}-ATPases found in slow-twitch muscle type (Wu & Lytton, 1993).

Maximal Ca^{2+}-activated activity, at optimal pH and ATP levels was found at a pCa around 5 which is similar to the rate found under normal conditions of pH and temperature, in leaky vesicles (i.e. in the presence of Ca^{2+} ionophore A-23187) or when the Ca^{2+} concentration is maintained by an EGTA-buffer (Korge & Campbell, 1994). However, the range of $[Ca^{2+}]_f$, over which a plateau in maximal Ca^{2+}-activated activity was reached, appeared to be more pronounced for certain muscle fibre types than others. This was especially true for the S, were Ca^{2+}-activated activity was not significantly different over a wide range of pCa values from 3.85 to 6.02. The fact that the plateau extends over this range of pCa values is not surprising since the K_m for the

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low affinity Ca^{2+} binding sites is in the much higher range of 1-3 mM and therefore an inhibitory effect would not be found at these lower pCa values. In the study of Simonides & van Hardeveld (1990) Ca^{2+}-Mg^{2+}-ATPase activity was not measured at $[Ca^{2+}]_f$ greater than pCa 5, but activites measured at the next higher pCa were only slightly lower than those measured at pCa 5, suggesting that the plateau does in fact begin at pCa values slightly over 5.

In the S, the initial activity measured before the addition of any exogenous Ca^{2+}, represented 43 % of the maximal activity, compared to 6.1 % and 10.7 % for the WGM and RGM, respectively. This difference between tissue in Ca^{2+}-dependency at low $[Ca^{2+}]_f$ was not observed between similar homogenate preparations of rat gastrocnemius and S in the experiments of Simonides and van Hardeveld (1990). Significant Ca^{2+}-Mg^{2+}-ATPase activity has been measured in vesicles incubated with ionophore, in a buffer containing no EGTA or exogenous Ca^{2+} (Korge & Campbell, 1994). Ca^{2+} contamination from the added SR vesicles was estimated at 4-5 μM. The level of Ca^{2+} contamination coming from the homogenate could have been higher for the S assay than for the WGM and RGM assays, since a volume of 33 μl was used for the S instead of 11 μl for the WGM and RGM. However, the presence of 1 mM EGTA, should have sequestered the contaminating Ca^{2+}.

Another possibility is that saturation of the high affinity sites could have occurred at a lower $[Ca^{2+}]_f$ because of the lower amount of the Ca^{2+}-Mg^{2+}-ATPase in slow and fast-twitch muscle fibre types. However, similar $[Ca^{2+}]$ at half-maximal activity ($Ca_{1/2}$) among the three muscle fibre types suggest that this was not the case (Table 3.1). These results also suggest that different proportions of SERCA isoforms

does not confer a particular affinity to the Ca^{2+}-Mg^{2+}-ATPase for $[Ca^{2+}]_f$ in the three muscle fibre types. Simonides and van Hardeveld (1990), employing the same homogenate preparation used in this study, reported a similar value of 0.6 μM for both rat gastrocnemius and soleus. $Ca_{1/2}$ between 0.2 and 2 μM (pH 7) have been reported in the literature for vesicle fractions, the variability depending on the Ca^{2+}-EGTA dissociation constant used (De Meiss & Vianna, 1979). Therefore, the results from COS expression system suggesting similar affinity to Ca^{2+} for the two isoforms (Lytton et al., 1992) is supported with the homogenate preparation. Finally the positive cooperativity between the two high affinity Ca^{2+} binding sites is confirmed with Hill coefficient (n_H) between 1.8 and 2.2.

3.4.4 Effect of pH on Ca^{2+}-Mg^{2+}-ATPase activity in homogenate

The effect of pH on the activity of the Ca^{2+}-Mg^{2+}-ATPase of isolated vesicles is described by a bell-shaped curve with an optimum near pH 7.0 (Martonosi, 1969; Bishop & Al-Shawi, 1988). In the present study, the activity of Ca^{2+}-Mg^{2+}-ATPase in homogenates was examined in single separate assays for three pH values : 6.4, 7.0 and 7.6. Maximal activity was observed at pH 7.0 and pCa 4.78 ([ATP] = 5 mM) for the RGM and WGM. However, for the S, the highest activity was found under a number of conditions, including the one above.

The depression in Ca^{2+}-Mg^{2+}-ATPase activity at low pH is explained by a decrease in the affinity for Ca^{2+} of both the low- and high-affinity Ca^{2+} binding sites (Meissner, 1973; Verjovski-Almeida & de Meis, 1977) as well as a reduced cooperativity of Ca^{2+} binding (Hill & Inesi, 1982; Henderson et al., 1994a). The

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increase in n_H at acidic pH values demonstrates that there is more inhibition by H^+ at the first Ca^{2+} binding site than at the second site (Forge et al., 1993a).

The decreased affinity is explained by a competition between the H^+ and Ca^{2+} for the Ca^{2+} binding sites (Pick & Karlish, 1982; Dixon & Haynes, 1990; Henderson et al., 1994a). The $Ca_{1/2}$ or K_m for Ca^{2+} has been reported to vary over three orders of magnitude with variation in pH from 5.5 to 8.0 (Haynes & Mandveno, 1983).The E_1 conformation of the enzyme has a high affinity for Ca^{2+} and a low affinity for H^+, whereas the E_2 conformation has a low affinity for Ca^{2+} and a high affinity for protons (Pick & Karlish, 1982). Therefore, the pH will determine the $E_1 \leftrightarrow E_2$ equilibrium which in turn will affect the rate of Ca^{2+} binding to the high affinity sites (Henderson et al., 1994b).

An increased affinity by the lumenal Ca^{2+} binding sites for Mg^{2+} (Bishop & Al-Shawi, 1988; Michalangeli et al., 1990) is one of the mechanisms that has been suggested to explain the reduction in activity observed at alkaline pH. Mg^{2+} plays many roles in the catalytic cycle of the Ca^{2+}-Mg^{2+}-ATPase. It activates the enzyme by direct binding. It is also essential for the binding of ATP to the Ca^{2+}-Mg^{2+}-ATPase, since Mg·ATP represents the physiological substrate (Meissner, 1973). It is also important in the translocation step from Ca_2·E_1~P to Ca_2·E_2~P (Yamada & Ikemoto, 1980) and in the dephosphrylation of E_2~P into E_2 + Pi (Guillain et al., 1984).

In the homogenate assay used in this study, the $[Mg^{2+}]$ had to be raised to 15 mM (10 mM $[Mg^{2+}]_f$), to maximise the activity of the coupling enzyme system of pyruvate kinase and lactate dehydrogenase under the high $[Ca^{2+}]_f$ used to inhibit the Ca^{2+}-activated activity and measure basal or Mg^{2+}-activated activity (Simonides & van

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Hardeveld, 1990). In assays of vesicle preparations, [Mg^{2+}] in high concentrations (Guillain et al., 1982; Forge et al., 1993a) has been shown to have an inhibitory effect on Ca^{2+}-Mg^{2+}-ATPase activity by competing for the high affinity Ca^{2+} binding sites (Guillain et al., 1982; Forge et al., 1993a) and lowering cooperativity between the two sites (Forge et al., 1993a). Surprisingly, in the homogenate assay used here, no difference was observed between the activities measured at 1 mM or 15 mM [Mg^{2+}] suggesting that this inhibitory effect was not happening under the specific conditions of this assay (Simonides & van Hardeveld, 1990).

Under alkaline pH, the effect of Mg^{2+} would appear to be amplified. In vesicle fractions, it was shown that while a free [Mg^{2+}] of 2 mM produced maximum activity at a pH of 7, it had an inhibitory effect on the kinetics of the enzyme at pH 8, where a [Mg^{2+}] of 0.2 mM was found to be optimal (Bishop & Al-Shawi, 1988). Mg^{2+} is suggested to reduce Ca^{2+}-Mg^{2+}-ATPase activity by binding to the lumenal low affinity sites and form a rate limiting dead-end complex $Mg_2 \cdot E_2 \sim P$ (Bishop & Al-Shawi, 1988). In the homogenate assay, the [Ca^{2+}]$_f$ were similar for the various conditions ranging from 9.99 to 10.1 mM. Therefore the difference in Ca^{2+}-Mg^{2+}-ATPase activity measured at pH 7.0 and 7.6 could be due to increase binding by Mg^{2+} to the low affinity sites at the more alkaline pH.

If Ca^{2+}-Mg^{2+}-ATPase activity is affected by Mg^{2+} through binding to the lumenal sites, the fact that the [Mg^{2+}] is found mostly on the outside of the vesicle, may diminished it's impact. This could explain why no difference in Ca^{2+}-Mg^{2+}-ATPase activity was found when the assay was run with either 1 mM or 15 mM [Mg^{2+}] (Simonides & van Hardeveld, 1990). The exchange of Mg^{2+} across the membrane is

slow unless the Ca^{2+} release channels are open (Lamb & Stephenson, 1991). However, the presence of Ca^{2+} ionophore might make the lumenal sites more available for binding by Mg^{2+}.

A second hypothesis to explain the role of pH on Ca^{2+}-Mg^{2+}-ATPase activity is through its competitive role with Ca^{2+} since H^+ is involved in counter-transport of Ca^{2+} (Dixon & Haynes, 1990; Da Costa & Madeira, 1994; Yu et al., 1994). Various models have proposed that 1 to 3 H^+ ions play a role in the counter-transport of 1 Ca^{2+} (Pick & Karlish, 1982; Forge et al., 1993b). At alkaline pH, the affinity of the lumenal sites for Ca^{2+} is increased (Verjovski-Almeida & De Meis, 1977) and removal from these sites constitute rate limitations which explain the pH dependence of the V_{max} of the enzyme (Dixon & Haynes, 1990). Counter-transport will therefore affect the kinetics of Ca^{2+} binding and/or dissociation. The relative importance of either V_{max} (counter-transport) or $Ca_{1/2}$ (competition for high affinity sites) at a certain pH is suggested to be dependent on the $[Ca^{2+}]$ (Dixon & Haynes, 1990). The rate of efflux of Ca^{2+} also depends on Mg^{2+} and K^+, and as for Ca^{2+}, the effect of these other ions is modulated by the pH. The rate of efflux is decreased by Ca^{2+}, Mg^{2+} and K^+ at pH 7.5 but not at pH 6.0 (Wolosker & De Meis, 1994). In the present experiment, the effect of alkaline pH on $[Mg^{2+}]$ is likely the mechanism explaining the reduction in activity, since in the presence of ionophore and EGTA, the effect of Ca^{2+} efflux is negligible. Counter-transport is likely to be more of a critical mechanism in conditions where back inhibition of lumenal Ca^{2+} is high and no Ca^{2+} chelator is present in the extravesicular space. This has been demonstrated in vesicle fraction experiments, where Ca^{2+} efflux is

more important when only low levels of oxalate are present (Korge & Campbell, 1994).

The modification of sulfhydryl (SH) groups has also been suggested as a mechanism to explain inactivation of the Ca^{2+}-Mg^{2+}-ATPase at alkaline pH (Xu et al., 1993). Lower Ca^{2+}-Mg^{2+}-ATPase activity and formation of phosphoenzyme intermediates were found when rabbit heart muscle vesicles were pre-incubated in a buffer-medium pH at 7.8 instead of 6.8 (Xu et al., 1993). These reductions at higher pH were prevented when dithiothreitol, a thiol reagent that was added to the pre-incubation medium. It was suggested that occupation of important SH groups on the Ca^{2+}-Mg^{2+}-ATPase by protons may prevent interaction with reactive oxygen or formation of disulfide bridges (Xu et al., 1993).

The difference in pH sensitivity of Ca^{2+}-Mg^{2+}-ATPase activity from homogenate of WGM, RGM and especially S could reflect the presence of different proportion of isoforms with distinctive pH sensitivities. These results, as opposed to what was found in COS 1 cells, would suggest that differences in pH sensitivity exist between SERCA 1a and SERCA 2a, expressed in fast- and slow-twitch muscle fibres, respectively. Although, the two isoforms have a similar number of amino acid residues and molecular weight, some structural differences in amino acid sequence has been demonstrated between Ca^{2+}-Mg^{2+}-ATPase isoforms (MacLennan et al., 1985; Brandl et al., 1986). The mapping of the amino-acid sequence of fast- and slow-type Ca^{2+}-Mg^{2+}-ATPase has revealed a structure that varies by approximately 15 % (Brandl et al., 1986). Variations in functional properties such as phosphorylation by Ca^{2+}/calmodulin-

dependent protein kinase (Hawkins et al., 1994) and inhibition by fluoride (Narayanan et al., 1991) have been observed between the SERCA 1 and SERCA 2a isoforms.

Since H^+ and Ca^{2+} are suggested to compete for the same binding sites on the surface of the enzyme, differences in the amino acid residue composition of these sites could modulate the sensitivity to pH. Site-directed mutagenesis experiments have suggested that the two high affinity Ca^{2+} binding sites of the Ca^{2+}-Mg^{2+}-ATPase are located in the middle of the transmembrane stalk 4 (Glu^{309}), 5 (Glu^{771}), (Asn^{796}, Thr^{799}, Asp^{800}) and 8 (Glu^{908}) (Clarke et al., 1989a; Vilsen et al., 1989; Clarke et al., 1990a). The transmembrane segment are highly conserved between the two isoforms and variations in amino acid residues in the hydrophobic transmembrane helices accounted for only 7.4 % of the total variation (Brandl et al., 1986). None of these variations affect the specific residues in the transmembrane stalks identified above by Clarke et al. (1990a) as the high affinity Ca^{2+} binding sites.

The possibility remains that the difference in pH sensitivity between isoforms is due to other distinctive amino acid residue sequences elsewhere in other domains of the enzyme that are affected by [H^+]. These domains appear to be highly interactive. For example, the binding of the MgATP complex is dependent upon pH, in the presence of Mg^{2+} (Lacapere et al., 1990) and accelerates the binding of Ca^{2+} (MacLennan et al., 1985). Phosphate incorporation and binding of fluorescein isothiocyanate to the nucleotide domain have been found to be reduced following short term intense activity (Luckin et al., 1991). Based on the experiments of MacLennan and co-workers (MacLennan, 1990), subtle changes similar to those induced by conservative

substitutions of amino acid residues by site directed mutagenesis, could possibly alter the normal conformational step of the Ca^{2+}-Mg^{2+}-ATPase (William & Klug, 1995).

The difference in pH sensitivity could also be the result of two populations of the same Ca^{2+}-Mg^{2+}-ATPase molecule with different conformations (Nakamura, 1989; Nakamura & Furukohri, 1994). Recent evidence suggests the existence of one Ca^{2+}-Mg^{2+}-ATPase molecule that is in a pH dependent equilibrium between E_1 and E_2 and another molecule that is pH independent and predominantly in the E_2 conformation (Nakamura & Furukohri, 1994). The role played by H^+ in the equilibrium of the two populations of Ca^{2+}-Mg^{2+}-ATPase molecules and in Ca^{2+} binding to those molecules was different (Nakamura, 1994). The distinctive sensitivity to pH displayed by the RGM and WGM, and S could reflect a different proportion of these two molecules in the muscle homogenate preparation. Finally, binding of H^+ by other proteins than by Ca^{2+}-Mg^{2+}-ATPases, present in the homogenate, cannot be dismissed.

3.4.5 Effects of ATP concentration on Ca^{2+}-Mg^{2+}-ATPase activity in homogenate

Until recently, reductions in ATP concentrations as a viable mechanism explaining the reduction of Ca^{2+}-Mg^{2+}-ATPase activity has been difficult to justify. The measurements of ATP cell concentration before and after fatigue, in humans subjected to electrical stimulation (Hultman & Sjoholm, 1983; Spriet et al., 1987), in "in situ" preparations (Dudley & Terjung, 1985a) or single fibre (Nassar-Gentina et al., 1978) reveal that the concentration of ATP never falls under 50 %, which is well above the K_m for the myosin ATPase (Hultman et al., 1988). For the Ca^{2+}-Mg^{2+}-ATPase, K_d for MgATP of between 3 and 16 μM have been reported depending on experimental

conditions (Meissner, 1973; Guillain; 1984; Lacapere & Guillain, 1993), values that are also very much lower than the average ATP concentration which stays above 1 mM even after extreme fatigue (Nagesser et al., 1992).The decreases in ATP (10-15 %) are even less in studies using more physiological model of muscle fatigue (Sahlin & Ren, 1989; Tesch et al., 1989). In a study examining levels of substrates, the concentration of ATP after approximately 5 min of intense running was unaltered in horses (Byrd et al., 1989b).

However, it has been suggested that lower concentrations may be obtained in the vicinity of the ATPases, where the local equilibrium between the supply and demand for ATP may be jeopardised , (Korge et al., 1993; Korge & Campbell, 1994; Stephenson et al., 1995). The ATP compartmentalisation hypothesis rests upon evidence demonstrating that glycolytic enzymes and creatine kinase are concentrated locally within the cell (Korge & Campbell, 1995).

In this study at each $[Ca^{2+}]_f$, ATP, for the most part, resulted in peak activity regardless of the concentrations (1, 3 & 5 mM). Minimum levels of Ca^{2+} (0.1-1 μM) are essential for the activation of ATP hydrolysis (De Meis & Inesi, 1982) and this could explain why increasing ATP concentrations made no difference on Ca^{2+}-Mg^{2+}-ATPase activity before the addition of external Ca^{2+}. Only for the RGM were the activities measured at 3 mM and 5 mM of ATP different than those measured at 1 mM and pCa 5.99.

Similar to our results, Belcastro et al. (1993) using an vesicle fraction preparation and an end-point assay based on the measurement of inorganic phosphate level, showed an approximately two fold increase in Ca^{2+}-Mg^{2+}-ATPase activity when

ATP concentration was increased from 1 to 5 mM. Comparison of the end-point-assay and coupled assay systems are difficult since the latter buffers ATP decline and prevents the accumulation of Pi. However, similar Ca^{2+} sensitivities and cooperativities have been demonstrated between the two assays for the myofibrillar ATPase of white fish muscle, despite a 25 % lower activity with the end-point assay (Parkhouse, 1992).

A possible mechanism explaining the lower activities observed at 1 mM for the RGM is likely to be related to differences in the kinetics of ATP binding and phosphorylation of the enzyme (Lacapere & Guillain, 1993). The lower activity at 1 mM ATP concentration could be the result of ATP binding that is slower than phosphorylation of the Ca^{2+}-Mg^{2+}-ATPase molecule (Lacapere & Guillain, 1993).

However, it is difficult to explain the lack of change in activities at this pCa for all three ATP concentrations for the WGM and S. The fact that both WGM and S displayed no changes in enzymatic activities over the ATP concentrations utilised suggests that isoform differences are not responsible for the difference in activity observed between RGM and the homogenates of the two muscles.

3.5   SUMMARY

The measurement of the sensitivity to pH of the Ca^{2+}-Mg^{2+}-ATPase in muscles of different fibre type composition suggests that the response to acidosis may vary slightly from one muscle to another. It is reported that 5 and 60 min were needed to obtain 50 % depression of Ca^{2+} uptake in fast frog muscle and slow rat muscle, respectively (Biedermann & Klug, 1992; Ward & William, 1994). An important consideration is that the depressed Ca^{2+}-Mg^{2+}-ATPase activity reported by some studies suggest that the effect of pH is not transient, since the measurements are

normally done under optimal conditions and neutral pH (O'Brien et al., 1991). However, the alterations are reversible since recovery in enzymatic activity is observed after 60-120 min of rest (Byrd et al., 1989b; Luckin et al., 1992). This suggests that if Ca^{2+}-Mg^{2+}-ATPase activity is inhibited by an increased $[H^+]$, the effect is through reversible alterations of the enzyme structure.

Variations in ATP concentration appear to play a minimal role in the inhibition of Ca^{2+}-Mg^{2+}-ATPase activity regardless of $[Ca^{2+}]_f$. However, its effect might be more important if it occurs in conjunction with other alterations in the cell's environment, such as a reduction in pH and/or an increase in $[Mg^{2+}]_f$ (Stienen et al., 1993).The effect of increases in other products of ATP hydrolysis were not examined in this study. However, the concentration of inorganic phosphate (Pi) through its effect on the free energy of hydrolysis could be the single most important modulator of Ca^{2+}-Mg^{2+}- ATPase activity (Stienen, 1993; Westerblad & Allen, 1993b). The effect of Pi is impossible to examine *in vitro* with the coupled assay enzymes because of the stimulating effect of Pi on the pyruvate kinase reaction. The use of a Ca^{2+}-Mg^{2+}- ATPase end-point assay based on Pi production is also impractical since level of Pi produced from ATP hydrolysis cannot be detected from the background Pi concentration used to inhibit the enzyme.

Finally, given that intracellular $[Ca^{2+}]_f$ is intimately linked to tension production in muscle and that a reduction in Ca^{2+} uptake will increase the intracellular $[Ca^{2+}]$, a failure in excitation contraction coupling leading to a depression in the Ca^{2+} release by the SR is likely to be the most important mechanism underlying muscle fatigue (Westerblad & Allen, 1996; Lannergren et al., 1996).

CHAPTER IV

FAILURE OF PROLONGED EXERCISE TO ALTER Ca^{2+}-Mg^{2+}-ATPASE IN BOTH HOMOGENATE AND ENRICHED FRACTIONS OF RAT MUSCLE

4.0   ABSTRACT

The purpose of this study was to examine the role of fractionation on exercised induce changes in Ca^{2+}-Mg^{2+}-ATPase activity. The procedures of Byrd et al. (1989a) were carefully replicated to minimize as much as possible the variability due to species and gender, exercise protocol, fibre type composition and analytical procedures. Pairs of rats (n = 32) were randomly assigned to CON and RUN groups. The RUN animals were run to fatigue on a graded treadmill (21 m . min^{-1} for the first 60 min; 25 m . min^{-1} from 60 to 120 min; 28 m . min^{-1} to exhaustion). The red gastrocnemius and vastus muscles of two animals were pooled together for the CON and RUN groups in order to obtain sufficient tissue for the isolation and assays. Ca^{2+}-Mg^{2+}-ATPase activity was measured in Hom, discarded fractions of first, second and third centrifugation (P_1, P_2, P_3), in a supernatant (SC) combining the supernatants of the fourth (S_4) , fifth (S_5) and sixth (S_6) centrifugation and in the final vesicle fraction (P_6). Marker enzymes for mitochondrial (citrate synthase, CS) and sarcolemmal (p-nitrophenyl phosphatase, p-NPP) contamination's were measured in the Hom, supernatants of the first (S_1), second (S_2), third (S_3) centrifugation's and in P_6, to detect alterations in the purity of the Hom and sub-fractions.

No differences in Ca^{2+}-Mg^{2+}-ATPase activity, CS, p-NPP or protein concentrations were found between the CON and RUN for the Hom or any of the sub-

fractions. These results indicate that exercise does not predispose the Hom to a differential effect by the fractionation procedure and that the discrepancy in results found in the literature (Byrd et al., 1989a; Chin & Green, 1996) cannot be explained on the basis of differences in rat species and gender, exercise protocol or isolation procedures since these variables were similar between the two studies.

4.1 INTRODUCTION

Contractile activity depends on increasing free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_f$). The increase in $[Ca^{2+}]_f$ is mediated by the sarcoplasmic reticulum (SR) which carries the dual function of controlling both release and uptake of $[Ca^{2+}]_f$. The release of Ca^{2+} occurs via a release channel distributed primarily in the terminal cisternae region of the SR. The uptake of $[Ca^{2+}]_f$ is an energy dependent process that is controlled by an ion-transport enzyme, the Ca^{2+}-Mg^{2+}-ATPase (de Meis & Vienna, 1979). Sustained activity, and particularly sustained high intensity activity necessitates that both Ca^{2+} release and Ca^{2+} uptake not be significantly altered.

Several studies have been published examining the effects of acute exercise on Ca^{2+} release (Favero et al., 1993) and Ca^{2+} sequestration (Byrd et al., 1989a,b, Luckin et al., 1991, Belcastro et al., 1993; Dossett-Mercer et al., 1994; Chin & Green, 1996) by the SR. The results, for the effects of exercise on SR Ca^{2+} sequestration properties and particularly on Ca^{2+}-Mg^{2+}-ATPase activity remain controversial. A depression in Ca^{2+}-Mg^{2+}-ATPase activity has been reported following single bouts of exhaustive exercise [Ml]by some investigators (Belcastro et al., 1981; Byrd et al., 1989a; Byrd et al., 1989b, Luckin et al., 1991; Belcastro et al., 1993), whereas others have been enable to show any alterations in activity (Fitts et al., 1982; Dossett-Mercer et al., 1994; Chin & Green, 1996).

Differences in analytical procedures between laboratories remains as a distinct possibility to explain the contradictory results regarding the effect of exercise on Ca^{2+}-Mg^{2+}-ATPase activity. Up until relatively recently, it was necessary to perform measurements of Ca^{2+}-Mg^{2+}-ATPase activity on enriched SR fractions that were

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obtained through differential centrifugation, a procedure that results in considerable loss of SR membranes. Homogenate procedures, where the measurements are performed on the whole tissue were not possible because of the contaminating effects of other cellular ATPases. However, in 1990, a homogenate procedure was published, allowing for measurement of Ca^{2+}-Mg^{2+}-ATPase activity via the selective inhibition of other ATPases (Simonides & van Hardeveld, 1990). Using the homogenate (Hom) procedure, our laboratory has not been able to document decrease in maximal Ca^{2+}-Mg^{2+}-ATPase activity with either induced prolonged (Chin et al., 1995) or with voluntary prolonged (Chin & Green, 1996) or intense (Dossett-Mercer et al., 1994) treadmill running in rats, regardless of the tissue type composition of the muscle. Moreover, the fractionation technique does not appear to explain the discrepancy in results (Chin & Green, 1996).

Several possibilities exist to explain the contradictory findings: the fibre composition of the muscle, the specific characteristics of the activity and the analytical techniques have all been suggested as potential factors to explain the discrepant results (Chin & Green, 1996). The analytical procedures and fibre type composition were examined by measuring Ca^{2+} uptake and Ca^{2+}-Mg^{2+}-ATPase activity in different SR fractions from rats subjected to exhaustive treadmill running (Chin & Green, 1996). To examine the analytical procedures, the study measured Ca^{2+}-Mg^{2+}-ATPase activity and Ca^{2+} uptake in homogenates and various fractions obtained by two different protocols of SR isolation (Byrd et al., 1989a; O'brien et al., 1990). The effect of muscle fibre type composition was examined by measuring the above parameters in two muscle tissue types. No differences in Ca^{2+}-Mg^{2+}-ATPase activity was found between the exercised

and control animals for any of the muscles and or fractions isolated by the two protocols (Chin & Green, 1996).

Evidence that could suggest a differential effect of the isolation procedures on Ca^{2+}-Mg^{2+}-ATPase activity comes from two sources. Initial reports about differences in oxalate supported Ca^{2+} uptake between Hom and vesicle fractions of control and ischemic cardiac tissue (Feher et al., 1980) prompted a closer examination of the isolation procedure (Rapundalo et al., 1986). The effect of ischemia on oxalate supported Ca^{2+} uptake in cardiac muscle showed that the isolation procedure was selecting certain sub-populations of Ca^{2+}-Mg^{2+}-ATPases (Rapundalo et al., 1986). As the length of the ischemic period increased, it was found that a greater fraction of SR able to accumulate Ca^{2+} was discarded during two centrifugations of the isolation protocol (Rapundalo et al., 1986). It was hypothesized the homogenisation preferentially released segments of the SR that were most susceptible to damage by the ischemia and that these segments were preferentially discarded during the above two centrifugations of the isolation procedure.

The most likely mechanism that has been used to explain the reduction in Ca^{2+}-Mg^{2+}-ATPase activity following both acute and chronic repetitive activity has been structural modifications of the nucleotide binding domain which renders part of the Ca^{2+}-Mg^{2+}-ATPase population unphosphorylatable (Leberer et al., 1987; Luckin et al., 1991; Matsushita & Pette, 1992). Evidence suggesting the inactivation of a specific population of Ca^{2+}-Mg^{2+}-ATPase was demonstrated after 4-day of chronic low frequency stimulation (Matsushita et al., 1991). Isolation with a sucrose gradient revealed that the proportion of vesicles displaying low oxalate supported Ca^{2+} uptake

was higher in the lighter fraction of stimulated than control muscle (Matsushita et al., 1991). The lower oxalate loading capacity was accompanied by a reduced Ca^{2+}-Mg^{2+}-ATPase activity and phosphoprotein formation implying that alterations of the nucleotide binding site was the underlying mechanism for the reduced enzyme function (Matsushita et al., 1991). If exercise affects the muscle tissue in a way that is similar to ischemia, it is possible that the difference in Ca^{2+}-Mg^{2+}-ATPase activity and in the distribution of inactive and active vesicles between control and stimulated muscles are not only due to the effects of exercise, but to steps in the analytical procedure. In other words, the effects of exercise could predispose a certain population of vesicles to adverse effects as a result of the homogenisation and isolation procedures. The accumulation or rejection of this population could then be accentuated by specific centrifugation procedures and explain in part the observed differences in Ca^{2+}-Mg^{2+}-ATPase activity between control and exercise animals.

The present study was designed with the intention of extending the work of Chin and Green (1996), namely to identify if the selectivity of the isolation protocol could be a contributing factor to the discrepancy in results that exist between laboratories concerning the effect of prolonged exhaustive exercise on Ca^{2+}-Mg^{2+}-ATPase activity. This was achieved by comparing Ca^{2+}-Mg^{2+}-ATPase activity of not only the homogenates and vesicle sub-fractions of exercised and control animals, but also the Ca^{2+}-Mg^{2+}-ATPase activity of the sub-fractions and supernatants that are normally discarded during the isolation procedure. It was hypothesized that exercise, similar to ischemia, would affect the distribution of active and inactive Ca^{2+}-Mg^{2+}-ATPases in all or some of the discarded sub-fractions and/or supernatants during the

fractionation procedure. This differential effect of the fractionation process on Ca^{2+}-Mg^{2+}-ATPase activity in vesicle sub-fractions and/or supernatants of control and exercise animals could provide an explanation for the discrepancy in the literature concerning the effect of exercise on the activity of this enzyme.

4.2   METHODS

4.2.1 Animal description and care

The thirty-two adult male Sprague-Dawley rats (195-220 g) used for this study were housed in an environmentally controlled room (27° C) with a reversed 12:12 light/dark cycle and were allowed free access to food (Purina rat chow) and water until the time of the experiment. Animal care was in accordance with the guidelines established by the Canadian Council on Animal Care. This study was approved by the University of Waterloo Animal Ethics Committee.

4.2.2 Experimental design .

Pairs of rats were randomly assigned to enter a control (CON) or exercised (RUN) groups in order to examine the effect of exhaustive running on sarcoplasmic reticulum (SR) function. The RUN animals were acclimatized to treadmill running with two sessions of 10 (4 % grade; 21 $m \cdot min^{-1}$) and 20 min long (4 % grade; 10 min at 21 $m \cdot min^{-1}$; 10 min at 25 $m \cdot min^{-1}$) , scheduled 5 and 2 days before the day of the experiment, respectively. The RUN animals were fatigued on a motor driven treadmill set at 4 % grade, 21 $m \cdot min^{-1}$ for the first 60 min, with increments in speed to 25 $m \cdot min^{-1}$ from 60-120 min and to 28 $m \cdot min^{-1}$ from 120-180+ min. Rats were run until they were incapable of avoiding the shock grid. This criteria was used to indicate exhaustion.

The diagram (Figure 4.1) provides an overview of the various steps of the SR isolation procedure based on the protocol of Heilman et al. (1977) as modified by

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Figure 1. Procedure for the isolation of skeletal sarcoplasmic reticululum

CON (n=2)　　　　　　　　　RUN (n = 2)

HOMOGENATE

1600 g_{max} * 10 min (Type 70.1 Ti = 4174 RPMs)

P_1　　　　　　　　　　　　　S_1 (p-NNP activity
　　　　　　　　　　　　　　　　　　citrate synthase activity)

(Resuspension: 11:1
Ca^{2+}-Mg^{2+}-ATPase activity)

10000 g_{max} * 18 min
(Type 70.1 Ti = 10435 RPMs)

(p-NNP activity
citrate synthase activity) S_2　　　P_2

(Resuspension: 11:1
Ca^{2+}-Mg^{2+}-ATPase activity)

10000 g_{max} * 18 min
(Type 70.1 Ti = 10435 RPMs)

(p-NNP activity
citrate synthase activity) S_3　　　P_3

(Resuspension: 11:1
Ca^{2+}-Mg^{2+}-ATPase activity)

50000 g_{max} * 52 min
(Type 70.1 Ti = 23333 RPMs)

(Resuspension in 10 ml buffer
S_4　　　　　P_4　with 0.6 M KCl)

50000 g_{max} * 52 min
(Type 70.1 Ti = 23333 RPMs)

SC

S_5　　　P_5

50000 g_{max} * 52 min
(Type 70.1 Ti = 23333 RPMs)
(Ca^{2+}-Mg^{2+}-ATPase activity)

50000 g_{max} * 52 min
(Type 70.1 Ti = 23333 RPMs)

S_6　　　　　P_6

(Ca^{2+}-Mg^{2+}-ATPase activity
p-NPP activity, Citrate synthase
activity)

Byrd et al. (1989a). Ca^{2+}-Mg $^{2+}$-ATPase activity was measured not only for the homogenate (Hom) and the vesicle sub-fraction of SR (P6) but also on the material that is normally discarded during the isolation procedure. The Ca^{2+}-Mg $^{2+}$-ATPase activity was measured in the discarded sub-fractions or pellets (P) of the first (P_1), second (P_2), third centrifugation (P_3) and on a discarded supernatant (SC) obtained from the combination of the supernatants produced from the fourth (S_4), fifth (S_5) and sixth (S_6) centrifugation. A contamination profile was established by assaying the activity of the following marker enzymes: citrate synthase (Chi et al., 1983) for mitochondria and K^+-stimulated p-nitrophenolphosphatase (Plough et al., 1992) for sarcolemma on Hom, enriched SR supernatants from the first centrifugation (S_1), second centrifugation (S_2), third centrifugation (S_3) and the vesicle sub-fractions P_6.

4.2.3 Experimental procedures

The animals were anesthetized with an intraperitonial injection of sodium pentobarbital (0.07 ml/g of tissue). The gastrocnemius and vastus of both hindlimbs were quickly excised from the animal and submersed in ice-cold homogenizing buffer containing: 250 mM sucrose, 10 mM NaN_3, 5 mM Hepes, 0.2 mM phenylmethylsulfonylfluoride (PMSF). The deep red portion of the gastrocnemius and vastus, containing a high proportion of a mixture of type IIa and type I, was separated from the white superficial compartments, composed of a majority of type IIb fibres (Baldwin et al., 1972) and used for this study. The specific selection of this tissue was based on the results of Byrd et al., (1989a), where depressed Ca^{2+}-Mg^{2+}-ATPase activity with treadmill running was found only for red muscles.

The excised muscles were rid of blood, fascia and fat. The muscles were blotted dry, weighed, replaced in ice-cold buffer, minced in small pieces with scissors and transferred to a test tube. All procedures were performed on ice. The tissue was then mechanically homogenized (11:1 dilution) with two 30-s bursts of a Polytron (Brinkmann Kinematica GmbH), at 60 % of maximal power, separated by a 30-s interval. A 2 ml volume of the pre-determined buffer volume was kept aside to rinse the head of the probe and then added to the initial volume of homogenate. A 40 µl sample of homogenate was pipetted into a labeled Eppendorf tube and stored on ice for the immediate assay Ca^{2+}-Mg^{2+}-ATPase activity. Aliquots of samples were pipetted into other tubes, dropped in liquid nitrogen and stored at -80° C for subsequent analyses of marker enzymes The remainder of the homogenate was transferred to 10 ml polycarbonate centrifugation tubes (Nalgene) and placed on ice.

The isolation protocol duplicated the procedures of Weidekamm and Brdiczka (1975) as modified by Byrd et al., (1989a). All isolations of vesicle sub-fractions were done using a Beckman Ultracentrifuge with the 70.1 Ti fixed angle rotor (Figure 1). The homogenate was centrifuged at 1600 g (4200 RPM) for 10 min at 4° C. The supernatant (S_1) was then passed through 4 layers of gauze and aliquots were pipetted into Eppendorf tubes and frozen in liquid nitrogen. The discarded pellets (P_1) were resuspended in a buffer similar to the homogenisation buffer but at pH 7 (11:1 dilution) with glass to glass homogenisation. Aliquots of the P_1 resuspension were transferred to Eppendorf tubes and also frozen in liquid nitrogen. The remaining supernatant was centrifuged at 10,000 g (10,400 RPM) for 18 minutes. Aliquots of the supernatant (S_2) and the resuspended pellets (P_2) were frozen in liquid nitrogen. The

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centrifugation was repeated and aliquots (S_3 and P_3) were frozen in liquid nitrogen. The remaining supernatant was centrifuged at 50,000 g (23,400 RPM) for 52 minutes. The supernatant (S_4) was frozen at - 20° C and centrifuged the following day. The pellets were resuspended in 10 ml of buffer, containing 0.6 M KCl, with a glass to glass homogeniser. The tubes were stirred on ice for 15 min and centrifuged again at 50000 g. The supernatant (S_5) was collected and stored at -20° C and centrifuged the next day. The remaining material was resuspended in 10 ml of buffer without KCl and the centrifugation repeated. The pellets (P_6) were resuspended in a volume of 800 μl. Aliquots, of this purified SR suspension, were pipetted in Eppendorf tubes, frozen in liquid nitrogen and stored at - 80° C for subsequent analyses.

4.2.4 Free Ca^{2+} determination

Extreme care was taken to prevent contamination of the buffer solutions by Ca^{2+}. All solutions were made with water filtered through a Milli-Q Water purification system (Millipore) and plastic wares were used to reduce Ca^{2+} leaching associated with glassware (Bers et al., 1994). The plastic wares were also soaked in a solution of EGTA (3 mM) and EDTA (1mM) (Meissner, 1973) and rinsed five times with ultra-pure water. A 10 mM $CaCl_2$ standard solution (Orion Research, Boston, Ma.) was used as the Ca^{2+} source. The free Ca^{2+} concentrations ($[Ca^{2+}]_f$) were determined using, Chelator, a Ca^{2+} software program (Schoenmakers, 1992) and taking in consideration the precise ionic strength, temperature (37° C) and pH of the buffers. Binding constants determined under standards conditions (0.1 M ionic strength, pH 7, 20° C) were adjusted to the specific experimental conditions of the present experiments.

The total $[Ca^{2+}]$ needed to get maximal Ca^{2+}-Mg^{2+}-ATPase activity was determined before hand through a Ca^{2+} dependency curve for both the homogenate and the vesicle fraction assay. Maximal Ca^{2+}-Mg^{2+}-ATPase activity was reached at total $[Ca^{2+}]$ of 0.969 mM for the homogenate and 0.909 mM for the vesicle assays. The $[Ca^{2+}]_f$ was calculated using the Ca^{2+} software program by taking in consideration the different chemical composition of the two buffers, mainly the KCl, Mg^{2+} and PEP concentration. The calculated $[Ca^{2+}]_f$ were 16.6 µM and 6.0 µM for the homogenate assay and the vesicle and other fraction assay, respectively. To insure that maximal activity was reached a second addition of Ca^{2+} was made to a $[Ca^{2+}]_f$ of approximately 162 µM, representing total $[Ca^{2+}]$ of approximately 1.2 mM for both assays.

To prevent the decrease in pH, produced by the release of protons from EGTA, following the addition of $CaCl_2$ volumes to the buffer, volumes of KOH were added. These volumes of KOH, needed to maintain the pH at the desired value, were determined before hand under similar medium conditions for each Ca^{2+} concentration at 37° C.

4.2.5 Assay for Ca^{2+}-Mg^{2+}-ATPase activity.

For the homogenate, total Ca^{2+}-Mg^{2+}-ATPase activity was measured with the homogenate method of Simonides and van Hardeveld (1990) consisting of the coupled enzyme assay which involves the pyruvate kinase and lactate dehydrogenase reactions. In this assay the disappearance of NADH is monitored spectrophotometrically. The chemical composition of the assay enables the measurement of Ca^{2+}-Mg^{2+}-ATPase activity through suppression of the contaminating

effects of others ATPase activities. The assay medium (Simonides & van Hardeveld, 1990) contained 20 mM Hepes, 200 mM KCl, 15 mM MgCl$_2$, 10 mM NaN$_3$, 1 mM EGTA, 10 mM phosphoenolpyruvate (PEP), 5 mM ATP, 18 U ml^{-1} each of purified pyruvate kinase and lactate dehydrogenase and 2 µM Ca^{2+} ionophore A23187 and pH at 7. The pH electrode was calibrated in pH buffers thermostatted at 37° and the pH of the assay buffer was adjusted at the same temperature (Tsien & Pozzan, 1989). Basal or Mg^{2+}-ATPase enzyme activity was determined at the end of the assay run by adding 2 M Ca^{2+} to a final concentration of 20 mM (Simonides & van Hardeveld, 1990). Ca^{2+}-activated activity was determined by subtracting basal activity from total activity.

In order to duplicate as much as possible the conditions of Byrd et al. (1989a), the Ca^{2+}-Mg^{2+}-ATPase activity, of the vesicle sub-fractions (P$_6$), was measured using the coupled enzyme assay method of Weidekamm and Brdiczka (1975) as modified by Byrd et al., (1989a). This assay was also used to measure Ca^{2+}-Mg^{2+}-ATPase activity of the intermediary discarded sub-fractions of the centrifugation process. The assay medium consisted of 25 mM Hepes, 100 mM KCl, 10 mM MgCl$_2$, 10 mM NaN$_3$, 1 mM PEP, 0.6 mM NADH, 7.5U of pyruvate kinase, 5 U of lactate dehydrogenase and was pH at 7 at 37° C.. However, as opposed to the original method, 1 mM EGTA was added to the assay medium to effectively control the [Ca^{2+}]$_f$. Therefore, the basal activity was not measured by the addition of EGTA, but as with the homogenate, by the addition of 2 M Ca^{2+} to a final concentration of 20 mM to saturate the low affinity Ca^{2+} binding sites (Simonides & van Hardeveld, 1990). A second trial was run with ionophore A23187, added after Ca^{2+}, to a concentration of 7.6 µM, to verify vesicle intactness (Byrd et al., 1989a).

The frozen fractions was left to thaw on ice for approximately 15 min. After the addition of 1 ml of buffer to a plastic cuvette, the buffer was allowed 8 min to equilibrate to 37° C. The enzymes and 11 μl of homogenate was added to the cuvette. After another 2 min of incubation, the reaction was followed spectrophotometrically (Shimadsu, UV-1601PC, Tek Science, Missisauga, Ont.) by measurement of NADH at 340 nm. Volumes of KOH and $CaCl_2$ were then added to obtain the pre-determined desired pH and $[Ca^{2+}]_f$ and the absorbance signal monitored for approximately 2 min. This step was repeated for the second $[Ca^{2+}]_f$ and for the addition of an aliquot of 2 M $CaCl_2$ to determine basal activity. The steepest portion of the sampled area was used to determine the activity. Two trials were run on the same sample and since assay time following the initial 10 min of incubation was approximately 8-10 min, both trials were completed within an h after being taken out of the freezer. The value of 6.27×10^{-3} M^{-1} · cm^{-1} of was used as the molar absorption coefficient of NADH to calculate the rate of hydrolysis.

4.2.6 Contamination profile, protein yield, membrane purity and Ca^{2+}-Mg^{2+}-ATPase activity in discarded fractions.

Contamination profiles were established using marker enzymes of the mitochondria [citrate synthase] and the sarcolemma [K^{+}-stimulated p-nitrophenolphosphatase (K^{+}-pNPP)] (Bers, 1979; Tibbits et al., 1981) and included measurements of activities in Hom, SR enriched supernatant (S_1, S_2, S_3) and the final vesicle sub-fractions (P_6). No analyses were done on the SR enriched sub-fractions of the fourth (P_4) and fifth (P_5) centrifugations because of the limited availability of samples for these pellets. Percent contamination of mitochondrial and sarcolemmal

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membranes was calculated as the ratio of activities in the supernatants and vesicle fractions to the Hom fraction expressed in mg of protein.

The method of Chi et al., (1983) was used to determine citrate synthase activity. The samples were appropriately diluted (Hom; 227:1, S_1; 75:1, S_2; 70:1, S_3; 60:1) to fall within the fluorescence of the citric acid standards, with diluting media containing 20 mM imidazole-HCl and 0.02 % charcoal to prevent inactivation of the enzyme (Passonneau & Lowry, 1993). For the vesicle sub-fraction (P_6), because of the variation in protein concentration, the dilution factor fluctuated between 15:1 and 10:1. The citrate synthase reaction was allowed to react at room temperature for an h in a buffer containing 50 mM tris-HCl, 0.4 mM acetyl-CoA, 0.5 mM oxaloacetate and 0.25 % of charcoal treated bovine serum albumin (BSA), pH 8.1 (Chi et al., 1983). The reaction was stopped by adding a volume of NaOH to a final concentration of 0.5 N, and excess oxaloacetate was destroyed by heating at 95° C.

One ml of citrate reagent containing: 100 mM Tris-HCl, 100 μM $ZnCl_2$, 0.01 % BSA, 30 μM NADH, 0.003 $U\,ml^{-1}$ citrate lyase and 3 $U\,ml^{-1}$ malate dehydrogenase was then added and the reaction allowed to proceed for 20 min at room temperature. HCl, to a final concentration of 1 N was then added to the tube to destroy NADH. After 10 min, strong NaOH (0.6 N) with imidazole (10mM) were added and quickly mixed to destroy NAD^+ and convert it to a highly fluorescent product (Passonneau & Lowry, 1993). The products of the reaction were then heated at 60° C for 20 min. The tubes were cooled at room temperature before reading since fluorescent has a negative T coefficient of 1.3 % per degree (Passonneau & Lowry, 1993). Triplicates were run for each sample.

The K^+-pNPP, an acid phosphatase of the sarcolemma which catalyzes the hydrolysis of the phosphate ester of nitrophenol to produce Pi. K^--pNPP was determined by the method of Ploug et al., (1992). Dilution for the Hom and vesicle sub-fractions were done based on the recommendations that 50 µg homogenate and 8 µg of membrane protein be used to run the assay (Ploug et al., 1992). For the Hom, the factor for the second dilution was 90.9:1. For the SR enriched supernatants (S_1, S_2), a dilution of 60:1 yielded absorbance readings within the range of the p-nitrophenyl phosphate (pNPP) standards used for the assay. For the vesicle fraction preparation, the dilution factor was calculated for each sample using the particular protein concentration to obtain the suggested 8 µg of membrane protein. The dilution factor fluctuated between extremes of 50:48 and 50:8, with most sample being diluted between 50:8 and 50:12.

The assay medium consisted of 50 mM Tris-HCl, 5 mM EGTA, 8.3 mM $MgCl_2$. The detergent saponin (208 µg . ml^{-1}) was also added to the assay medium to counteract the vesiculation of plasma membrane that occurs during the isolation (Ploug et al., 1992). The sample was added to 300 µl of buffer and left at room temperature for 15 min. The tubes were then incubated at 37° C for 10 min. KCl (250 mM) and water were added to determine total and basal activity, respectively. The substrate p-NPP (50 mM) was then added at 15 s intervals and the reaction allowed to proceed for 30 min (Ploug et al., 1992). Ice cold NaOH (0.2 M) with EDTA (20 mM) was added to stop the reaction and after rewarming tubes to room temperature, absorbance was read at 410 nm (Ploug et al., 1992). The K^+-stimulated activity was obtained by subtracting the basal activity from the activity in the presence of KCl.

A sample was run with the same buffer with 16.7 mM of ouabain, a compound that inhibits the K^+ stimulated phosphatase (Ploug et al., 1992). The ouabain-inhibitable K^+-dependent activity was determined by subtracting the activity measured in the presence of KCl by the activity measured in the presence of both KCl and ouabain (Ploug et al., 1992). The ouabain-inhibitable K^+-dependent activity was not measured in P_6 because of the limited amount of vesicles. Duplicates were run for each samples.

Protein concentration was determined by the method of Lowry et al. (1951) as modified by Schacterle and Pollock (1973). Protein yields for the vesicle sub-fractions were expressed as μg per g of tissue (Chin & Green, 1996). Membrane purity was assessed by dividing Ca^{2+}-Mg^{2+}-ATPase activity ($\mu M \cdot g^{-1}$ protein $\cdot$ min^{-1}) in P_6 by the activity measured in the Hom. Ca^{2+}-Mg^{2+}-ATPase activity in the presence of Ca^{2+} ionophore A-23187 was used for these calculations (Chin & Green, 1996).

4.2.7 Data and statistical analysis

The data was collected with Personal Spectroscopy Sofware (Shimadzu Scientific Instruments Inc.) program. Regression analyses were used to determine the slope of the steepest portion of the absorbance signal curve. One way Analyses of Variance (ANOVA) procedures were used to assess the main effects due to condition (Con vs. Run) on Ca^{2+}-Mg^{2+}-ATPase, total and basal (Mg^{2+}-stimulated) activity for the Hom, discarded sub-fractions and SC, and P_6. Since the data were not significantly different for the main effect due to condition, the Con and Run data were pooled together and a one way ANOVA was used to analyze fractions (P_1, P_2, P_3, SC and P_6) as the repeated measure. If the F ratio were significant, planned comparisons were used

on the repeated measure (fraction) to determine differences between means. Similar procedures were used for the activity of the two marker enzymes: p-nitrophenyphatase and citrate synthase in the Hom, SR enriched supernatants and vesicle sub-fractions. Significance was accepted at the level of $P < 0.05$.

4.3. RESULTS

4.3.1 Ca^{2+}-Mg^{2+}-ATPase activity in homogenate, discarded sub-fractions and vesicle sub-fractions.

No differences were observed for total, basal and maximal Ca^{2+}-stimulated activity between the control and run animals for the Hom, discarded sub-fractions and SC, and vesicle sub-fractions (Table 4.1 & Figure 4.2). The maximal Ca^{2+}-stimulated activity in the presence of Ca^{2+} ionophore A-231187, expressed in Table 2 as a % of homogenate activity, was also similar between the two groups.

To examine the differences among the homogenate, sub-fractions and SC, the statistical analysis was done by pooling the Con and Run group together and treating the fractions as a repeated measure. Total Ca^{2+}-Mg^{2+}-ATPase activity, measured in the presence of ionophore was different between many of the 6 fractions (Hom,P_1, P_2, P_3, SC and P_6) (Figure 4.3A). No difference in total activity was found between Hom and P_1 fractions. Hom and P_1 had the lowest activity of all fractions followed by P_2, P_3, and P_6 and SC (Hom = P_1 < P_2 < P_3 < SC = P_6).

Basal or Mg^{2+} stimulated activity was lower in the Hom, followed by the P_1 and P_2 fractions which displayed similar values (Figure 4.3B). The activities of the P_3, SC and P_6 were also similar, but more than a two-fold difference over the activity measured in P_2 (Hom < P_1 = P_2 < P_3 = SC = P_6). For the Ca^{2+} -activated ATPase activity, the relationship between the fractions was similar to what was found for total activity. The activity of the Hom was similar to the activity found in the P_1 sub-fraction. The latter was lower than the activity of the P_2 and P_3 sub-fractions which displayed no differences (Figure 4.3C). The activities in the SC and P_6 fractions were similar and higher than the

Table 4.1. Total, Ca^{2+}-activated and basal (Mg^{2+}-activated) ATPase activity measured in homogenate, discarded sub-fractions and supernatant combined and vesicle sub-fractions in Con and Run animals.

	Fractions	n	Total ATPase activity	Ca^{2+}-activated ATPase activity	Basal ATPase activity
Con	Hom	8	405 ± 46.6	364 ± 37.7	45.9 ± 10
	P_1	8	455 ± 64.9	351 ± 51.4	103 ± 19
	P_2	7	1251 ± 246	1048 ± 181	198 ± 61
	P_3	8	1897 ± 229	1669 ± 225	229 ± 41
	SC	5	4740 ± 1484	4383 ± 1429	357 ± 89
	P_6	8	3282 ± 367	2944 ± 363	375 ± 41
Run	Hom	8	423 ± 57.9	376 ± 62.1	45.6 ± 9.5
	P_1	8	523 ± 70.0	372 ± 47.4	152 ± 38
	P_2	7	1648 ± 468	1439 ± 385	208 ± 84
	P_3	8	1495 ± 127	1124 ± 127	370 ± 98
	SC	5	3975 ± 910	3585 ± 889	390 ± 67
	P_6	8	2984 ± 271	2558 ± 276	437 ± 103

Values are means ± SE expressed in $\mu mol . g^{-1}$ protein . min^{-1}; Hom; homogenate, P_1; pellets from centrifugation 1, P_2; pellets from centrifugation 2, P_3; pellets from centrifugation 3, SC; combined supernatant from centrifugation 4-5-6, P_6; pellets from centrifugation 6.
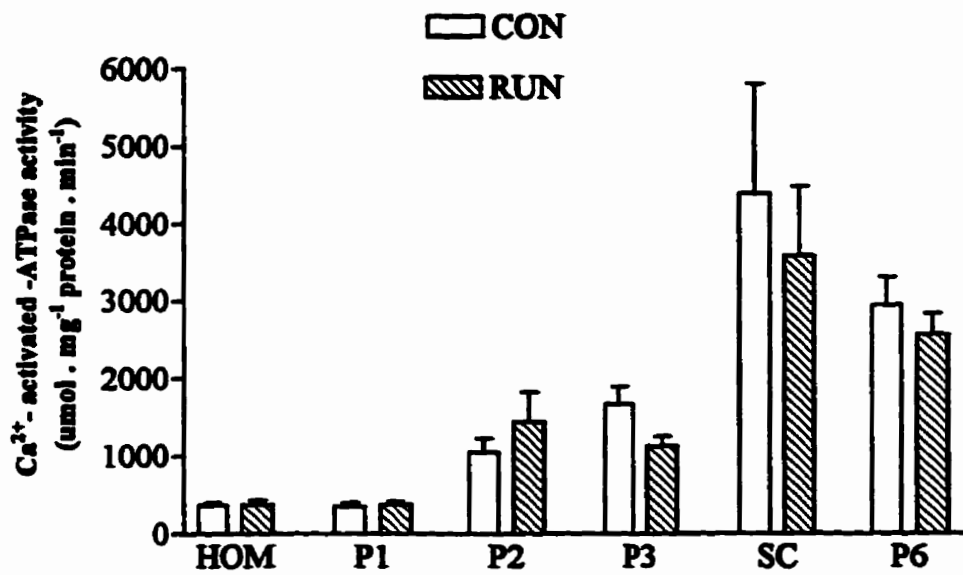
Figure 4.2. Ca^{2+}-activated adenosinetriphosphatase (ATPase)activity in homogenate (hom) discarded sub-fractions of centrifugation 1 (P_1), 2 (P_2) and 3 (P_3), discarded supernatant combined (Supernatant 1; S_1, supernatant 2; S_2 and supernatant 3; S_3), and vesicle sub-fraction (P_6) in Con and Run animals. Values are means $\pm$ SE.
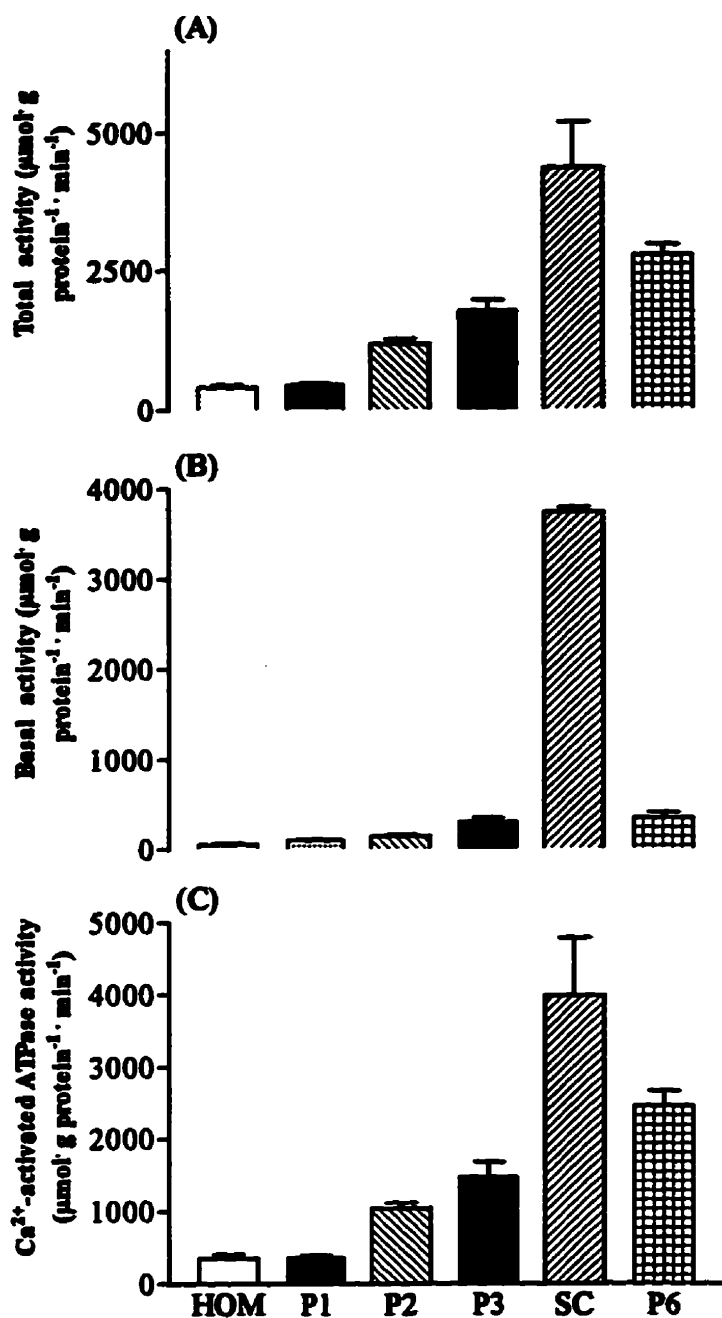
Figure 4.3.Total (A); basal (B); and Ca^{2+}-activated (C); adenosinetriphosphatase (ATPase) activity in homogenate (Hom), vesicle fraction (P_6), discarded sub-fractions (P_1, P_2, P_3) and discarded supernatant combined $(SC = S_4 + S_5 + S_6)$. Values are means ± SE and are based on average of pooled Con and Run animals (n = 10). Significant main effects for fraction/supernatant for total ATPase (Hom = $P_1 < P_2 < P_3 < SC = P_6$), for basal ATPase (Hom < $P_1 = P_2 < P_3 = SC = P_6$)for Ca^{2+}-activated ATPase activity (Hom = $P_1 < P_2 = P_3 < SC = P_6$). $P < 0.05$.

activities of the Hom and discarded sub-fractions (Hom < P_1 < P_2 = P_3 < SC = P_6). For the Ca^{2+}-activated ATPase activity in the presence of Ca^{2+} ionophore A-23187, the activity in the Hom was similar to the activity found for P_1 and the activity in P_3 was higher than the activity in P_2 (Table 4.2).

4.3.2 Contamination profile, protein yield, membrane purity and Ca^{2+}-Mg^{2+}-ATPase activity in discarded fractions.

No differences were observed between citrate synthase (CS) activity, a marker enzyme for mitochondrial contamination, between the Con and Run groups for any of the fractions analyzed (Hom, S_1, S_2, S_3 & P_6) (Table 4.3). Although, CS activity was similar for the Hom, S_1, S_2 and S_3, the level of activity in P_6 was reduced to insignificant levels with the final centrifugation. The activities represented 0.2 % for the Con and 0.9% for the Run groups of the activity measured in the Hom (Hom = S_1 = S_2 = S_3 > P_6) (Figure 4.4).

As with CS, no differences were found for the K^+-pNPP, a marker of sarcolemmal contamination between the Con and Run groups for any of the fractions(Hom, S_1, S_2 & P_6) (Table 4.4). The highest activity was found in S_2 (Hom = S_1 < S_2; P_6 < S_1) (Figure 4.5A). No differential effect of exercise was observed on the ouabain suppressible activity for the Hom, S_1 and S_2 (Table 4.4) When the data of the Con and Run groups were pooled together, no differences in activity were found between Hom and the two SR enriched supernatants (Figure 4.5B).

Protein concentration was significantly affected by the isolation procedure. However, there were no differences between the Con and Run groups for the homogenate or any of the sub-fractions (Table 4.5). With the exception of fraction S_1

Table 4.2. Membrane purity and Ca^{2+}-Mg^{2+}-ATPase activity in the presence of Ca^{2+} ionophore A-23187 in homogenate, discarded sub-fractions and supernatant combined, and vesicle sub-fractions in Con and Run animals.

Fractions	n	Ca^{2+}-Mg^{2+}-ATPase activity in homogenate		
		Con	n	Run
Hom	8	363 ± 38	8	370 ± 35
P_1	8	434 ± 72	8	489 ± 88
P_2	7	2307 ± 251	5	2067 ± 331
P_3	7	3962 ± 564	8	3242 ± 365
SC	5	6103 ± 1452	5	5542 ± 1713
P_6	8	5377 ± 676	8	5064 ± 1087

Values are means ± SE of Ca^{2+}-Mg^{2+}-ATPase activity ($\mu M . g^{-1}$ protein . min^{-1}). P_1; pellets from centrifugation 1, P_2; pellets from centrifugation 2, P_3; pellets from centrifugation 3, SC; combined supernatant from centrifugation 4-5-6, P_6; pellets from centrifugation 6. Significant main effect was found for fractions (Hom = $P_1 < P_2 < P_3 =$ SC = P_6) ($P < 0.05$).

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Table 4.3. Citrate synthase activity in homogenates, SR enriched supernatants and vesicle sub-fractions.

	Fractions	n	Enzyme specific activity [†]
Con	Hom	8	11.0 ± 1.6
	S_1	7	12.1 ± 0.8
	S_2	8	12.8 ± 0.9
	S_3	8	13.5 ± 0.7
	P_6	8	0.02 ± 0.02 [*]
Run	Hom	8	11.1 ± 0.9
	S_1	7	12.0 ± 0.8
	S_2	8	12.1 ± 0.8
	S_3	8	12.6 ± 0.7
	P_6	8	0.1 ± 0.05 [*]

Values are means ± SE [†]expressed in $\mu mol . hr^{-1} . mg^{-1}$ protein. Hom; homogenate. S_1; supernatant from centrifugation 1, S_2; supernatant from centrifugation 2, S_3; supernatant from centrifugation 3, P6; pellets from centrifugation 6. [*] Significantly different from other fraction. ($P < 0.05$).
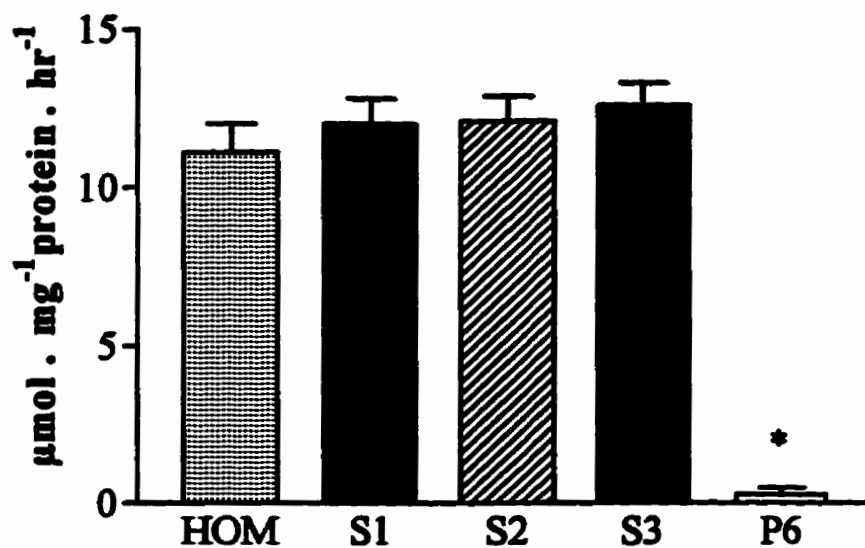
Figure 4.4. Citrate synthase activity in homogenate (Hom), SR enriched supernatant 1 (S_1), 2 (S_2) and 3 (S_3), and vesicle fraction (P_6). Values are mean ± SE and are based on average of pooled Con and Run animals (n = 14). *Significantly different from Hom and SR enriched supernatants. $P < 0.05$.

Table 4.4. P-nitrophenylphosphatase K^+-stimulated and ouabain suppressible activity [†] and corresponding purification indexes in homogenate, SR enriched supernatants and vesicle sub-fractions.

	Fractions	n	NPP-K^+-stimulated activity[†]	NPP-K^+-ouabain suppressible activity
	Hom	8	3.1 ± 0.3	4.8 ± 0.7
CON	S_1	7	5.5 ± 0.4	3.5 ± 1.3
	S_2	8	8.4 ± 1.1	5.2 ● 1.6
	P_6	8	2.6 ± 1.1	—
	Hom	8	3.7 ● 0.6	4.1 ± 0.6
RUN	S_1	7	5.0 ± 1.0	3.5 ± 1.3
	S_2	8	9.4 ± 1.8	5.9 ± 1.6
	P_6	8	1.8 ± 0.9	—

Values are means ± SE expressed in [†]μmol . hr^{-1} . mg^{-1} protein .Hom; homogenate, S_1; supernatant from centrifugation 1, S_2;supernatant from centrifugation 2, P6; pellets from centrifugation 6.
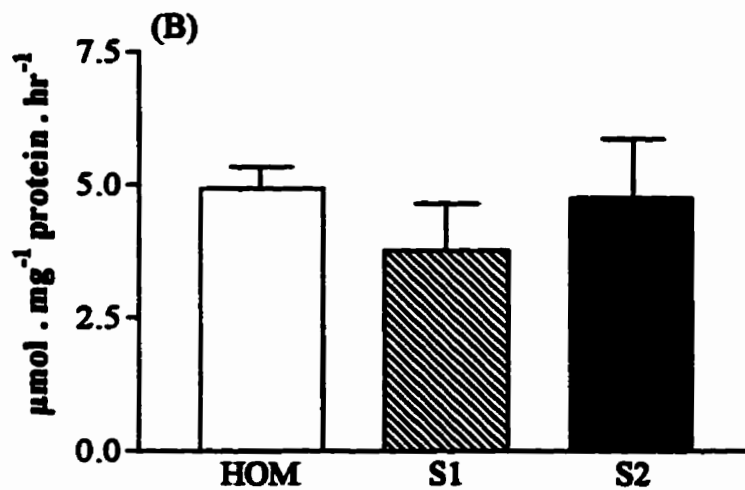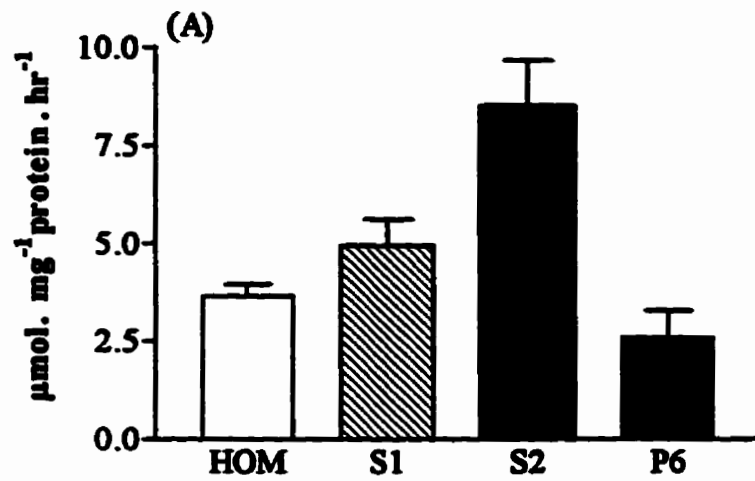
Figure 4.5. K^+-stimulated p-nitrophenolphosphatase (p-NPP) (A); and Ouabain-suppressible (B); activity in homogenate (Hom), supernatant 1(S_1) and supernatant (S_2). Values are mean ± SE and are based on average of pooled Con and Run animals. There were significant main effects for fraction/Sr enriched supernatant for K^+-pNPP (n = 12) ($P_6 < S_1 < S_2$; Hom $< S_2$). $P < 0.05$.

and S_2 which had similar protein concentration values, progressive reductions were observed for Hom, S_1, S_2, S_3 and P_6 ($P_6 < S_3 < S_2 = S_1 < Hom$) (Figure 4.6A). Similar results were observed for the discarded sub-fractions and SC ($P_6 < SC < P_3 < P_2 < P_1 < Hom$) (Figure 4.6B). Protein yield for the vesicle sub- fraction was very low (Table 4.6), but again no differences were observed between the Con and Run group.
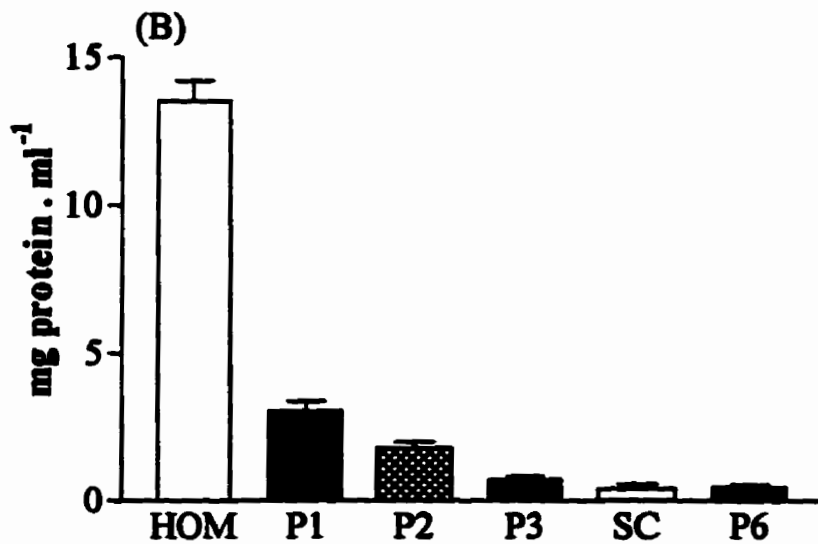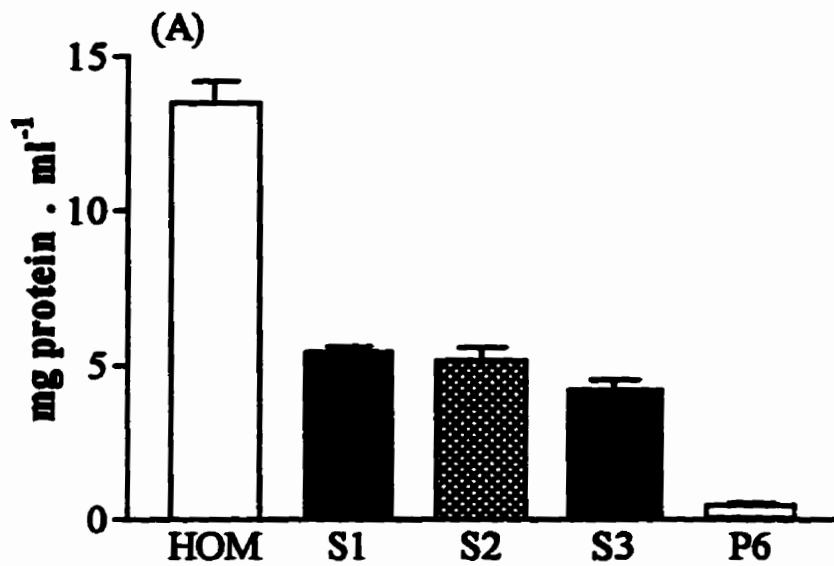
Figure 4.6. Comparison of protein concentration between homogenate (hom) and vesicle sub-fractions (P_6) with SR enriched supernatants ($S_1, S_2, \& S_3$) (A); and discarded sub-fractions ($P_1, P_2 \& P_3$) and discarded supernatants combined (SC; $S_4, S_5 \& S_6$) (B).

Table 4.5. Protein concentration for homogenate, discarded sub-fractions and supernatant combined, SR enriched supernatants and vesicle sub-fractions for Con and run animals.

| Fractions | n | Protein concentration | |
		Con	Run
Hom	8	13.7 ± 0.9	$13.4 \bullet 1.2$
P_1	8	3.1 ± 0.5	2.8 ± 0.5
S_1	7	5.3 ± 0.1	5.5 ± 0.3
P_2	7	1.8 ± 0.3	1.7 ± 0.3
S_2	8	4.9 ± 0.2	5.4 ± 0.4
P_3	8	0.7 ± 0.1	0.8 ± 0.2
S_3	8	$4.2 \bullet 0.2$	4.2 ± 0.3
SC	5	0.6 ± 0.3	$0.2 \bullet 0.05$
P_6	8	0.4 ± 0.1	0.6 ± 0.1

Values are means $\pm$ SE expressed in mg protein . ml^{-1}. Hom, homogenate,P_1 ; pellets fom centifugation 1, S_1; supernatant from centrifugation 1, P_2; pellets from centrifugation 2, S_2; supernatant from centrifugation 2, P_3; pellets from centrifugation 3, S_3; supernatant from centrifugation 3, SC; supernatant combined from supernatant 4, 5, and 6, P6; pellets from centrifugation 6.

Table 4.6. Protein yield for vesicle sub-fractions (P_6) for Con and Run animals.

Fractions	n	Muscle tissue (g) (wet weight)	Volume of P_6 (ml)	Protein concentration $(mg . ml^{-1})$	Protein yield $\mu g . g^{-1}$
Con	8	$7.94 \bullet 0.25$	0.775 ± 0.025	0.370 ± 0.01	0.38 ± 0.09
Run	8	8.65 ± 0.40	$0.631 \pm 0.0 67$	$0.580 \bullet 0.015$	0.39 ± 0.07

Values are means $\bullet$ SE.

4.4 DISCUSSION

The results of this study demonstrated that Ca^{2+}-Mg^{2+}-ATPase activity of exercised and control animals was similar not only in the homogenates and vesicle sub-fractions, but also in the sub-fractions and supernatants that are normally discarded during the isolation procedure. Therefore, assuming that the amount of 110 kDa Ca^{2+}-Mg^{2+}-ATPase protein, relative to total protein content did not change for the discarded sub-fractions, SC and vesicle fractions, the hypothesis that exercise would affect the distribution of active and inactive Ca^{2+}-Mg^{2+}-ATPases in the discarded sub-fractions and/or supernatants during the fractionation procedure, must be rejected.

The similar contamination by sarcolemma (pNPP activity) and mitochondria (citrate synthase activity) in Hom, SR enriched supernatants (S_1, S_2,S_3) and vesicle sub-fractions in the Con and Run groups confirmed the results of Byrd et al. (1989a) and Chin and Green (1996) reporting no effects of the running exercise on these parameters. Similar to the results of Chin and Green (1996), the fractionation procedure resulted in about a 12-fold increase in Ca^{2+}-Mg^{2+}-ATPase activity in the vesicle sub-fractions over the Hom activity. Measurements of Ca^{2+}-Mg^{2+}-ATPase in the discarded sub-fractions and SC, suggest that a high proportion of SR membrane is loss during the centrifugation procedure. The activity in P_3 and in SC was 10- and 16-fold higher than the Hom activity. However, protein yields found in this study were about one third of what is normally (1 mg . g^{-1} muscle wet weight) reported for this centrifugation procedure (Byrd et al., 1989a; Chin & Green, 1996). This is probably due to the collection of SR enriched supernatants after centrifugation steps 1 (S_1), 2 (S_2) and 3 (S_3) for the contamination assays.

Results from the present investigation confirmed previous published results (Dossett-Mercer et al., 1994; Chin & Green, 1996) from this laboratory that found no depression in Ca^{2+}-Mg^{2+}-ATPase activity following running exercise. As with previous studies, these results contradict the findings of other studies (Belcastro et al., 1981; Byrd et al., 1989a; Byrd et al., 1989b, Belcastro et al., 1993) that demonstrate reductions in maximal Ca^{2+}-Mg^{2+}-ATPase activity ranging from 18 to 50 % with this type of exercise. Therefore the discrepancy in the literature concerning the effect of exercise on the activity of the Ca^{2+}-Mg^{2+}-ATPase in Hom (Dossett-Mercer et al., 1994) and vesicle sub-fractions (Byrd et al., 1989a) is not explained by the differences in yields in vesicle fractions isolated from the muscle of control and exercised animals, as suggested by Dossett-Mercer et al. (1994).

Results from the present investigation also support the findings of Chin and Green (1996) that reported no change in the maximal Ca^{2+}-Mg^{2+}-ATPase activity in Hom, and different vesicle fractions obtained by two different protocols of isolation. It also suggests that the shorter time of 20 min, instead of 60 min, used by Chin and Green (1996) for the second centrifugation at 10,000 g_{avg} had minimal impact in the pelleting of the vesicle sub-fraction.

In the present study, the original protocol of Byrd (1987) was duplicated as closely as possible. The time of each centrifugation were determined from the original times (Byrd, 1987; Byrd et al., 1989a), the k factors of the Sorval SS34 (personal communication with G. Klug) fixed angle rotor used by Byrd et al. (1989a) and the k factor of the Beckman fixed angle Type 70.1 Ti used for the present experiments.. Our results support the conclusions of Chin and Green (1996) and eliminate any suspicion

that the divergent results could be due to the difference in centrifuging time between the isolation protocols.

Other possibilities, to explain the conflicting results in addition to the analytical procedures have been suggested in studies (Dossett-Mercer et al., 1994; Chin & Green, 1996) reporting no changes in Ca^{2+}-Mg^{2+}-ATPase activity with exercise to explain their divergent results. These possibilities include the species and gender of the animal, the exercise protocol, the muscle fibre type composition and a reduction in basal (Mg^{2+})-stimulated activity. In the present study, as opposed to Chin & Green (1996), who used Wistar rats, Sprague-Dawley rats were used, to eliminate any differences due to species with the study of Byrd et al. (1989a). This left one or a combination of the three other factors to explain the large the discrepancy in results.

The exercise protocol used in this study to produce muscular fatigue was similar to the one used by Chin and Green (1996). The running time to exhaustion in the present study was 162 ± 47 min ($x \pm SE$) with many animals running close to or more than 3 h. In the study of Chin and Green (1996), only 3 animals out of 22 ran up to the imposed limit of 180 min before being sacrificed. Average time excluding these three animals was 115 ± 10 min. In the study of Byrd and colleagues (Byrd, 1987; Byrd et al., 1989a), the speed was maintained at 21 m . min^{-1} with a 10 % grade, resulting in a running time to exhaustion that averaged 140 ± 55 min ($x \pm SE$). The longer running time of the animals in the present study compared to the running time of the animals in the study of Byrd et al. (1989a) and Chin and Green (1996) could be attributable to the lower treadmill slope of 4 %. However, when comparing the present study with Byrd et al., (1989a), the lower slope used in this study would be in part off-

set by the higher treadmill speed (21 m . min^{-1} after 60 min to 25 m . min^{-1} from 60-120 min and 28 m . min^{-1} from 120-180+ min). In the study of Chin and Green (1996) since the same treadmill protocol was used, the lower running time to exhaustion is likely to be due to the 4 % difference in treadmill slope.

One interesting qualitative observation that was made during the course of the present study was that the running patterns varied among the exercised animals. Certain animals preferred maintaining a steady pace throughout the run, whereas others had a tendency to sprint to the top of the treadmill and then rest until they were brought back to the bottom again. Could this difference in running pattern be an overlooked variable that could help explain the wide variability in Ca^{2+}-Mg^{2+}-ATPase activity observed in the present study ? For practical reasons it is difficult to examine the validity of this hypothesis in our study since the muscles of two animals were pooled together in order to secure the amount of tissue needed for the analytical procedures.

The pattern of glycogen depletion in the working muscles is often used to categorize the intensity of the exercise protocol and provide an indication of the level of recruitment demanded by the activity. Glycogen was not measured in the present study, however, Chin and Green (1996) reported glycogen depletion amounting to approximately 65 % of initial levels. The standard error of the mean (SEM) of approximately 10 % also suggest that variation in glycogen depletion from one animal to the next were minimal and therefore that probably similar recruitment of the working musculature took place independently of the running "strategy" adopted by the animals. In addition, fibre type differences among animals, an important factor to consider when examining glycogen depletion pattern, would not have made an important contribution

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in this case since the level of depletion was similar in both red and white gastrocnemius (Chin and Green, 1996).

In the study of Byrd et al. (1989a), a slightly more pronounced depletion was observed in the deep red fibres of the combined vastus and gastrocnemius (80-90%) than in the superficial white fibres of the same muscles, where the depletion ranged from 80 % in the vastus to 50-60 % in the gastrocnemius. Since a depressed Ca^{2+}-Mg^{2+}-ATPase activity was only found in vesicle fractions isolated from the deep muscles, it has been suggested by the authors (Byrd et al., 1989a) and by Chin and Green (1996) that the greater depletion in these muscle could be responsible for the decrease in enzymatic activity. However, caution should be exercised in the interpretation of this data. The lower depletion in the superficial gastrocnemius was significant after 20 min of exercise, but contrary to the other three muscles (red gastrocnemius and vastus and white vastus) it was similar to initial levels at exhaustion. The depletion was, however, greater at exhaustion than at 20 min and the lack of significance appears to be the result of the high variability of this specific value and the limited number of animals (n = 4) (Byrd et al., 1989a). Therefore, the levels of exercise intensity based on glycogen utilization would appear to be similar between these two muscle fibre types.

The differential effect of exercise on Ca^{2+}-Mg^{2+}-ATPase of red and white muscles found by Byrd et al. (1989a) suggest that muscle fibre type composition, independent of glycogen depletion, could in part, explain the differences in results between studies. The large reduction (70 %) in Ca^{2+}-Mg^{2+}-ATPase activity observed within the initial 2 min of running (25 $m \cdot min^{-1}$, 10 % grade) provides another line of evidence suggesting that the reduced enzymatic activity is not dependent on glycogen

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levels (Belcastro et al., 1993). This finding is corroborated by the results of another study reporting a decrease of 50 % in Ca^{2+}-Mg^{2+}-ATPase activity in horses after only 4-5 min of intense running at VO_2max (Byrd et al., 1989b). The importance of muscle fibre type is also suggested by the lower depression (18 %) reported by Belcastro et al. (1993). The depression reported by this group is well below the 50 % reduction reported by Byrd et al. (1989a) and the 40 % reduction reported by Luckin et al. (1991). This difference could be related to the fact that these investigators used the entire gastrocnemius muscle for the isolation of vesicle fractions of SR, instead of selectively excising the red and white portions.

The difference in Ca^{2+}-Mg^{2+}-ATPase activity in these two studies (Byrd et al., 1989a; Belcastro et al., 1993) suggest that muscle fibre type composition is an important factor that modulates the response of the enzyme to exhaustive running exercise. The metabolic potential of a muscle is largely dependent upon it's fibre composition (Meyer et al., 1980; Dudley & Terjung, 1985a) and differences in levels of metabolic by-products could provide a reason for the differential effect of exhaustive exercise observed on Ca^{2+}-Mg^{2+}-ATPase activity of red and white muscles. Reversal of Ca^{2+}-Mg^{2+}-ATPase has been demonstrated with single cardiac muscle fibres immersed in a solution mimicking the intracellular milieu changes that occur during short-term hypoxia (Zhu & Nosek, 1991). Reversible, subtle alterations of the Ca^{2+}-Mg^{2+}-ATPase structure during acute exercise could provide the mechanism by which Ca^{2+}-Mg^{2+}-ATPase activity is reduced (Byrd et al., 1989b, Luckin et al., 1991; Williams & Klug, 1995).

In two studies were no alterations in Ca^{2+}-Mg^{2+}-ATPase activity were found, for either muscle fibre types, short intense (Dossett-Mercer et al.,1994) and prolonged exhaustive exercise (Chin & Green, 1996) resulted in only mild metabolic perturbations. It was only with short term high intensity running in horses that a documented reduction in Ca^{2+}-Mg^{2+}-ATPase activity was concomitantly observed with a decrease in muscle pH and an increase in muscle temperature (Byrd et al., 1989b). Possible differences in response due to differences in muscle fibre type composition were not examined in the study with horses (Byrd et al., 1989b)

The results observed with short term high intensity running in horses (Byrd et al., 1989b) and rats (Belcastro et al., 1993) are not equivocal. In the study of Dossett-Mercer et al. (1994) mentioned above, rats subjected to 15-20 min of interval running with bouts of 2.5 min of sprint running interspersed with 2 min pause, showed no change in Ca^{2+}-Mg^{2+}-ATPase activity. In another study using rats and running exercise, treadmill speed was increased by 5.4 $m\,min^{-1}$ every min from an initial speed of 21.5 $m\,min^{-1}$ (grade of 0 %) (Bonner et al., 1976). After reaching a maximal speed 53.6 $m\,min^{-1}$ (6 min), the grade was increased by 2.5 % per min to a maximum of 10 %. Under such extreme conditions, Ca^{2+}-Mg^{2+}-ATPase activity displayed a tendency to increase (Bonner et al. 1976).

In a recent study, a progressive increase in Ca^{2+}-Mg^{2+}-ATPase activity of up to 30 % was observed after 2 h of running (19 m . min^{-1}; 75 min, 16 m . min^{-1}; 45 min), followed by fast walking (42min:10 m . min^{-1}) until sacrifice of the animal (Ferrington et al., 1996). For animals sacrificed immediately, and after 18 min of fast walking, the increase in Ca^{2+}-Mg^{2+}-ATPase activity was not significantly different than the activity

found for the control group. Some differences with other studies (Byrd et al., 1989a; Green & Chin, 1996) examining the effect of exercise on Ca^{2+}-Mg^{2+}-ATPase activity that could explain the divergent results include a lower exercise intensity and a low number of animals (n = 2) per exercise groups. Another important difference is that the increased activity was observed in animals that were not exhausted, since partial recovery was achieved during the 42 min of walking. A possible hypothesis suggested by the authors to explain these results was that the exercise activated a population of Ca^{2+}-Mg^{2+}-ATPase that was dormant. Recently, Ca^{2+}-Mg^{2+}-ATPase activity in both soleus and extensor digitorum longus muscles was shown to increase with ischemia and a similar hypothesis was advanced to explain the results. The existence of two different conformations of chemically equivalent Ca^{2+}-Mg^{2+}-ATPase molecules and their different sensitivity to pH and Ca^{2+} binding (Nakamura & Furukohri, 1994) may provide clues regarding the underlying mechanisms that determine the activation or inactivation of a proportion of the enzyme's population.

A factor that could contribute to the difference in Ca^{2+}-Mg^{2+}-ATPase activity in the present study and Byrd et al. (1989a) is the increase in basal (Mg^{2+}-stimulated activity) in the latter study. This reduction in basal activity has not been observed in any of the studies conducted in our laboratory (Dossett-Mercer et al., 1994; Chin & Green, 1996). Byrd et al (1989a) have suggested that contamination by the t-tubular membrane which possesses a high Mg^{2+} stimulated activity could be responsible for the higher basal activity observed with vesicle fractions isolated from exercised animals. However, this remains to be demonstrated, as discussed by Chin and Green (1996), since

contamination by other membranes sarcolemmal and mitochondrial appears not to be affected by exercise.

A second important consideration relating to analytical procedure outside of the isolation protocol is the Ca^{2+}-Mg^{2+}-ATPase assay. The spectrophotometric assay of Simonides and van Hardeveld (1990) was used to measure Ca^{2+}-Mg^{2+}-ATPase activity in Hom. Despite differences in the buffer composition, this homogenate assay was validated against another assay for vesicle fractions (Simonides and van Hardeveld, 1990) and used in our laboratory previously (Dossett-Mercer et al., 1994). For the other fractions, including the final pellet (P_6), the assay of Weidekamm and Brdiczka (1975) as modified by Byrd (1987) was used with one difference; the addition of 1 mM of EGTA to effectively control the $[Ca^{2+}]_f$.

In the studies (Dossett-Mercer et al. 1994; Chin and Green, 1996) where no changes in Ca^{2+}-Mg^{2+}-ATPase activity were observed, the $[Ca^{2+}]_f$ has been suggested as a possible factor that could explain the discrepancy in results with other studies showing a reduction (Belcastro et al., 1981; Byrd et al., 1989a; Byrd et al., 1989b, Luckin et al., 1991; Belcastro et al., 1993). Chin and Green (1996) estimated the $[Ca^{2+}]_f$ to be 2.5 μM. However, the $[Ca^{2+}]_f$ was estimated with a Ca^{2+} software program (Kurzmack) that is not designed to adjust Ca^{2+} binding constants for changes in temperature. The program uses binding constants set for a 25° C temperature, while the assay used by Chin and Green (1996) was run at 37° C.

Problems in estimation of $[Ca^{2+}]_f$ also appeared to be present in the study of Byrd et al. (1989a), where a range of $[Ca^{2+}]_f$ between 0.6 and 2 μM was reported to be required to get maximal Ca^{2+}-Mg^{2+}-ATPase activity. However, no Ca^{2+} chelator

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was added to the buffer, with the exception of EGTA at the end to measure basal activity. Therefore, it is unlikely that precise levels of $[Ca^{2+}]_f$ were measured in the absence of EGTA or another Ca^{2+} chelator. The Chelator software program (Schoenmakers, 1992) used in the present study does not provide calculations when no chelator is used to control free Ca^{2+}. However, if an EGTA concentration of 1 mM is used and the appropriate entries for ionic strength, pH and temperature are made, the total $[Ca^{2+}]_f$ needed to obtain the reported $[Ca^{2+}]_f$ values of 0.6 and 2 μM in the assay buffer (Byrd et al.,1989a) are 0.47 and 0.75 mM, respectively, instead of the 1 and 1.6 mM reported in the principal author's thesis (Byrd, 1987). If the $[Ca^{2+}]_f$ are calculated from the total $[Ca^{2+}]$ of 1 and 1.6 mM provided in the thesis (Byrd, 1987), then the $[Ca^{2+}]_f$ become 20 and 370 μM, respectively, instead of 0.6 and 2 μM. These are important differences and they were obtained in the presence of 1 mM EGTA. Since no EGTA was used in the study of Byrd et al. (1989a), the actual $[Ca^{2+}]_f$ were probably much higher. Nevertheless, despite these problems in the estimation of $[Ca^{2+}]_f$, the reported values of Ca^{2+}-Mg^{2+}-ATPase activity were maximal since a Ca^{2+} titration was done for each trial to insure that maximal activity was reached.

In the present investigation, the $[Ca^{2+}]_f$ was pre-determined with pilot assays by adding Ca^{2+} until maximal Ca^{2+}-Mg^{2+}-ATPase activity was reached. Maximal Ca^{2+}-Mg^{2+}-ATPase activity was reached at $[Ca^{2+}]_f$ of 14 μM for the Hom assay (Simonides and van Hardeveld, 1990) and 6.4 μM for the modified assay of Byrd et al. (1989a) used for the other fractions. To insure that maximal activity had been reached, a second addition of Ca^{2+} to a $[Ca^{2+}]_f$ of 162 μM was made.

4.5   SUMMARY

Results from this study demonstrate that exercise, as opposed to ischemia (Rapundalo et al., 1986) does not predispose the Hom to a differential effect by the fractionation procedure. Ca^{2+}-Mg^{2+}-ATPase activity of sub-fractions and supernatants discarded during the isolation procedure were found to be similar in both control and exercised animals. The discrepancy on the effect of prolonged exercise between Byrd et al. (1989a) and studies from our laboratory cannot be explained on the basis of differences in species of rats, exercise protocol or isolation procedure, since these variables were similar in the two studies.

CHAPTER V

CONCLUSION

1.0   CONCLUSION

Many years have passed since the first study of this thesis, using the *in vitro* diaphragm preparation, was completed. Considerable knowledge has been accumulated on the cellular aspects of muscular fatigue through experiments using the single intact fibre preparation. In studying the involvement of specific sites in fatigue, the single fibre preparation offers many advantages over a whole muscle preparation. Perhaps, the greatest advantage of this preparation is that it allows the measurement of $[Ca^{2+}]_i$ in conjunction with tension, through the use of Ca^{2+} fluorescent dyes. Assessing the fluctuations of $[Ca^{2+}]_i$ that occur during and following repetitive activity has become an essential measurement in our search for the mechanism(s) underlying muscular fatigue. With this measurement not only can $[Ca^{2+}]_i$ be directly linked to fatigue but the role of the SR, both Ca^{2+} release and Ca^{2+} uptake, in contributing to the depression in $[Ca^{2+}]_i$ can be assessed.

In the series of studies reported in this thesis we did not have access to the technology needed to measure $[Ca^{2+}]_i$. As a consequence, we have attempted to rely on pharmacologic agents, such as caffeine and direct measurement of Ca^{2+}-Mg^{2+}-ATPase activity in order to determine the role of $[Ca^{2+}]_i$ and the SR in the fatigue process.

In the first study, the objective was to create fatigue and an altered metabolic environment (lower phosphorylation potential and increases in metabolic by-products

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such as ADP, Pi and H$^+$) by using short term repetitive stimulation and then to examine the role of Ca^{2+} release in fatigue by manipulating selected aspects of the intracellular environment. The strategy consisted in manipulating the experimental conditions during the recovery period, in an attempt to determine the role of the phosphorylation potential (ATP, PCr) and the metabolic by-products (Pi, ADP, H$^+$).

In the first condition, stimulation was resumed for a brief period to maintain the metabolic environment created by the electrical stimulation and the role of Ca^{2+} release examined by adding caffeine, an agent known to stimulate Ca^{2+} release from the Ca^{2+} channels of the SR.

In the second condition, 5 min of passive recovery was introduced after the initial 3 min of stimulation which would allow the recovery of the high energy phosphate systyem but not pH. Since normalization of pH is a relatively slow process, the condition allowed for independent examination of acidosis on Ca^{2+} release and fatigue.

In the final condition, the 20 min anoxic period was designed to allow for the normalisation of pH but to inhibit recovery of the phosphorylation potential. This manipulation allowed for the study of the role of a depressed phosphorylation potential and elevated levels metabolic by-products such as Pi and ADP on Ca^{2+} release and fatigue.

In this first study, direct measurements of the cellular metabolites were not made. Despite, the fact that measurements of muscle metabolite concentrations following repetitive activity in whole muscle is of limited value, because of fibre

heterogeneity, the information remains pertinent if we are to understand how muscle fatigue modulates $[Ca^{2+}]_i$. Unfortunately, the loss of frozen tissue prevented measurements of the metabolic profile in the different states. Evidence from experiments using induced stimulation conducted in this laboratory and by other investigators allow a fairly accurate picture of the metabolic environment that would be expected for the specific fatigue protocol used. However, the omission of these measurements must be recognised as a serious limitations in the first study. Therefore it is recommended that such measurements be made to confirm that the experimental manipulations created the intracellular environment that was intended during each of the three conditions.

A third factor that can be used to determine how metabolic perturbations modulate $[Ca^{2+}]_i$ is the use of pharmacological agents that act upon the structures that regulate $[Ca^{2+}]_i$. By comparing tension and $[Ca^{2+}]_i$ with and without the presence of these drugs, conclusions can be derived on the involvement of specific structures in the fatigue process. In this first study, caffeine, a known potentiator of Ca^{2+} release, was used to determine the effect of selected metabolic perturbations on the Ca^{2+} release channels of the SR. Ca^{2+} channel blockers or other drugs acting on the other important structure regulating $[Ca^{2+}]_i$; the Ca^{2+}-Mg^{2+}-ATPase, can also be used to determine the effect of metabolic perturbations on the Ca^{2+} regulating structures. However, the diaphragm, because of its mixed fibre composition, may not be the most appropriate muscle to use. Other muscles that have a more homogeneous fibre type composition may be more appropriate for examining the interactions of drugs and metabolic

perturbations. *In vivo* or *in situ* preparations may have to be used for these other muscles to insure their viability over the length of the experiment.

The two additional studies completed for this thesis have attempted to understand the role of Ca^{2+}-sequestration in fatigue by examining the Ca^{2+}-Mg^{2+}-ATPase. In one study, the measurements were conducted *in vitro* using different concentrations of Ca^{2+}, H^+ and ATP. In a second study, the effect of prolonged voluntary activity on the Ca^{2+}-Mg^{2+}-ATPase activity was examined.

The rational behind the one study was to examine if the expression of different SERCA isoforms of the Ca^{2+}-Mg^{2+}-ATPase play a role in modulating Ca^{2+} uptake in tissues by displaying differences in sensitivity to the metabolic by-products generated during muscle contraction. The finding that Ca^{2+}-Mg^{2+}-ATPase activity in homogenates of WGM and RGM is more sensitive than in S homogenates to H^+ concentration suggests that the two SERCA isoforms display a different sensitivity to metabolic acidosis. In contrast, the SERCA isoforms in COS expression system do not show this difference in sensitivity. Although, findings obtained during this study will have to be reproduced before a definite conclusion can be reached, the results suggest that the native environment of the Ca^{2+}-Mg^{2+}-ATPase may be important in modulating the response of the enzyme to the metabolic environment.

Organic phosphate (Pi) would certainly be the candidate of choice to further examine the difference in dependency by the SERCA isoforms, since it is considered to be a major metabolite involved in the regulation of $[Ca^{2+}]_i$. However, the measurement of Ca^{2+}-Mg^{2+}-ATPase activity under different concentrations of Pi is complicated by

the direct effect of Pi on the assay used to measure the Ca^{2+}-Mg^{2+}-ATPase. In the

coupled assay system of pyruvate kinase and lactate dehydrogenase, Pi is a substrate of

the pyruvate kinase reaction, and therefore increasing the background concentration of

Pi accelerates the kinetics of this reaction, independently of the amount of Pi produced

by the Ca^{2+}-Mg^{2+}-ATPase reaction. The use of an end-point assay (Parkhouse, 1992)

for measuring Ca^{2+}-Mg^{2+}-ATPase activity through the liberation of Pi in the protein-

free supernatant is also problematic because of the interference of the background Pi

with the colorimetric nature of the reaction.

The major obstacle in the dependency studies resides in obtaining accurate

$[Ca^{2+}]_f$ so that the activity of the Ca^{2+}-Mg^{2+}-ATPase under various conditions can be

compared on the same basis and the specific effect of a metabolite isolated.

Determination of $[Ca^{2+}]_f$ in buffers is difficult because of contamination and other

factors (please see Appendix IIA). A recommendation is to avoid measurements of the

Ca^{2+}-Mg^{2+}-ATPase where accurate $[Ca^{2+}]_f$ are required. A compromise may be to assess

the effect of metabolites on Ca^{2+}-Mg^{2+}-ATPase activity under saturating level of $[Ca^{2+}]_f$

where maximal activation of the enzyme is obtained (Parkhouse, 1992). If measurement

of Ca^{2+}-Mg^{2+}-ATPase activity under various accurate $[Ca^{2+}]_f$ is desired, the $[Ca^{2+}]_f$ must

be determined with a Ca^{2+} software program that takes in consideration the chemical

composition of the buffer as well as the specific concentration of Pi or other

metabolites used to inhibit Ca^{2+}-Mg^{2+}-ATPase activity.

For the final study, the effect of prolonged exercise on the activity of the Ca^{2+}-

Mg^{2+}-ATPase was examined. In this type of experiment, animals are subjected to an

exhaustive bout of running-exercise and the Ca^{2+}-Mg^{2+}-ATPase activity is measured *in vitro*, using homogenate or vesicle fraction preparations, under optimal environmental conditions. This study was conducted with the specific objective of examining if the differential effect of exercise on the fractionation procedure is responsible for the discrepancy observed in the literature for the effect of prolonged exercise on Ca^{2+}-Mg^{2+}-ATPase activity . (Byrd et al., 1989a; Chin & Green, 1996).

Results showed no differences in Ca^{2+}-Mg^{2+}-ATPase activities between control and exercised animals for any of the fractions assayed. In addition, the similar activities of marker enzymes and similar protein concentrations suggested that the purity of the fractions was not altered by the exercise. However, additional information on Ca^{2+}-Mg^{2+}-ATPase activity was obtained in the third study and not presented in this thesis. This data contained Ca^{2+}-Mg^{2+}-ATPase activities measured from tissues subjected to a different homogenisation procedure, namely glass to glass hand homogenisation, instead of mechanical homogenisation. It is possible that the method of homogenisation could affect the changes in Ca^{2+}-Mg^{2+}-ATPase activity observed with exercise. If a decrease in activity is observed for the exercised group, this would suggest that the more violent mechanical homogenisation may actually mask the effects of exercise on Ca^{2+}-Mg^{2+}-ATPase activity. The assay may proved to be sensitive to the homogenisation technique. Byrd et al. (1989a) showed a 50 % decrease in Ca^{2+}-Mg^{2+}-ATPase activity, measured in vesicle fractions, under optimal conditions, despite using a mechanical device to homogenise the muscle tissue. However, perhaps the blender used

by Byrd et al., (1989a) does not inflict the same damage to the muscle tissue than the damage produced by the polytron, the apparatus used so far in our laboratory.

Factors such as the species and gender of the rat, muscle fibre type composition and isolation procedure, given the similarity between the experimental procedures followed by Byrd et al. (1989a) and the present investigation, do not appear to explain the contradictory results. The recent study of Ferrington et al. (1996), showing an increase in Ca^{2+}-Mg^{2+}-ATPase activity in two rats, suggest that some forms of active recovery (fast-walking) following the exhaustive exercise could influence the outcome of the results. A possible hypothesis suggested by the authors to explain these results was that the exercise activated a population of Ca^{2+}-Mg^{2+}-ATPases that were dormant. It is possible that the existence of a mechanism where a large population of enzymes could be turned on or turned off, depending on certain specific conditions, could explain the divergence of results on the effect of exercise on Ca^{2+}-Mg^{2+}-ATPase activity observed in the literature.

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APPENDIX I

APPENDIX IA: DEFINITION OF ISOMETRIC CONTRACTILE PROPERTIES

1) Twitch peak tension (P_t)

Definition : the maximal tension developed by a muscle in response to a   single
supramaximal stimulus.

Units: mg, g, mN, N or if tension is normalised to cross-sectional area: $mg . cm^{-2}$,
$g . cm^{-2}$, $mN . cm^{-2}$, $N . cm^{-2}$.

Physiological basis: the twitch is dependent upon events of Excitation-Contraction
Coupling (ECC) as well as shortening of the series elastic elements
(SEE). Factors from ECC that could affect the twitch include:
quantity of Ca^{2+} release from the sarcoplasmic reticulum, the cycling
of the actin-myosin cross-bridges, the affinity of Ca^{2+} for troponin
and its binding to other cytosolic Ca^{2+} binding proteins. Factors that
will affect the twitch by altering both processes of ECC and the
SEE: length of the muscle.

2) Contraction time (CT)

Definition : the time from the onset of tension needed to reach peak tension and for that
matter it is also called time to peak tension. It represents the first half of the
Ca^{2+} transient.

Units: ms

Physiological basis: it is affected by the active processes involve in tension
development, chiefly the release of Ca^{2+} by the sarcoplasmic
reticulum and the cycling of the actin-myosin cross-bridges.

3) Half-relaxation time ($RT_{1/2}$)

Definition : the time needed for twitch tension to decline by one half, representing the
second half of the Ca^{2+} transient.

Units: ms

Physiological basis: it affected by the active process involved in tension decay, chiefly
the re-uptake of Ca^{2+} by the sarcoplasmic reticulum and the
myoplasmic Ca^{2+} buffers, the dissociation of Ca^{2+} from troponin and
the detachment of the actin-myosin cross-bridges.

4) Maximum rate of tension development (+dP/d t_{max})

Definition : the maximum of the first derivative of tension development

Units: g . s^{-1}, mg . s^{-1}, mN . s^{-1}, N . s^{-1} or if normalised to cross-sectional area: g . cm^{-2} . mg . cm^{-2} s^{-1}, mN . cm^{-2} . s^{-1}, N . cm^{-2}. s^{-1}.

Physiological basis: the rate limiting step in +dP/d t_{max} appears to be the transition between the weakly bound, low tension state to the strongly bound, high tension state cross-bridges. In the fatigued state, +dP/d t_{max} could be limited by the rate of Ca^{2+} release (Thompson et al., 1992a).

5) Maximum rate of tension decline (-dP/d t_{max})

Definition : the maximum of the first derivative of tension decline

Units: g . s^{-1}, mg . s^{-1}, mN . s^{-1}, N . s^{-1} or if normalised to cross-sectional area: g . cm^{-2} . mg . cm^{-2} s^{-1}, mN . cm^{-2} . s^{-1}, N . cm^{-2}. s^{-1}.

Physiological basis: several processes are suggested as possible limiting factor for the depression in -dP/d t : (1) the off-rate of actin and myosin binding, (2) the dissociation of Ca^{2+} from troponin C, (3) Ca^{2+} binding to parvalbumin and (4) the Ca^{2+}-Mg^{2+}-ATPase (Thompson et al., 1992b)

APPENDIX IB: ESTIMATED DIAPHRAGM MUSCLE CROSS-SECTIONAL AREA AND ITS TENSION EXPRESSED IN NEWTON CENTIMETER SQUARE

Sample calculation

Given: Muscle density : 1.056 g . ml^{-1} (g . cm^{-3})
Given: Acceleration due to gravity: 9.81 m . s^{-2}

Measured: Diaphragm muscle length : 1.37 cm
Diaphragm muscle weight: 0.01796 g
Diaphragm muscle force: 3.891 g

1. Estimated diaphragm muscle cross-sectional area (CSA)

CSA   = Diaphragm muscle weight (g) / (diaphragm muscle density (g . cm^{-3})
• diaphragm muscle length (cm))

$$= \ 0.01796 \ g \ / (1.056 \ g \ . \ cm^{-3} \bullet 1.37 \ cm)$$

$$= \ 0.01241 \ cm^{-2}$$

2. Tension per cross-sectional area (TCSA)

$$TCSA = (tension \ (g) \ / \ CSA \ (cm^{-2}) \ ) * (1 \ (kg) \ / \ 1000 \ (g)) \bullet 9.81 \ m . \ s^{-2}$$

$$= (3.891 \ g \ / \ 0.01241 \ cm^{-2}) * (1 \ (kg) \ / \ 1000 \ (g)) * 9.81 \ m . \ s^{-2}$$

$$= 3.075 \ N \ (kg . m . s^{-2}) . \ cm^{-2}$$

APPENDIX IC: SUPPLEMENTARY TENSION-FREQUENCY DATA FOR RAT *IN VITRO* DIAPHRAGM STUDY AT 10 HZ, 20 HZ AND 50 HZ

The remaining tension-frequency data from the study presented in chapter II at 10, 20 and 50 Hz are presented in this appendix. The statistical procedures used to analyze these data was similar to the analysis used in chapter II (see section 2.2.5). The use of "different" in the result section means that a significant difference was found. Significance was accepted at $P < 0.05$.

A.1.C.1 Resting contractile measurements

No differences were observed for resting tension, maximum rate of tension development ($+dP_t/dt_{max}$) or rate of tension relaxation ($-dP_t/dt_{max}$) with a few exceptions at the 50 Hz stimulation frequency (Table A1C.1, A1C.2 & A1C.3). At 50 Hz, the $-dP_t/dt_{max}$ for the caffeine-2 min stimulation group was different than the values for the no caffeine-2 min stimulation and the caffeine-5 min recovery groups.

A.1.C.2 Effect of 3 min stimulation protocol

The 3 min stimulation protocol produced a reduction in tension for all the groups and at all three frequencies of stimulation (Table A1C.1, A1C.2 & A1C.3). The magnitude of the reduction was inversely related to the frequency of stimulation: 40 % at P_{10Hz} (Table A1C.1), 36 % at P_{20Hz} (Table A1C.2) and 27 % at P_{50Hz} (Table A1C.3). For the dynamic related tetanic contractile properties, the $+dP_t/dt_{max}$ was reduced at all frequencies of stimulation (Table A1C.1, A1C.2 & A1C.3). The $-dP_t/dt_{max}$ was also

Table A1C.1. Resting isometric tetanic contractile data at 10 Hz stimulation frequency for the six experimental groups.

Group	0 min	3 min
P_{10}, N. cm^{-2}		
No caf.-2MS	3.02 ± 0.38	1.75 ± 0.25[a]
No caf.-5MR	4.25 ± 0.56	2.63 ± 0.46[b]
No caf.-20MR	3.12 ± 0.73	1.72 ± 0.42[b]
Caf.- 2MS	4.23 ± 0.76	2.25 ± 0.32[a]
Caf.-5MR	2.83 ± 0.36	1.84 ± 0.27[a]
Caf.-20MR	2.63 ± 0.54	1.79 ± 0.27[a]
+dP$_t$/d t_{max}, mN. cm^{-2}. s^{-1}		
No caf.-2MS	165 ± 26	78 ± 14[a]
No caf.-5MR	229 ± 34	112 ± 13[b]
No caf.-20MR	181 ± 35	75 ± 12[a]
Caf.- 2MS	232 ± 41	110 ± 22[a]
Caf.-5MR	138 ± 24	85 ± 10[a]
Caf.-20MR	171 ± 32	97 ± 11[a]
-dP$_t$/d t_{max}, mN. cm^{-2}. s^{-1}		
No caf.-2MS	67 ± 9	39 ± 11[a]
No caf.-5MR	118 ± 16	38 ± 4[a]
No caf.-20MR	78 ± 14	26 ± 4[a]
Caf.- 2MS	97 ± 21	32 ± 5[b]
Caf.-5MR	68 ± 9	29 ± 2[a]
Caf.-20MR	69 ± 13	33 ± 4[a]

Values represent means ± SE. No caf.; no caffeine, caf.; caffeine, 2MS; 2 min stimulation, 5MR; 5 minute recovery, 20MR; 20 minute recovery. P$_o$; peak tetanic tension, +dP$_t$/dt; peak rate of tension development, -dP$_t$/dt; peak rate of tension decline. [a] Significantly different from Caf.-2MS ($P <$ 0.05). [a] Significantly different from control ($P <$ 0.01), [b] Significantly different from control ($P <$ 0.05).

Table A.1.C.2. Resting isometric tetanic contractile data at 20 Hz stimulation frequency for the six experimental groups.

Group	0 min	3 min
P_{20}, N. cm^{-2}		
No caf.-2MS	4.92 ± 0.63	3.14 ± 0.50 [a]
No caf.-5MR	7.25 ± 0.85	4.61 ± 0.56 [b]
No caf.-20MR	5.59 ± 1.38	3.22 ± 0.68 [a]
Caf.- 2MS	6.36 ± 0.96	3.93 ± 0.51 [a]
Caf.-5MR	4.57 ± 0.31	3.43 ± 0.28 [a]
Caf.-20MR	5.16 ± 0.92	3.31 ± 0.38 [a]
+dP_t/d t_{max}, mN . cm^{-2} . s^{-1}		
No caf.-2MS	150 ± 21	77 ± 11 [a]
No caf.-5MR	204. ± 30	111 ± 13 [a]
No caf.-20MR	175 ± 34	81 ± 14 [a]
Caf.- 2MS	207 ± 33	111 ± 22 [a]
Caf.-5MR	129 ± 19	81 ± 10 [a]
Caf.-20MR	169 ± 34	92 ± 11 [a]
-dP_t/d t_{max}, mN . cm^{-2} . s^{-1}		
No caf.-2MS	89 ± 11	60 ± 11 [a]
No caf.-5MR	140 ± 20	54 ± 9 [b]
No caf.-20MR	114 ± 26	45 ± 7 [a]
Caf.- 2MS	129 ± 33	54 ± 10
Caf.-5MR	86 ± 7	50 ± 5 [a]
Caf.-20MR	101 ± 17	52 ± 5 [a]

Values represent means ± SE. No caf.; no caffeine, caf.; caffeine, 2MS; 2 min stimulation, 5MR; 5 minute recovery, 20MR; 20 minute recovery. P_o ; peak tetanic tension, +dP_t/dt; peak rate of tension development, -dP_t/dt; peak rate of tension decline. [a] Significantly different from Caf.-2MS ($P < 0.05$). [a] Significantly different from control ($P < 0.01$), [b] Significantly different from control ($P < 0.05$).

Table A.1.C.3. Resting isometric tetanic contractile data at 50 Hz stimulation frequency for the six experimental groups.

Group	0 min	3 min
P_{50}, N . cm^{-2}		
No caf.-2MS	8.49 ± 0.64	6.41 ± 0.91 [a]
No caf.-5MR	11.20 ± 1.21	8.34 ± 0.96 [b]
No caf.-20MR	8.88 ± 1.63	6.32 ± 1.21 [a]
Caf.- 2MS	11.62 ± 1.50	7.50 ± 0.97 [a]
Caf.-5MR	7.33 ± 0.73	5.93 ± 0.45 [a]
Caf.-20MR	8.46 ± 1.36	6.56 ± 0.75 [a]
+dP$_t$/d t_{max}, mN . cm^{-2} . s^{-1}		
No caf.-2MS	159 ± 25	87 ± 14 [a]
No caf.-5MR	208 ± 31	113 ± 13 [a]
No caf.-20MR	173 ± 42	79 ± 16 [a]
Caf.- 2MS	220 ± 35	111 ± 17 [a]
Caf.-5MR	132 ± 20	81 ± 9 [a]
Caf.-20MR	171 ± 32	100 ± 11 [a]
-dP$_t$/d t_{max}, mN . cm^{-2} . s^{-1}		
No caf.-2MS	198 ± 14 [a]	105 ± 23 [a]
No caf.-5MR	279 ± 33	111 ± 16 [a]
No caf.-20MR	246 ± 44	93 ± 13 [a]
Caf.- 2MS	369 ± 62	110 ± 15 [a]
Caf.-5MR	188 ± 29 [a]	87 ± 10 [a]
Caf.-20MR	214 ± 35	103 ± 7 [a]

Values represent means ± SE. No caf.; no caffeine, caf.; caffeine, 2MS; 2 min stimulation, 5MR; 5 minute recovery, 20MR; 20 minute recovery. P$_o$; peak tetanic tension, +dP$_t$/dt; peak rate of tension development, -dP$_t$/dt; peak rate of tension decline. [a] Significantly different from Caf.-2MS ($P < 0.05$). [a] Significantly different from control ($P < 0.01$), [b] Significantly different from control ($P < 0.05$).

reduced with the exception of one group at the 20 Hz stimulation frequency (Table A1C.2).

A.1.C.3 Effect of experimental conditions on caffeine induced contractile response

A.1.C.3.1 Additional 2 min of stimulation (2MS)

Tension at 10 Hz (P_{10Hz}), 20 Hz (P_{20Hz}) and 50 Hz (P_{50Hz}) was not affected by the additional 2 min of stimulation at 15 Hz (Figure A1C.1A, A1C.2A & A1C.3A). The addition of caffeine before the supplementary stimulation produced increases of 64 % for P_{10Hz} and 29 % for P_{20Hz} representing 88 %, and 80 % of the initial resting tension, respectively. At the 50 Hz stimulation frequency, caffeine produced the opposite effect causing a reduction of 13 % in P_{50Hz}.

Maximum rate of tension development ($+dP_t/dt_{max}$) was increased at 20 Hz (39 %) and 50 Hz (37 %) (Figure A1C.2B & A1C.3B). A strong tendency towards an increase was observed at 10 Hz (39 %) but significance was not obtained because of the high variability associated with the data (Figure A1C.1B). Rate of tension decline was decreased by 35 % for $-dP_t/dt_{max}$ at 50 Hz (Figure A1C.3C).

A.1.C.3.2 Five min of passive recovery (5MR)

Tensions at 10 Hz (P_{10Hz}) and 20 Hz (P_{20Hz}) did not recover from the 3 min stimulation with the 5 min recovery period (Figure A1C.4A & A1C.5A). At 50 Hz, P_{50Hz} had recovered by 26 % from its post-fatigue value (Figure A1C.6A), translating into a value equivalent to 94 % of its pre-fatigue value. The addition of caffeine

Figure A.1.C.1. Effect of caffeine after two min of additional stimulation (2MS) on 10 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE ($P < 0.01$). ‡ Significantly different from no-caffeine ($P < 0.01$).

Figure A.1.C.2 Effect of caffeine after two min of
additional stimulation (2MS) on 20 Hz tension (A);
rate of tension development (B); rate of tension
decline (C). PRE & POST, before and after addition
of caffeine and stimulation. Data are means $\pm$ SE.
** Significantly different from PRE ($P < 0.01$) $\ddagger$
Significantly different from no-caffeine ($P < 0.01$).

Figure A.1.C.3 Effect of caffeine after two min of additional stimulation (2MS) on 50 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE ($P < 0.01$), *($P < 0.05$). ‡ Significantly different from no-caffeine ($P < 0.01$).† ($P < 0.05$).

Figure A.1.C.4. Effect of caffeine after 5 min of recovery (5MR) on 10 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE ($P < 0.01$). ‡ Significantly different from no-caffeine ($P < 0.01$).† ($P < 0.05$).

Figure A.1.C.5. Effect of caffeine after 5 min of recovery (5MR) on 20 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE (*P* < 0.01), * (*P* < 0.05) ‡ Significantly different from no-caffeine (*P* < 0.01), † (*P* < 0.05).

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Figure A.1.C.6. Effect of caffeine after 5 min of recovery (5MR) on 50 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE ($P < 0.01$), * ($P < 0.05$). ‡ Significantly different from no-caffeine ($P < 0.01$).

produced increases of 148 % for P_{10Hz}, 131 % for P_{20Hz} and 32 % for P_{50Hz}, representing 160 %, 140 % and 107 % of initial resting values, respectively.

Caffeine produced very high increases in $+dP_v/dt_{max}$ independent of the stimulation frequency: 135 % at 10 Hz, 143 % at 20 Hz and 133 % at 50 Hz (Figure A1C.4B, A1C.5B & A1C.6B). Increases were also observed for the control strip at 20 Hz (Figure A1C.5B). At 20 Hz, the $+dP_v/dt_{max}$ of the caffeine group after administration of the drug was higher than for the control group, whereas before the administration both groups displayed no differences.

A.1.C.3.3 Twenty min of anoxic recovery (20MR)

As for the twitch results presented in the first chapter, the 20MR condition produced no increase in tension for either the control or caffeine diaphragm at the lower stimulation frequencies of 10 Hz and 20 Hz (Figure A1C.7A & A1C.8A). For the tension produced at the 50 Hz stimulation frequency, a reduction of 27 %, similar to the one recorded at 100 Hz (see chapter II) was observed (Figure A1C.9A).

Maximum rate of tension development ($+dP_v/dt_{max}$) displayed an increase (127 %) for the caffeine diaphragm at 10 Hz, but no change for the control diaphragm (Figure A1C.7B). At 20 Hz, $+dP_v/dt_{max}$ increased in both control and caffeine stimulated diaphragms by 36 % and 27 %, respectively. A similar occurrence took place at 50 Hz with the control diaphragm displaying a 46 % increase and the treatment diaphragm a 32 % increase (Figure A1C.9B). The maximum rate of tension decline ($-dP_v/dt_{max}$) was increased at 10 Hz (63 %) and 50 Hz (60%) in the control diaphragm (Figure A1C.7C & A1C.9C) but unaffected by caffeine at 10 Hz, 20 Hz and 50 Hz (Figure A1C.7C, A1C.8C & A1C.9C).
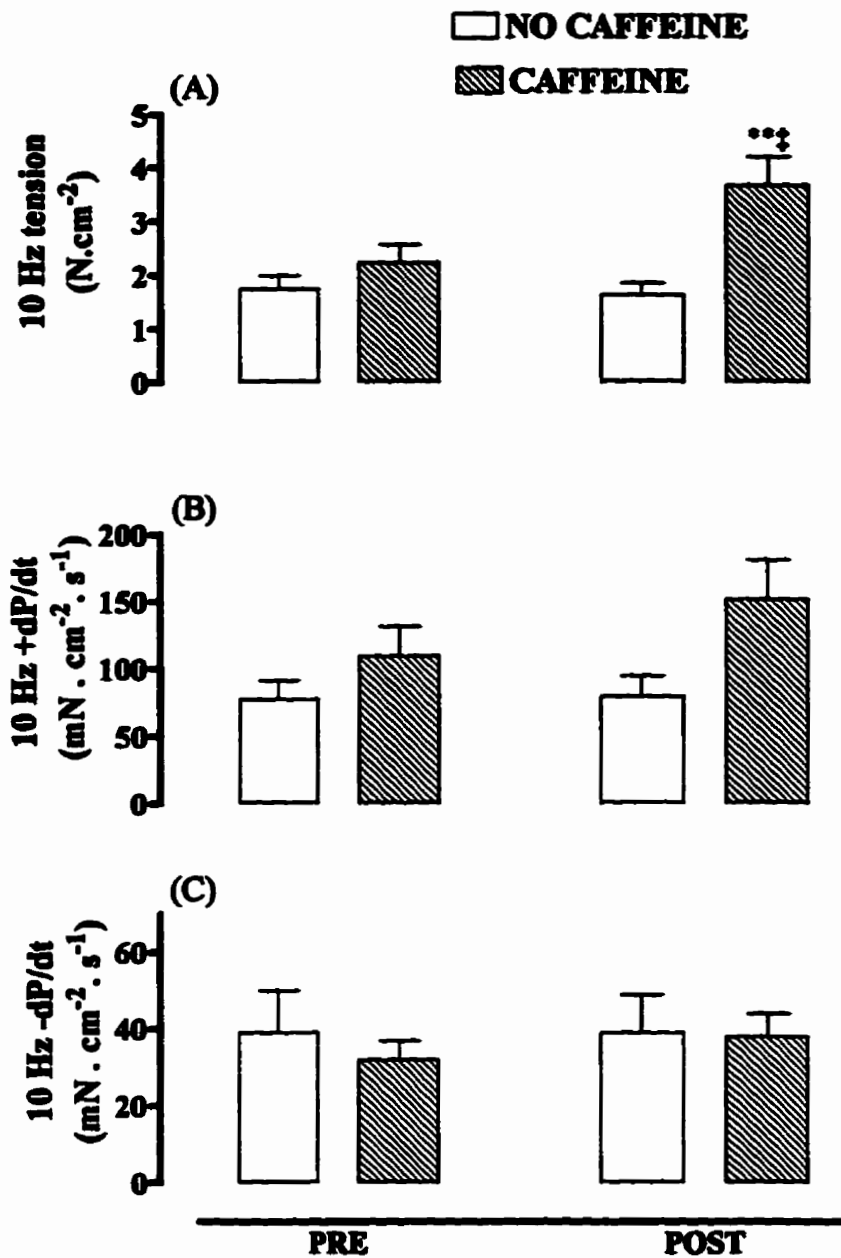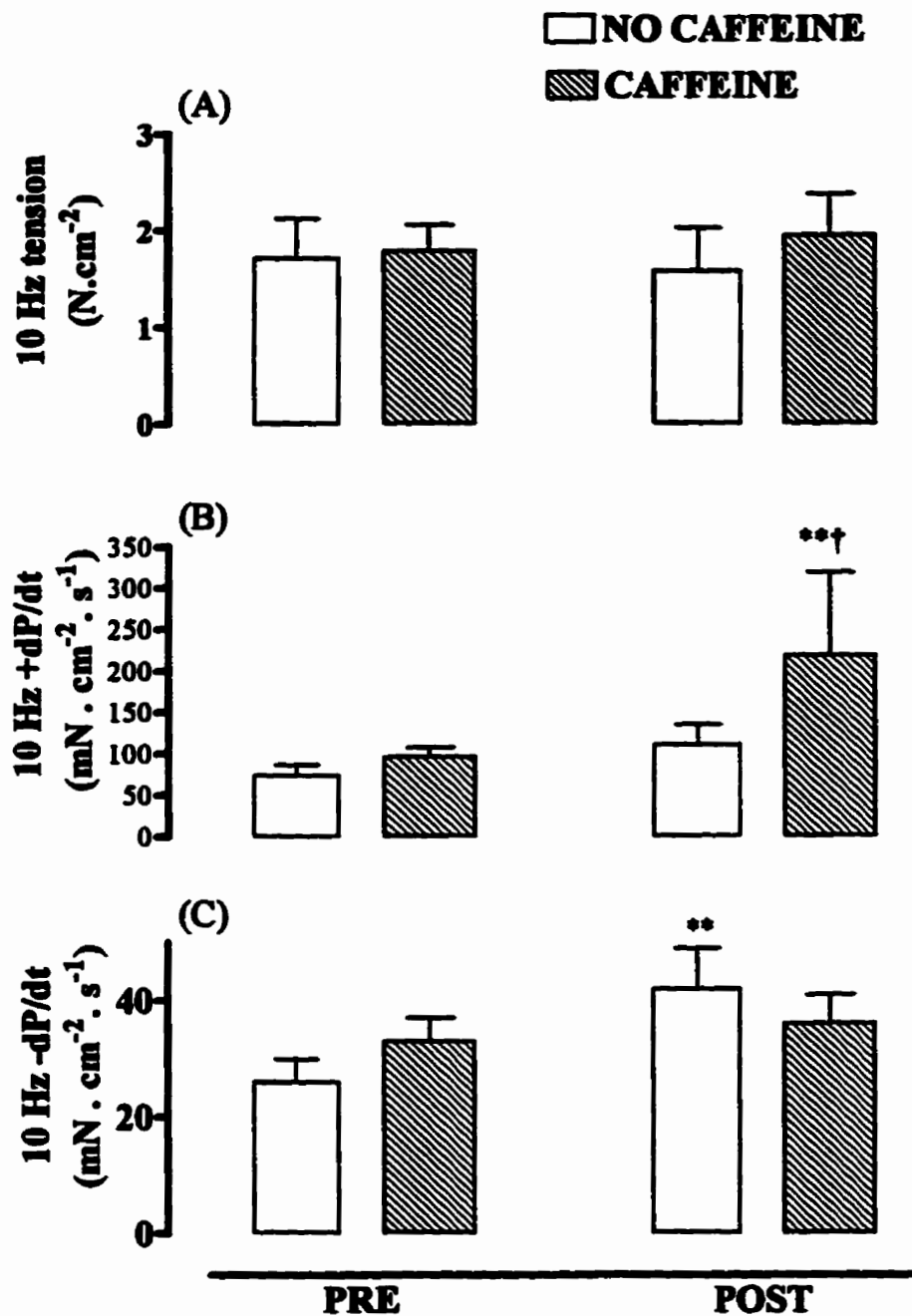
Figure A.1.C.7. Effect of caffeine after 20 min anoxic recovery (20MR) on 10 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. **‡** Significantly different from PRE ($P < 0.01$) † Significantly different from no-caffeine ($P < 0.05$).
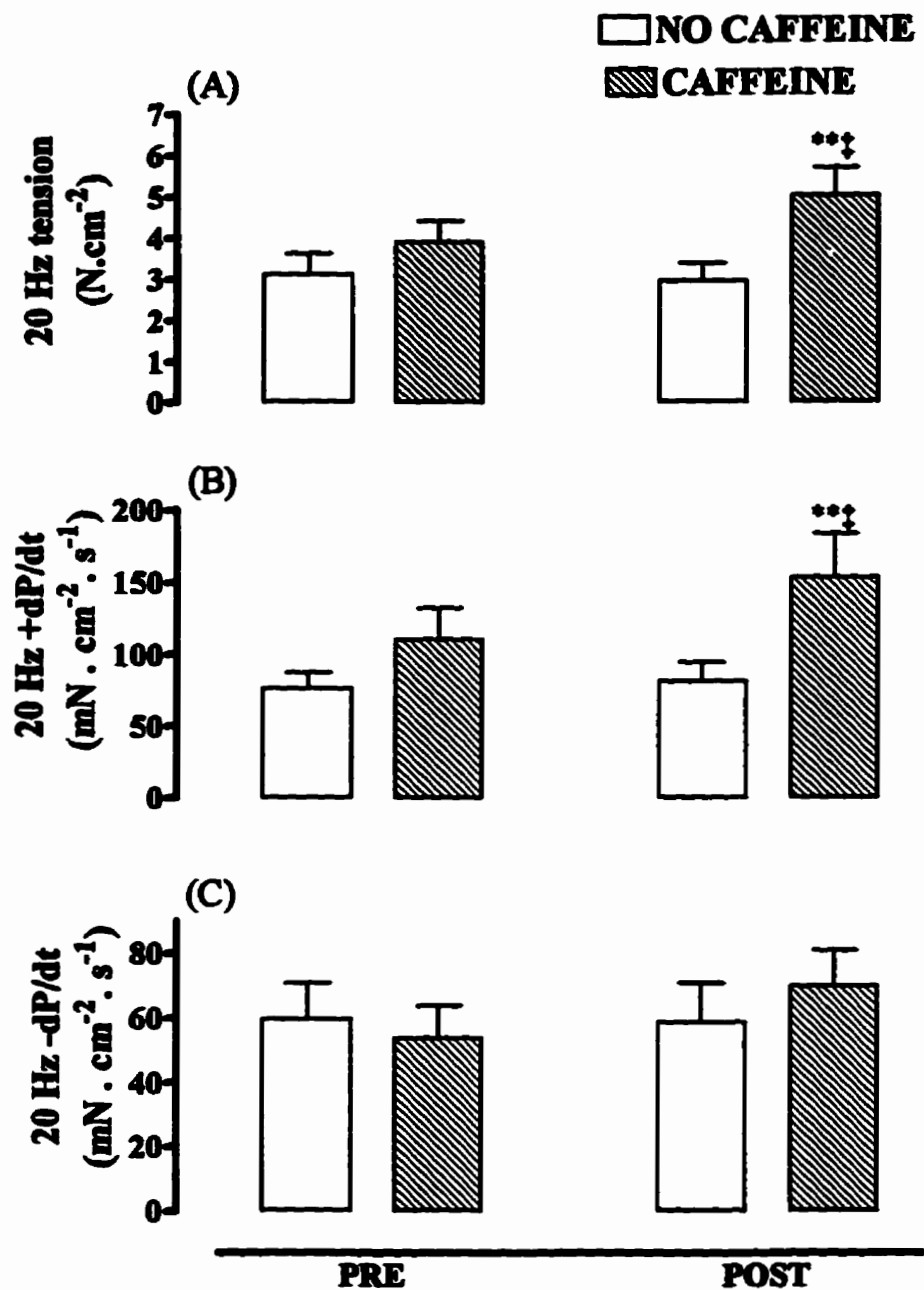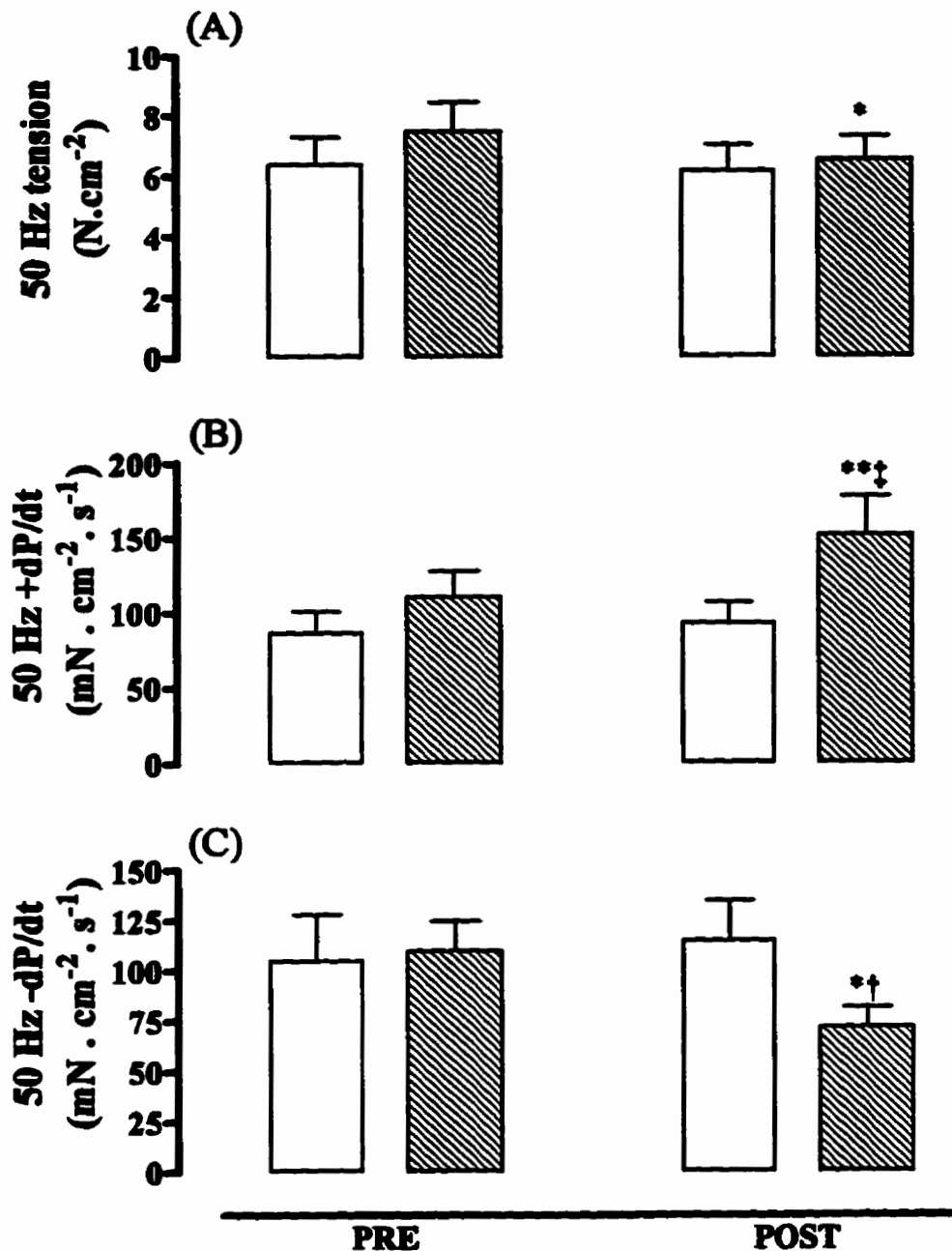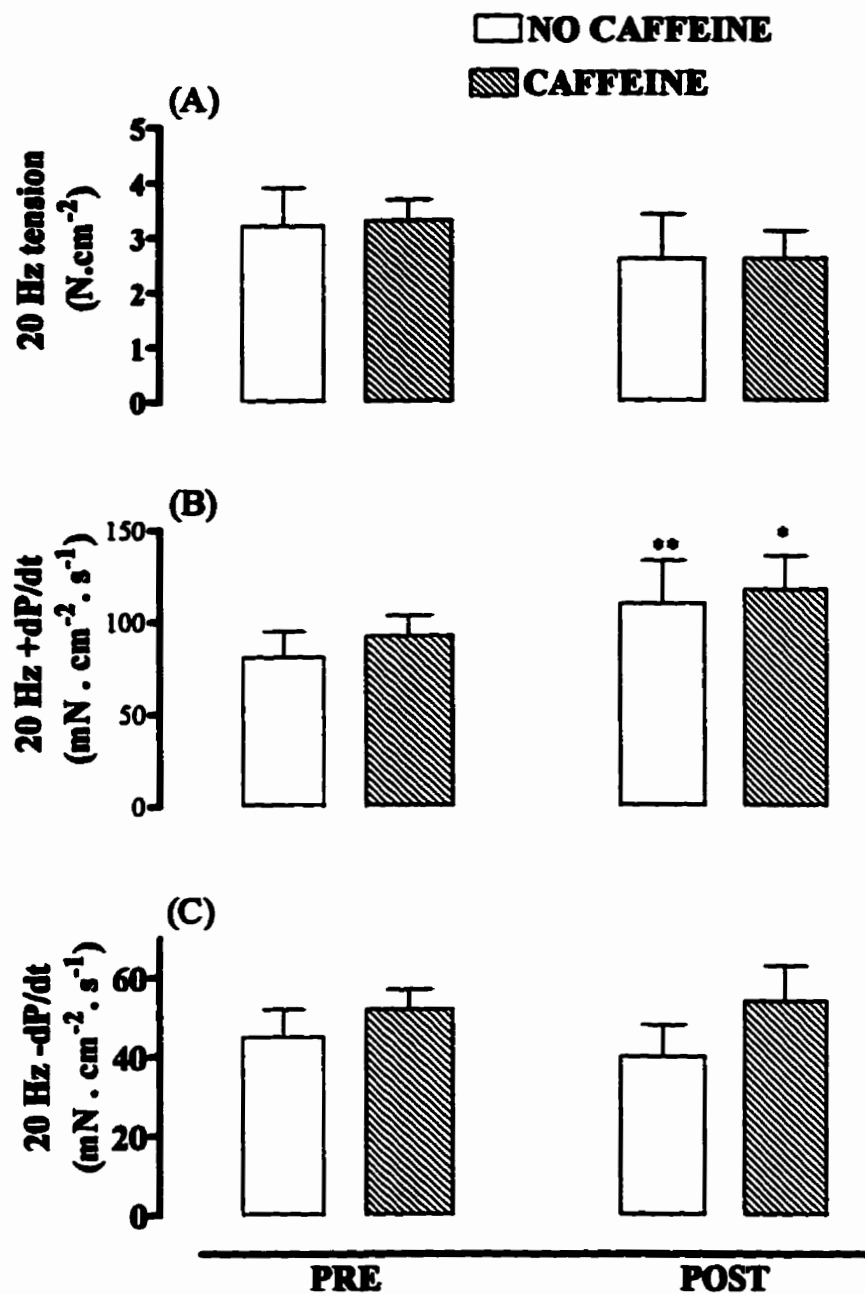
Figure A.1.C.8. Effect of caffeine after 20 min anoxic recovery (20MR) on 20 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE ($P < 0.01$),* ($P < 0.05$).
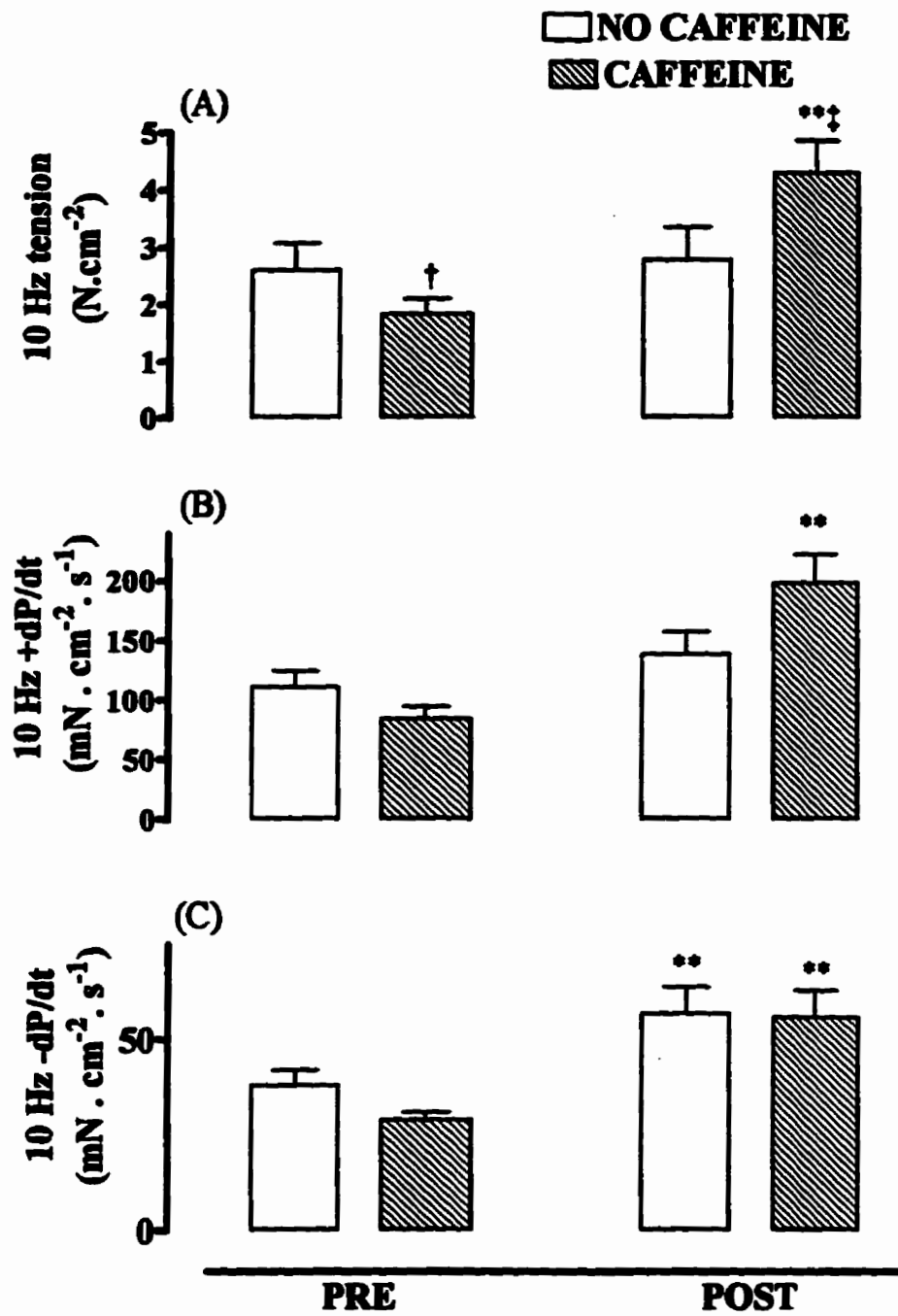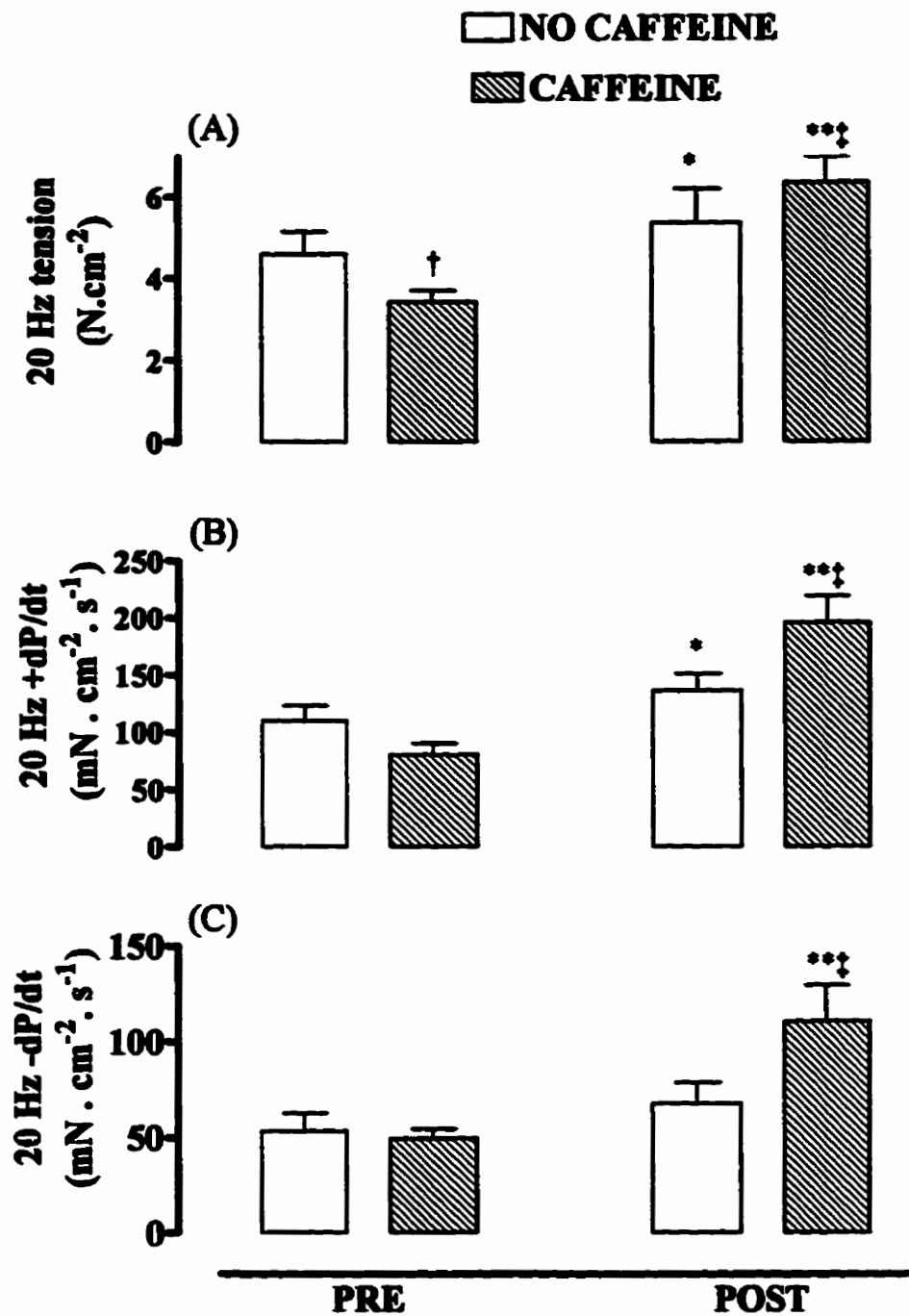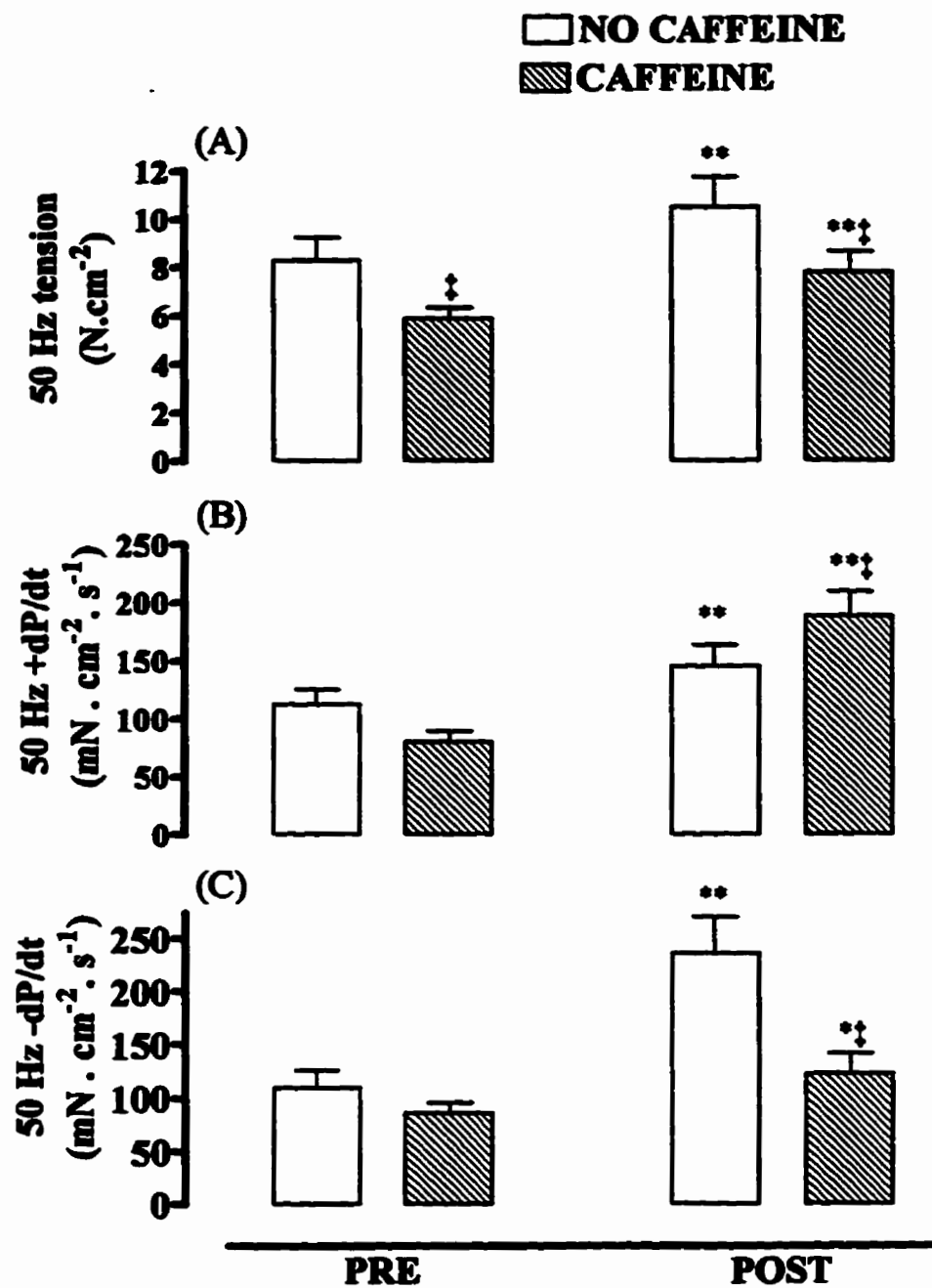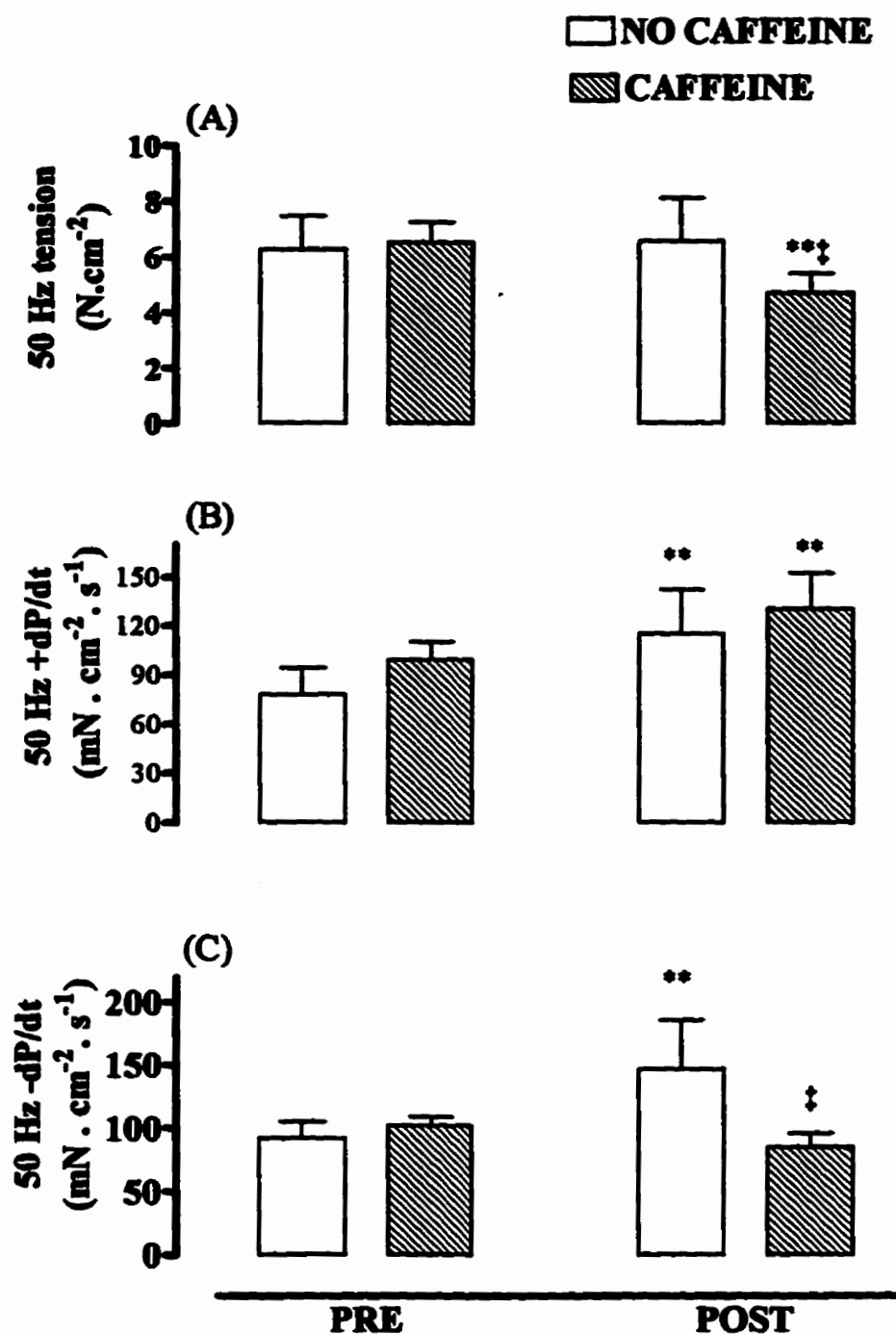
Figure A.1.C. 9. Effect of caffeine after 20 min anoxic recovery (20MR) on 50 Hz tension (A); rate of tension development (B); rate of tension decline (C). PRE & POST, before and after addition of caffeine and stimulation. Data are means ± SE. ** Significantly different from PRE ($P < 0.01$). ‡ Significantly different from no-caffeine ($P < 0.01$).

Appendix II

APPENDIX IIA: DETERMINATION OF FREE Ca^{2+} IN BUFFERS

The second study of this thesis was designed to examine the effect of Ca^{2+}, H^+, and ATP on the activity of the Ca^{2+}-Mg^{2+}-ATPase of different muscle fibre types using an hom preparation (Simonides & van Hardevelt, 1990). The *in vitro* assay with hom was used to control experimental conditions and therefore, isolate the specific action of Ca^{2+}, H^+, and ATP enzymatic activity. However, to precisely isolate the specific action of a metabolite, control of the other variables of the assay medium is critical, but not easily achieved.

For example, it is not adequate to examine the effect of H^+ on the kinetics of the enzyme, by simply comparing the activity of the enzyme before and after the additions of small volumes of a weak acid or base to the assay medium. Although, enzymatic activity may be altered if the change in pH is significant, it may be difficult to precisely determine the mechanism by which the H^+ affected this activity. The mechanism underlying the decrease in activity could be through a competitive inhibition between H^+ and ionizable groups of the high affinity Ca^{2+} binding sites or of the Mg^{2+} binding sites or of the nucleotide binding domain. However, a modification of the various association constants, mainly the Ca^{2+}- Ca^{2+}-ligand association constant, will also play a role by altering levels of $[Ca^{2+}]_f$ that is available for binding to the high affinity sites. This in turn will affect the affinity of the Ca^{2+}-Mg^{2+}-ATPase for Ca^{2+} and the cooperativity of Ca^{2+} binding. A Ca^{2+}-ligand system, as was learned through trials and errors during the course of this thesis (see below), is essential in achieving

some sort of control on the level of $[Ca^{2+}]_f$ in the assay buffer. However, choosing the proper Ca^{2+} buffer for a given set of conditions and attempting to control contamination by exogeneous Ca^{2+} sources are two parameters that must be given serious considerations in experiments where the control of $[Ca^{2+}]_f$ is an important factor.

Controlling the level of $[Ca^{2+}]_f$ is also critical if comparisons are to be made between the different conditions to which the assay medium is subjected to. The $[Ca^{2+}]_f$ resulting from a set total $[Ca^{2+}]$ is different at pH 6.4 and 7.6 and if no adjustments are made it is impossible to know for certain if the change in enzymatic activity observed with the variation in H^+, is due to the H^+ or to the change in $[Ca^{2+}]_f$. This appendix outlines some of the procedures that were attempted to control $[Ca^{2+}]_f$. Although, suggestions are made, the goal of this appendix is not to serve as a troubleshooting guide, but to make the experimenter aware of some of the potential problems that may be encountered when making Ca^{2+} buffers.

A.2.A.1 Determination of free Ca^{2+} ($[Ca^{2+}]_f$) in buffer solutions.

McGuigan et al. (1991) defined Ca^{2+} buffer solution, as a solution that resists changes in $[Ca^{2+}]_f$ on the addition of small amount of Ca^{2+}. The use of buffers of precise low $[Ca^{2+}]_f$ is important to study intracellular reactions where $[Ca^{2+}]_f$ are typically in the 100 nm range (Bers et al., 1994). It also essential in the calibration of Ca^{2+}-indicators used in fluorometry. Although, some excellent papers have provided detailed methods for the preparation of Ca^{2+} buffers (Tsien & Pozzan, 1989; McGuigan et al., 1991; Bers et al., 1994), it remains a tedious and sensitive task for a number of factors. Contamination, purity and specificity of the chelating agent, and

temperature, pH and ionic strength of the buffer are some of the factors that may posed potential problems when dealing with buffers of low $[Ca^{2+}]_f$.

A.2.A.1.1 Contamination

Contamination remains a major problem. The first source of contamination is the water used to make the buffers and rinsed the glassware. Laboratory distilled water could have trace contamination ranging from 500 nM to 3 μM (Bers et al, 1994). Figure A.2.A.1 shows the Ca^{2+}-activated ATPase activities in the absence of a Ca^{2+} chelator measured in vesicle fractions, isolated by a discontinuous sucrose gradient procedure (Rousseau et al., 1992). Despite the use of a Ca^{2+} software program to take in account the chemical environment (Kurzmack, unpublished) and the fact that the water was distilled, deionized and filtered through a water purification system (Barnstead | Thermolyne Corp., Iowa) and displayed the low resistivity of 18 M ohm . cm^{-1}, the activity was similar at the five different $[Ca^{2+}]_f$, suggesting that the level of $[Ca^{2+}]_f$ contamination was too elevated to obtain the desired levels of free Ca^{2+}.

Levels of 1-3 μM are typically found in "Ca^{2+} free solutions" (Bers et al., 1994) although certain investigators report levels of up to 15 μM (Forge et al., 1993a). Concentrations of up to 10 μM have been suggested to have no effect on calculated values of $[Ca^{2+}]_f$ (McGuigan et al., 1991). The contamination can be measured with a Ca^{2+} micro-electrode (Bers, 1982) or through the use of atomic absorption spectrometry (Bers et al., 1994). Using a commercial Ca^{2+} micro-electrode (Microelectrodes, Inc., Bedford, NH) a low contamination of approximately 0.9 μM was obtained in a solution made with water filtered through a Milli-Q Water Purification System (Millipore) (Figure A.2.A.2).

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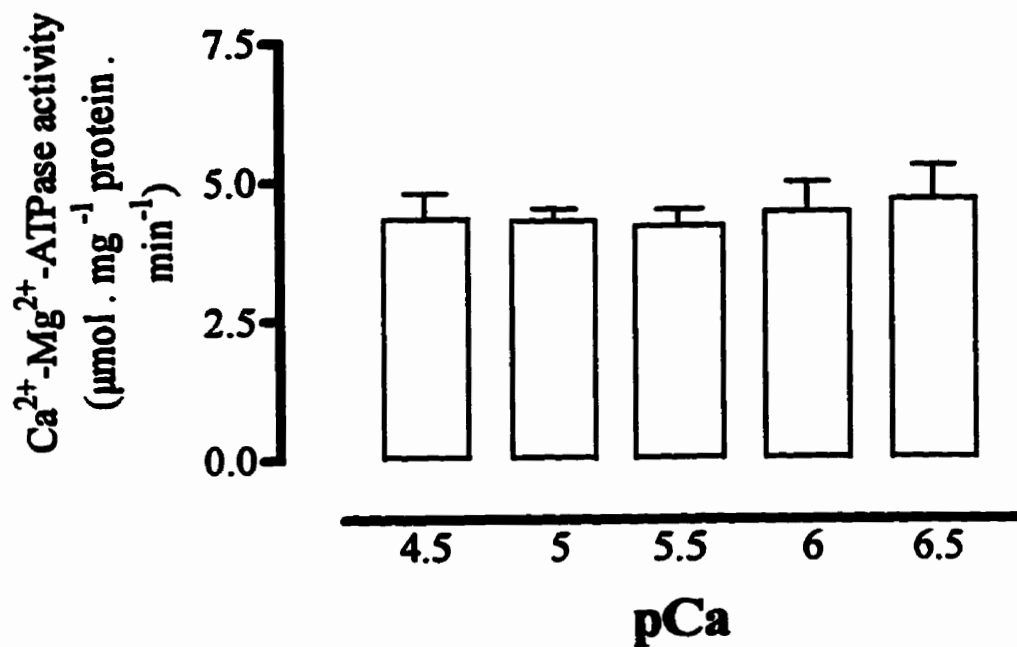
Figure A.2.A.1. Ca^{2+} dependency of Ca^{2+}-Mg^{2+}-ATPase activity in vesicle fractions of red muscles (rat gastrocnemius and vastus, n = 4) isolated on a discontinuous sucrose gradient. Ca^{2+}-Mg^{2+}-ATPase assay contained no Ca^{2+} buffer.

This type of commercial micro-electrode is robust and give similar results to commercial macro-electrode. However, its sensitivity is down to approximately $[Ca^{2+}]_f$ of 700 nM..

Atomic absorption spectroscopy uses extremely high temperatures (3600°C) to ionize the compound of interest and make it sensitive to spectrometry. However, problems with contamination appeared to be present during this procedure since it was impossible to obtain baseline values, even if a new graphite chamber was used.. Rinsing the graphite chamber with a solution containing a Ca^{2+} chelator and burning the chamber many times helped in achieving a somewhat reproducible baseline. Because of the high fluctuations observed between the trials, the data were of limited value.

Contamination can also occur through Ca^{2+} leaching from the glassware, especially if the containers are use for long term storage of the buffer solutions and to reduce this leaching it is recommended to use plasticware (Bers et al., 1994). It is recommended to soak glassware for 24 h in commercial detergent (see McGuigan et al., 1991) or laboratory-made solutions containing Ca^{2+} chelators. The solution that was used with plasticware was composed of a mixture of EGTA (1mM) and EDTA (3mM) (Meissner et al., 1973). The plasticware was rinsed at least 5 times with volume of ultra-pure water to eliminate the chelator or soap.

A.2.A.1.2 Choice of the Ca^{2+} chelator to make buffer

Two issues are of critical importance in the choice of the chelator used to make the buffer solution. First, choosing the appropriate chelator based on the level of $[Ca^{2+}]_f$ desired and the pH of the buffer. Second, and this according to some investigators (Miller & Smith, 1984; Schoenmakers, personal communication) may represent the single most important factor: the purity of the specific stock of chelator used to make

the buffer solution. A third issue should also be given some considerations, the possibility that the chelator will have a direct effect on one of the processes that is being studied (Bers et al., 1994).

Similar to pH, where the choice of an appropriate buffer is important, the choice of an appropriate Ca^{2+} chelator to buffer Ca^{2+} is critical. For pH buffering, the pK_a of the buffer should be closed to the desired working pH. For a Ca^{2+} buffer, the dissociation constant (K_d) should be closed to the desired level of $[Ca^{2+}]_f$ since the buffer will be more effective in absorbing and releasing ions and therefore maintaining the desired concentration (Bers et al., 1994).

Bers et al. (1994) suggest that the K_d should not differ from the desired $[Ca^{2+}]$ by more than a factor of 10. The second criteria is the selectivity of the chelator for the specific ion over other ions present in the medium. Ethylene glycol bis(ß-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) is one of the most commonly used chelator to buffer Ca^{2+} . It fulfills the above criteria by displaying at pH 7, an apparent dissociation constant that is closed to the $[Ca^{2+}]_i$ (0.4μM) and a selectivity for Ca^{2+} over Mg^{2+} that is 10^5 higher (Bers et al., 1994). This is especially important for work in cells where Mg^{2+} is 10,000 times more abundant than Ca^{2+} (Bers et al., 1994).

For these reasons and because the effects of other chelators on the Ca^{2+}-Mg^{2+}-ATPase activity are not well documented, EGTA was the chelator chosen to buffer solutions used in the experiments presented in this thesis. At a pH of 7.0, EGTA proved to be a good buffer over pCa values ranging from 5.9 to 6.9, but outside of that range the buffering is poor (Blinks et al., 1982).

The loops on the calibration curves demonstrate the poor capacity of EGTA between pCa values between 4 to 6 at pH 7 (See Figure A.2.A.2) The $[Ca^{2+}]_f$ in the area of the loop are very different than the initial chosen $[Ca^{2+}]_f$ levels. For example, the electrode potential recorded for 10^{-5} M (10000 nM) suggest that the concentration of $[Ca^{2+}]_f$ is actually 29700 nM. If we look at 2×10^{-6} M (2000 nM) a value outside the loop and in the range measured by the electrode, the $[Ca^{2+}]_f$ based on the electrode reading is similar to the actual value.

A solution to prevent this loop is to mix chelators that have buffering capacity over a wide range of $[Ca^{2+}]_f$ (Schoenmakers et al., 1992). This was attempted on two occasions by adding 0.5 mM of NTA to 0.5 mM of EGTA and in this particular case an even bigger loop was obtained. Although, this approach was unsuccessful, it warrants to be attempted again in the future. Schoenmakers et al. (1992) obtained accurate $[Ca^{2+}]_i$ with a buffer containing 0.5 mM of EGTA, HEDTA and NTA, and 3 mM of ATP and a high correlation with values measured by a commercial Ca^{2+} selective electrode (RH 44/2-SD/1; Philips) and with values obtained fluorometrically with Fura-2. The drawback, as mentioned above, is that these others Ca^{2+} chelators could have a direct effect on the Ca^{2+}-Mg^{2+}-ATPase.

The choice of a chelator must also take in considerations other characteristics of the medium. At alkaline pH, EGTA gives erratic measurements, because its affinity for Ca^{2+} is too high, even higher than that of the Ca^{2+}-Mg^{2+}-ATPase (Forge et al., 1993a). As was mentioned above for the micro-electrode measurement of $[Ca^{2+}]_f$, a slight error in total Ca^{2+} or EGTA will alter the binding curve. Underestimating total Ca^{2+} leads to an underestimation of $[Ca^{2+}]_f$ which in turn will increase the apparent affinity of

the Ca^{2+}-Mg^{2+}-ATPase and the cooperativity of Ca^{2+} binding (Forge et al., 1993a). The following example provided by Forge et al. (1993a) illustrates the importance of this effect:

"For instance, say that at pH 8, ATPase display a half-saturation concentration of 0.1 μM and a binding cooperativity of 1.3, an underestimation of total Ca^{2+} of 100 μM instead of 110 μM will lead to a binding curve displaying a half-saturation concentration of 0.3 μM and a binding cooperativity as high as 3.5"

One can see that under such circumstances, contaminating amount of Ca^{2+} could have catastrophic effects on the $[Ca^{2+}]_f$. The situation has now been resolved by using another chelator, derived from EGTA, called 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (Forge et al., 1993a). BAPTA's affinity for Ca^{2+} is lower than the affinity for the Ca^{2+}-Mg^{2+}-ATPase and its affinity is constant for pH values over 7 (Tsien, 1980). (Note: BAPTA is approximately 40 times more expensive than EGTA).

The second issue of major importance is the determination of the purity of the specific stock of chelator used to make the buffer solution. Although, a value of purity is provided by the manufacturer, this value tends to be different than the actual purity. In the above situation where the concentrations of EGTA and total Ca^{2+} are similar, a lower purity will have important consequences on the resulting levels of $[Ca^{2+}]_f$.

Bers (1982) proposed a way to determine $[Ca^{2+}]_f$ in solutions using the Ca^{2+} mini- electrode. The method is used to precisely calibrate the Ca^{2+} electrode beyond the Nerstian range. The method determines the purity of the EGTA and the precise value of the Ca-EGTA binding constant and uses those values to obtain more accurate $[Ca^{2+}]_f$.

Free Ca^{2+} is first measured in a solution similar to the actual buffer (HEPES, KCl and NaN$_3$) but without EGTA to check if the electrode is responding according to the Nerstian slope (29.5 mV at 25° C and 30.7 mV at 37° C /pCa unit. The average slope of the curve in Figure A.2.A.2. is -26.5 mV but the slope (-28.6) between the two highest concentrations (10 mM and 1 mM) was used instead of the average. The measurements are made at 37° C, pH 7 and at a ionic strength of 0.210, representing the ionic strength of the monovalent ions only (Schoenmakers, 1992) which in the specific buffer used during the experiments of this thesis included KCl and NaN$_3$.

A second calibration, is done under the same conditions but in the presence of EGTA (1 mM) (Figure A.2.A.2). The concentration of 1 mM is based on the assumption that EGTA is 100 % pure. The software spreadsheet program of Bers uses an association constant for Ca^{2+}-EGTA of log K = 6.4634. Corrections to this association constant are made for temperature, ionic strength and pH (Harrison & Bers, 1989)

The software spreadsheet program (A.2.A.3) provided by Bers (Bers et al., 1994) uses the potential values measured by the electrode (assuming that the electrode is behaving ideally: 30.7 mV per pCa unit at 37° C) at the various $[Ca^{2+}]_f$ and the slope of the curve obtained from the calibration in non-EGTA solutions to recalculate the $[Ca^{2+}]_f$. For example at 7×10^{-7} M, the voltage is 27.9 mV. At 10^{-3} M the potential is 110.8 mV. So - 83.3 (-111.2 mV-(-27.9 mV) is divided by -28.6 mV giving 2.912 pCa units. This means that the concentration at this voltage is 3 + 2.912 = 5.912 pCa units. This represents a concentration of 1224×10^{-7} M instead of the initial concentration of 7×10^{-7} M.

Figure A.2.A .2. Semi-logarithmic plot of Ca^{2+} electrode calibration without and with EGTA (1mM).

Figure A.2.A.3. Spreadsheet for calibration calculations (Bers et, 1994)

Ca Calibration
3/22/93

Solution Conditions
For Entry of Free [Ca]

7 pH
0.21 M Ionic Equiv (0.5*sum z_i, C_i)
37 degrees C
1 mM EGTA
40 ml bottle

Kd= 3.44E-07 M   or   0.3440 uM

K'Ca Calculation (see A32..G47)
B= 5.769515
Gamma H= 0.738823
Log [H] = -6.86854
[H] (M) = 1.35E-07
K'Ca Assn= 2907311   Log K'Ca= 6.463492
K'Ca Dissn=3.44E-07

H activity coefficient
Range for Linear Regression for
Scatchard Must be selected "/DR"
6.299464=Log K'Ca from Scatchard
5.02E-07 =K'Ca Dissociation from Scatchard

Regression Analysis (see I32..M40)
0.960126[EGTA]tot (mM)
0.98395 r^2

Initial pCa	Ca-free (nM)	Ca-total (mM)	ml 100 mM CaCl2	V-Ca (mV)	Ca-free (M)	Ca-free (nM)	Ca-Bound (mM)	B/F	Regressn line B/F	inter mediate	Recalculated (nM)	pCa
7.0000	100	0.225268	0.090107	13.7	3.90E-07	389.8604	0.224878	576.8167	1465.203	-0.000735	153.6896	6.8134
6.6990	200	0.367747	0.147099	18.1	5.56E-07	555.5911	0.367192	660.9029	1181.6	-0.000593	311.0932	6.5071
6.3979	400	0.537878	0.215151	23.7	8.72E-07	872.0863	0.537006	615.7716	843.1945	-0.000423	637.5025	6.1955
6.1549	700	0.670993	0.268397	27.9	1.22E-06	1222.961	0.66977	578.6225	578.6225	-0.00029	1157.897	5.9363
6.0000	1000	0.744814	0.297925	31.7	1.66E-06	1660.656	0.743153	447.5056	432.3848	-0.000216	1718.142	5.7649
5.6990	2000	0.854963	0.341985	39.6	3.14E-06	3136.92	0.851826	271.5486	215.8205	-0.000106	3915.179	5.4072
5.3979	4000	0.924502	0.369801	52.7	9.01E-06	9006.28	0.915496	101.6508	88.94018	-3.61E-05	10047.32	4.9979
5.1549	7000	0.959836	0.383935	64.7	2.37E-05	23666.06	0.93617	39.5575	47.73989	-7.92E-07	21554.18	4.6665
5.0000	10000	0.976415	0.390566	70.3	3.71E-05	37147.54	0.939268	25.28479	41.56745	1.58E-05	31394.06	4.5032
4.3979	40000	1.031133	0.412453	80.6	8.51E-05	85127.51	0.946006	11.11281	28.13971	7.05E-05	77206.87	4.1123
4.0000	100000	1.096229	0.438492	87.2	1.45E-04	144823.5	0.951406	6.569414	17.37839	0.000136	139543.3	3.8553
3.0000	1000000	1.999312	0.799725	110.9	9.76E-04	976136.4	1.023176	1.04819	-125.645	0.001039	1039649	2.9831

10 mM   10 mM   139.8
1 mM   1 mM   111.2
100 uM   100 uM   86.8
                    26.5
Avg slope=   28.6
Slope (mV)=   111.2
mV offset at 1 mM Ca=

From Regression
[EGTA]tot   K-Ca-EGTA
0.960126mM   1.9928x 10^6 /M
96.0196% pure 6.299464= log K

Temperature and Ionic Strength Correction

	Std Cond	Ionic Str incl T eff	Final 37 Deg C	Final		Delta H kcal/mol	valance 2*x*y
Temp	20						
I-Eq	0.100	0.210	0.210 M				
Stoich Const	Log K	Log K'	Log K'	K' (M)			
K1	9.47	9.247445	9.010245	1.02E+09		-5.8	8
K2	8.85	8.683084	8.445884	2.79E+08		-5.8	6
K3	2.66	2.548722	2.548722	353.7712		0	4
K4	2	1.944361	1.944361	87.97539		0	2
KCa	10.97	10.52489	10.19363	1.56E+10		-8.1	16
KCa2	5.3	4.966167	4.966167	92505.45		0	12
Log f	0.109225	0.137044					
Temp	293	310					
A	0.507424	0.523568					
epsil	80.1057	74.14832					

Regression Output:

Constant   1913.34 Y intercept
Std Err of Y Est   90.91
R Squared   0.98395
No. of Observations   8
Degrees of Freedom   6

X Coefficient(s) -1992.8/mM=-K'Ca (slope)
Std Err of Coef.   103.9

Next, Bers estimates the apparent association constant (K_d*) and the purity of the EGTA. From the total Ca^{2+} added to the solution and these recalculated $[Ca^{2+}]_f$, the bound Ca^{2+} is determined. A Scatchard plot which examines the relationship between bound and the ratio of bound to $[Ca^{2+}]_f$ is made (Figure A.2.A.4) The slope (-1993 . mM^{-1}) of this plot yields the apparent binding constant whereas the intercept of the x axis divided by the slope provides the purity of the EGTA (96 %). Using the new association constant and purity, the $[Ca^{2+}]_f$ concentrations are readjusted. For example, the initial concentration of $7 \times 10(-7)$ M (700 nM) was estimated at 1223 nM from the electrode reading. The $[Ca^{2+}]_f$ recalculated using the new association constant and purity, becomes 1158 nM.

Although simple to apply, the method of Bers is not without problems. First, other than the known problems associated with Scatchard plots (violation of the assumptions of linear regression) only the linear portion of the curve must be used to determine the regression equation. In practice, choosing the linear portion of the curve is difficult, especially in the presence of the loop described above. In the above example, the loop was not too pronounced, probably because of the low Ca^{2+} contamination contained in the water, and 8 values of the Scatchard plot ($r^2 = 0.99$) were chosen for the regression analysis. If the values of bound/free and bound Ca^{2+} at 7×10^{-7} M were ignored for the regression analysis, the slope became -2196 . mM^{-1} and the purity (95.6 %). If instead, the values at 10^{-4} were ignored than the slope was -1978 and the purity 96.2 %. Although, both estimates of purity determined with 7 values are closed to the purity determined with 8 values, this is not always the case.

Figure A.2.A.4 Scatchard Plot Ca-EGTA
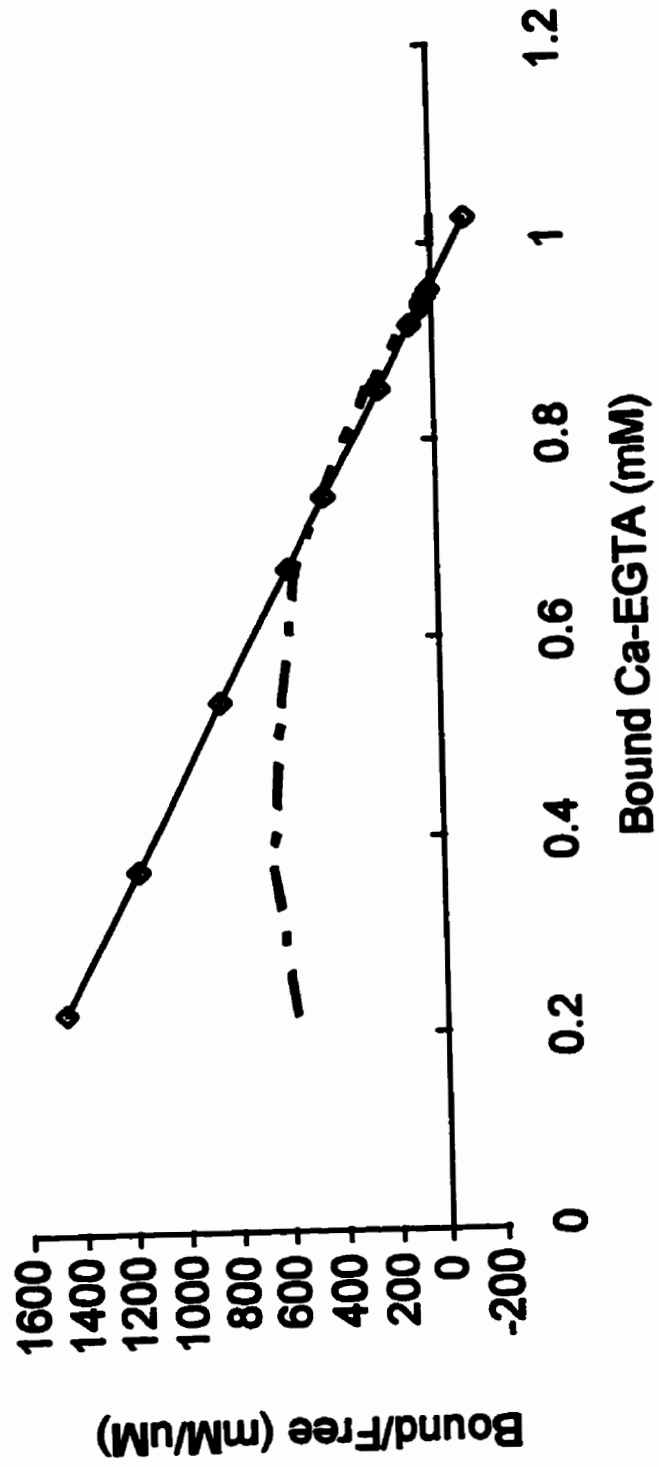
Perhaps the most important limitation of Bers's method and the software spreadsheet program is that it does not allow the determination of the purity of the chelator in a solution that mimics closely the actual buffer used to run the assay. Magnesium, for example, is not considered by Bers in the determination of the total Ca^{2+} in the presence of EGTA. The 15 mM of Mg^{2+} used in the assay of Simonides and van Harveveld (1990) is likely to affect the $[Ca^{2+}]_f$ of the medium by binding EGTA and therefore leaving less EGTA available for binding Ca^{2+}. It is suggested that the calibration curve determined from the recalculated value of $[Ca^{2+}]_f$ be used to determine the potential values of the desired $[Ca^{2+}]_f$. The potential obtained from the buffers with ATP and PEP can then be readjusted by adding either ATP or EGTA to match the potentials derived from the calibration curve.

Although this appears as a good idea in theory, it does not work very well in practice. The presence of other chemicals than EGTA in the buffer reduces the sensitivity and response time of the micro-electrode to $[Ca^{2+}]_f$, making it difficult if not impossible to accurately titrate the buffer and reach the potential value established previously in the buffer containing only EGTA.

A.2.A.2 Recommendations for the determination of $[Ca^{2+}]_f$ in buffer solutions

In addition to the to the precautions mentioned above, the following recommendations are suggested.

1. The use of a more sensitive electrode to measure lower $[Ca^{2+}]_f$ would be helpful in calibrating the Ca^{2+} buffer solutions and determining levels of contamination. In an EGTA buffer, the portion of the curve above the loop

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(lower $[Ca^{2+}]_f$) could possibly be avoided, increasing the accuracy of the method of Bers (1982).

2. Micro-electrodes made from polyethylene tubes (Hove-Madsen & Bers, 1992; 1993; Bers et al., 1994) in the absence of proteins demonstrate a Nerstian response (28.5 ± 0.3 mV/decade) down to a $[Ca^{2+}]_f$ of 30 nM for more than two weeks (Hove-Madsen & Bers, 1992). The electrode also drift less than 0.1 mV/h and the response is 95 % complete in less than 1 min above $[Ca^{2+}]_f$ of 30 nM.

3. A second recommendation that was already discussed above is to use more than one chelator in the buffer to avoid change in $[Ca^{2+}]_f$ at concentrations that are close to saturation. Blink et al. (1982) suggest not to use a single Ca^{2+}-buffer system over more than one log unit of $[Ca^{2+}]$. The presence of more than one chelator should also reduce the effect of slight day to day variations in total Ca^{2+} (contamination) and pH, and prevent, or at least reduce the kink found in Ca^{2+} solutions buffered with only EGTA (Bers, personal communication).

4. The purity of commercial EGTA could also be increased by solvent extraction and recrystallization (Randhawa, personal communication).

5. A good pH electrode is essential since small variations in pH will have enormous effect on the K_d^* of the chelator for Ca^{2+}. In buffers using EGTA the pH must be controlled very carefully, since EGTA is especially

dependent upon pH (Tsien & Pozzan, 1989; Bers et al., 1994). This is why the pH was readjusted immediately after the addition of Ca^{2+} during the dependency experiments (see Chapter III). It is advisable that a pH electrode be set-up exclusively for the purpose of making the Ca^{2+} buffers. The pH is temperature dependent and therefore the solution must be calibrated at the temperature at which the experiment is conducted (Tsien & Pozzan, 1989).

6. The measurement of the K_d^* of EGTA for Ca^{2+} in the actual Ca^{2+} buffer used for the Ca^{2+}-Mg^{2+}-ATPase assay with the method of Bers may be too complicated and impractical because of the high number of variables (Bers, personal communication) and the loss of sensitivity in the presence of other chemicals or organic material. Proteins, for example has been showed to affect the K_d^* of indo-1 for Ca^{2+}, by binding to the Ca^{2+} fluorometric dye (Hove-Madsen & Bers, 1992). The shift in K_d^* becomes more pronounced at high $[Ca^{2+}]_f$.

7. Ca^{2+} software programs correct published stability constants to reflect specific experimental conditions (pH, temperature and ionic strength). The use of Ca^{2+} software programs remains a "black box" approach, but with increasing numbers of chelators, the equilibrium calculations are very complex. Although, Ca^{2+} software programs provide H^+ and Ca^{2+} binding constants for most of the commonly used Ca^{2+} chelators, some software programs (Chelator, Schoenmakers et al., 1992; MaxC, Bers et al., 1994) allow the possibility of entering new ligands. These ligands may not bind

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Ca^{2+} directly but may affect the composition of the buffers, by modifying pH or the ionic strength of the medium.

APPENDIX IIB: Macro in Visual Basic for analysis of Ca^{2+}-Mg^{2+}-ATPase data

Macro (JRmacro) written by Paul Guy in Visual Basic on Micro-soft Excell to analyse through linear regression Ca^{2+}-Mg^{2+}-ATPase data obtained from UVPC Kinetics Personal Software version 2.5 (Shimadzu, Scientific Instruments, Inc., 1994).

```
Option Explicit
Sub try3()

    Dim currentcell As Object
    Dim expcol As Integer, exprow As Integer, datasheet As Variant, resultsheet As Variant,
            yourbook As Variant
    Dim regsheet As Variant, resultstitle As Variant, moveover As Integer, dataendrow As Integer
    Dim timerange As Object, absrange As Object, timeaddress As Variant, absaddress As Variant.
     resultsrow As Integer
    Dim macrostring As Variant, resultscolumn As Integer

    'set everything up, initialize
    moveover = 0     'use this to move to right # of columns to next set of data

    'get a title
    resultstitle = InputBox("Enter title for this set of data", "auto-thing", "junk")

    'pick up trial number on data sheet (you select this point to start)
    'let's use this as the start point, and determine things using this point as a reference
    Set currentcell = ActiveCell     'save start position
    expcol = ActiveCell.Column       'position of exp #
    exprow = ActiveCell.Row
    datasheet = ActiveSheet.Name
    yourbook = ActiveWorkbook.Name

     'define a new sheet for results, and define place for results. Place title and date on this result
     sheet
    resultsrow = 1
    resultsheet = "Sheet2"
    Sheets(resultsheet).Select

    resultscolumn = 1                          'start results in column 1
    While Not (IsEmpty(Cells(resultsrow, resultscolumn)) And (IsEmpty(Cells(resultsrow + 1,
     resultscolumn))))  'if there's already something there, shift over
        resultsrow = resultsrow + 1
    Wend

    resultsrow = resultsrow + 1
    Cells(resultsrow, resultscolumn).Value = resultstitle
    Cells(resultsrow, resultscolumn + 1).Value = yourbook
    Cells(resultsrow, resultscolumn + 2).Value = Time
    Cells(resultsrow, resultscolumn + 3).Value = Date
    resultsrow = resultsrow + 1
    Cells(resultsrow, resultscolumn).Value = "exp. #"
    Cells(resultsrow, resultscolumn + 1).Value = "intrcpt"
```

```vba
Cells(resultsrow, resultscolumn + 2).Value = "slope"
Cells(resultsrow, resultscolumn + 3).Value = "slope err"
resultsrow = resultsrow + 1


'copy current exp # to result sheet
  repeat: Sheets(datasheet).Select
Cells(exprow, expcol + moveover).Select
If IsEmpty(Cells(exprow, expcol + moveover)) Then GoTo finished
Selection.Copy
Sheets(resultsheet).Select
Cells(resultsrow, resultscolumn).Select
ActiveSheet.Paste



'select time and ABS data
Sheets(datasheet).Select
Cells(exprow + 2, expcol - 2).Select
Selection.End(xlDown).Select                    'drop to bottom of data (same as cntrl down
                                                 arrow)
dataendrow = ActiveCell.Row
Set timerange = Range(Cells(exprow + 2, expcol - 2), Cells(dataendrow, expcol - 2))    'excel
  range object
Set absrange = Range(Cells(exprow + 2, expcol - 1), Cells(dataendrow, expcol - 1))     'excel
  range object
timeaddress = timerange.Address(ReferenceStyle:=xlR1C1)    'get string of range in R1C1
                                                            format
absaddress = absrange.Address(ReferenceStyle:=xlR1C1)     'get string of range in R1C1 format



'run regression
Sheets(datasheet).Select
'make up macro string
macrostring = "REGRESS([" + yourbook + "]" + datasheet + "!" + absaddress + ",  [" +
  yourbook + "]" + datasheet + "!" + timeaddress
macrostring = macrostring + ", FALSE, FALSE, , """", FALSE, FALSE, FALSE, FALSE,
  , FALSE, )"
'do it
Application.ExecuteExcel4Macro String:=macrostring

'Application.ExecuteExcel4Macro String:= _
'   "REGRESS([R31406.XLS]Sheet1!R5C3:R24C3,  [R31406.XLS]Sheet1!R5C2:R24C2,
 FALSE, FALSE, , """", FALSE, FALSE, FALSE, FALSE, , FALSE, )"
regsheet = ActiveSheet.Name



'pick out y intercept
Range("B17").Select
Selection.Copy
Application.CutCopyMode = False
Selection.Copy

'copy it to sheet 2
Sheets("Sheet2").Select
Cells(resultsrow, resultscolumn + 1).Select
```

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```
ActiveSheet.Paste

'pick out slope
Sheets(regsheet).Select
Range("B18").Select
Application.CutCopyMode = False
Selection.Copy

'copy it to sheet 2
Sheets("Sheet2").Select
Cells(resultsrow, resultscolumn + 2).Select
ActiveSheet.Paste

'pick out slope error
Sheets(regsheet).Select
Range("C18").Select
Application.CutCopyMode = False
Selection.Copy

'copy it to sheet 2
Sheets("Sheet2").Select
Cells(resultsrow, resultscolumn + 3).Select
ActiveSheet.Paste


'clean out regression sheet
Application.DisplayAlerts = False
Sheets(regsheet).Delete
Application.DisplayAlerts = True

'do next set of data (loop back to repeat line)
resultsrow = resultsrow + 1     'new row for results
expcol = expcol + 3             'move over to the next set of input data
GoTo repeat



finished:  'this is the end
    currentcell.Select          'go back to original start cell
End Sub
```