The role of Calcineurin in skeletal muscle differentiation

By

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Contents

ABSTRACT

1. Strong evidence suggests that Calcineurin levels are higher in fast muscle fibers compared to slow-twitch in resting skeletal muscles. Activation of the Calcineurin in L_6 skeletal muscle myocytes selectively up-regulates slow-fiber-specific gene promoters through a mechanism involving the transcription factor NFATc1. The Calcineurin pathway itself was down-regulated when rat skeletal muscles were chronically stimulated at 10 Hz for a period of 3 weeks illustrating adaptation.
2. Skeletal muscles that received chronic stimulation treatment showed a significant increase in mitochondrial content. Histochemical studies detected a change towards the slow phenotype, through the decrease of fast-twitch Type IIb fiber content in fast skeletal muscles. Metabolic activity was not significantly affected through this period of chronic stimulation.
3. Cyclosporin A was not able to prevent this initial transition towards the slow phenotype, even though 3 weeks of 10 Hz chronic stimulation was insufficient to cause marked changes in the skeletal muscle metabolism. This suggests an incomplete fast-to-slow transformation was elicited by these conditions.
4. Stimulation of L6 myocytes with the calcium ionophore 4-Bromo-A23187 (10^{-6} M) a partial fast-to-slow transformation occurred. It is likely that this change was brought about by a number of processes including NFAT translocation to the nucleus.

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Abbreviations

$[Ca^{2+}]_i$	Free intracellular calcium ion concentration
AP-1	Activator protein 1 (c-fos and c-jun).
CaM	Calmodulin
CamK	Ca^{2+}/calmodulin dependent protein kinase
CnA	Calcineurin A
CnB	Calcineurin B
CsA	Cyclosporin A
ERK	Extracellular signal regulated kinase
GATA2	Skeletal muscle transcription factor binding a GATA DNA sequence
GM-CSF	Granular macrophage colony-stimulating factor
G-protein	Guanine nucleotide-bindinG-protein
GSK-3	Glycogen synthase kinase-3
IFN-g	Interferon-g
IGF	Insulin like growth factor
IL-2/4/5/13	Interleukin-2/4/5/13
Jak/STAT	Janus kinase/signal transducers and activators of transcription
JNK	N-terminal kinase
MAPK	Mitogen activated protein kinases
MEF2	Myogenic enhancing factor 2
MHC	Myosin heavy chain
MLC	Myosin light chain
MRF	Muscle regulatory factor
MyoD	Myogenic regulatory factor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
PIP_2	Phosphatidylinositol 4,5 bisphoshate
PKC	Protein kinase C

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DECLARATION

This Thesis is an original and authentic piece of work produced in fulfilment of my Masters degree regulations. I have fully acknowledged and referenced all my secondary sources. This thesis has not been submitted in whole or part for assessment in another module at this or any other University. I have read the Academic Regulations and I am fully aware of the potential consequences of any breach of them.

Signed.....PANAGIOTIS MANOLOPOULOS

Date: .....

CHAPTER 1- INTRODUCTION

CHAPTER 1- Introduction

1.1 Muscle Structure and Histology

1.1.1 Muscle microstructure and macrostructure

For a human, as for other animals, to move is to survive. Apart from thinking, every human activity requires a movement, or at least a muscle contraction, whether it is for walking, running, swimming, and breathing, or even standing still (McComas, 1996). The pumping of blood depends on the continual activity of cardiac muscle, while smooth muscle controls such processes as the movement of food through the gastrointestinal tract (peristalsis), the variations in the cross-sectional area of blood vessels and the vascular tone (Bullock et al., 1986). Each skeletal muscle is an organ that contains muscle tissue, connective tissue nerves, and blood vessels. Like connective tissue elsewhere in the body, muscle consists of fibres embedded in an amorphous ground substance. Most of the connective tissue fibres are made of collagen. There are at least five immunologically distinct types, while the remaining fibres are elastin (McComas, 1996). The connective tissue in muscle has three anatomical parts, one of which is *Epimysium* the fibrous connective tissue that covers more than 430 skeletal muscles and continuous with the tendons at the ends of the muscle (Figure 1.1) (Baechle, 1994). It contains tightly woven bundles of collagen fibres, from 600 to 1800 nm in diameter, which have a wavy appearance and connected to the *perimysium* (McComas, 1996).

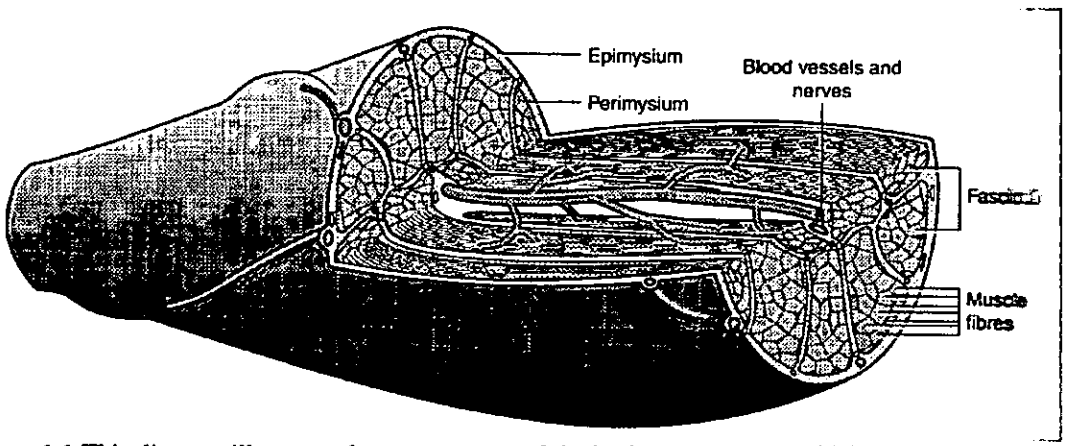


Figure 1.1 This diagram illustrates the arrangement of the basic components, which make up a typical skeletal muscle (as cited in Burkitt, Young, and Heath, 1993).

The tendon is attached to bone *periosteum*, contraction of the muscle pulls on the tendon and, in turn, the bone. Limb muscles have two attachments to bone: proximal (closer to the trunk) and distal (farther from the trunk). The two attachments of trunk muscles are termed superior (closer to the head) and inferior (closer to the feet). By convention, the origin of a muscle is defined as the attachment of the muscle that is more proximal or superior, and the insertion is defined as the attachment that is more distal or inferior (Baechle, 1994).

1.1.2 The muscle fibre

A single skeletal- muscle cell is known as a muscle fibre. Each muscle fibre is formed during development by the fusion of a number of undifferentiated, mononucleated cells, known as *myoblasts*, into a single cylindrical, multinucleated muscle fibre. This stage of muscle differentiation is completed around the time of birth, and the differentiated fibres continue to increase in size with the growth of the organism (Vander et al., 1994). In addition, it has been shown that fibre-type-specific programs of gene expression can be detected at early stages of myogenic development in the embryo (DiMario et al., 1993; Ontell et al., 1993; Stockdale 1997), but remain plastic in adults, in whom they are subjected to modification as a function of contractile load

(e.g., exercise training), hormonal shifts, or systemic diseases (Holloszy and Coyle 1984; Massie et al., 1988; Ianuzzo et al., 1991; Sabbah et al., 1993; Williams and Neufer 1996). Sometimes skeletal muscle fibers are destroyed as a result of injury. It is now generally known that that these damaged muscle fibers cannot be replaced from existing muscle fibers. New fibres can be reformed, however, from undifferentiated cells known as satellite cells. It must be noted however that this mechanism cannot restore a severely damaged muscle to full strength (Vander et al., 1994). In addition, there is a different mechanism, which can make up to a point for a loss of muscle tissue, through increased growth in the size of the remaining muscle fibres (Vander et al., 1994). Muscle fibers are long cylindrical cells 50 to 100 μm in diameter. The nuclei in these fibers are known to be located in the periphery of the cell's cylindrical structure. Under the epimysium, the muscle fibres are grouped in bundles (called *fasciculi*) that may consist of up to 150 fibres, with the bundles surrounded by the connective tissue mentioned before, called the *perimysium*. Each muscle fibre is surrounded by connective tissue called *endomysium*, which encircles and is continuous with the fibre's membrane, or *sarcolemma*. All the connective tissue- epimysium, perimysium, and endomysium- is continuous with the tendon, so tension developed in one muscle cell can develop tension in the tendon (Baechle, 1994) (see Figure 1.1). In addition, perimysium provides the pathway for the major blood vessels and nerves to run through the muscle belly.

Underneath the coarse perimysial sheets of connective tissue is a looser and more delicate network in which collagen fibrils run in all directions, some being connected to the endomysium. The arterioles and venules are found in these regions, often with intramuscular nerve branches. Figure 320 (in Appendix 1) illustrates the blood supply of the lower leg.

The interior structure of a muscle fibre is depicted in figure 1.2 following. The *sarcoplasm*- the term used for the cytoplasm of a muscle fibre- contains contractile components, which consist of protein filaments; other proteins; stored glycogen and fat particles; enzymes; and specialized structures such as mitochondria and the *sarcoplasmic reticulum* (Baechle, 1994). The most striking feature seen when observing a muscle fibre through a light microscope (Figure 1.3) is a series of light and dark bands perpendicular to the long axis of the fibre. Both skeletal and cardiac muscle fibres have this characteristic banding and are known as striated muscles, while smooth muscle cells do not show a banding pattern. The striated pattern in skeletal and cardiac fibres, results from the presence of numerous thick and thin filaments in the cytoplasm that are organized into approximately cylindrical bundles (1 to 2 μm in diameter) known as Myofibrils (Figure 1.4).

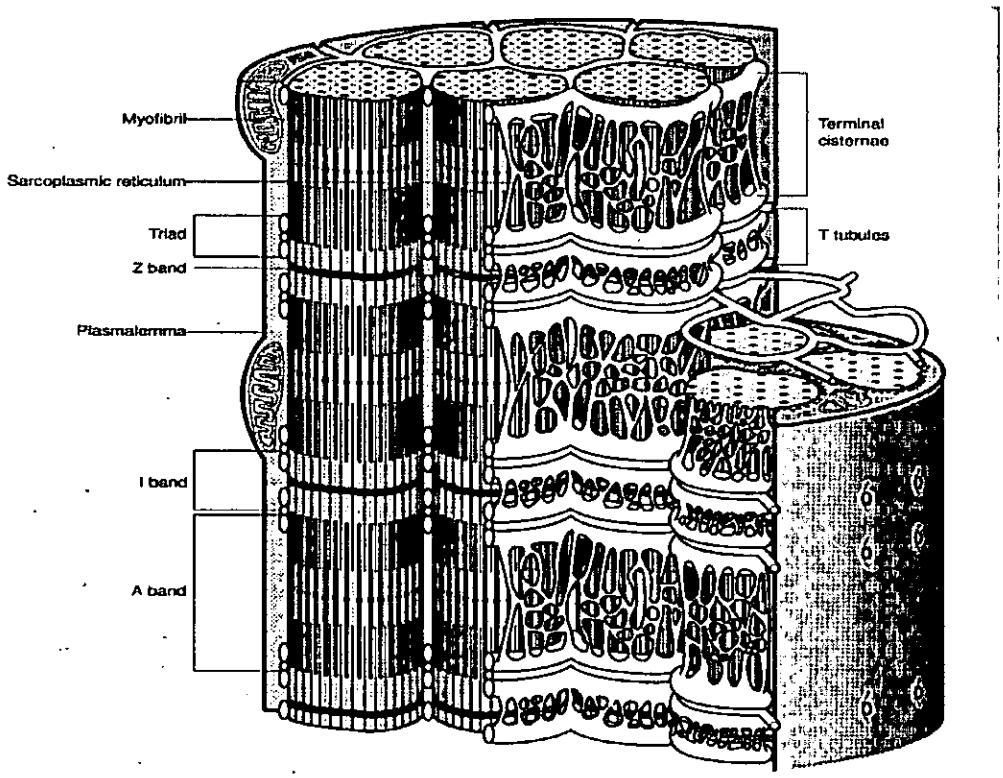


Figure 1.2 The interior structure of a muscle fiber (source: Burkitt, Young, and Heath, 1993).

Most of the cytoplasm is filled with myofibrils, each of which extends from one end of a fibre to the other (Vander et al., 1994). Each myofibril is composed of *thick* and *thin filaments* arranged in a repeating pattern along the length of the myofibril. One unit of this repeating pattern is known as a *sarcomere*. The thick filaments are composed almost entirely of the contractile protein *Myosin* (Vander et al., 1994).

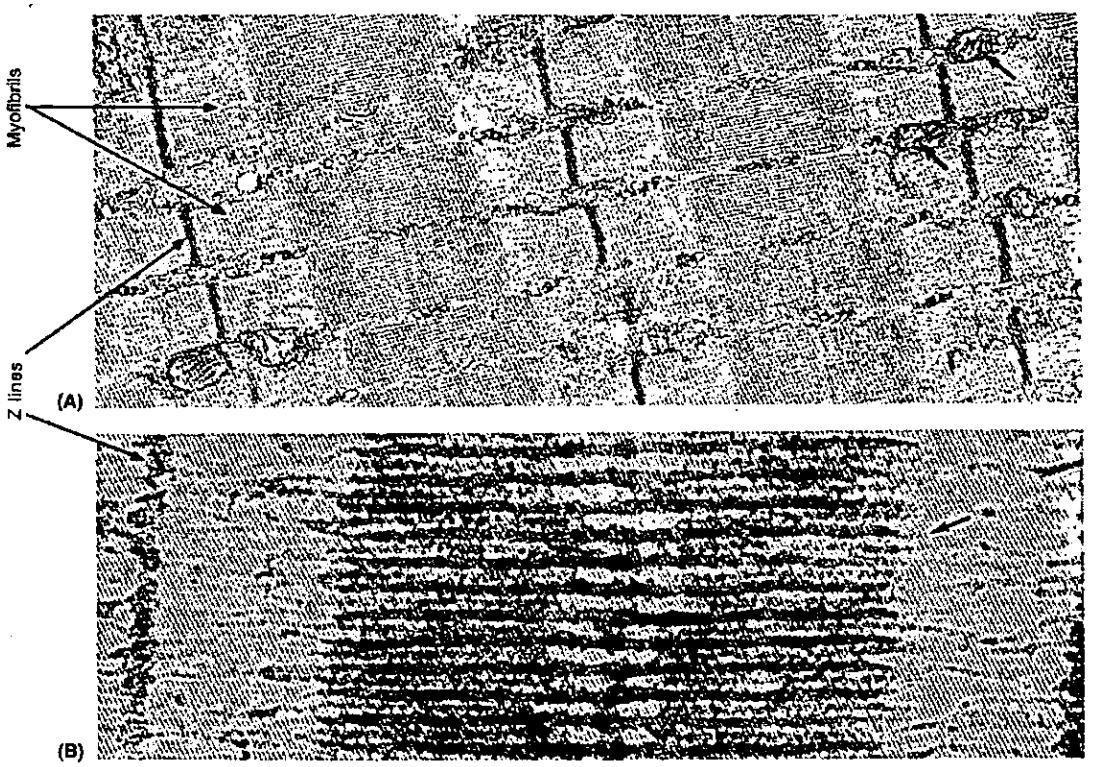


Figure 1.3 (A): This electron micrograph of mammalian skeletal muscle cut section demonstrates the main elements of the conducting system. (B): High magnification of a single sarcomere within a single myofibril (arrow at the right of A band indicates end of a thick filament) (Source: Vander et al., 1994).

The myosin filaments (thick filaments about 16 nm in diameter) contain up to 200 myosin molecules (Baechle, 1994). On the other hand the thin filaments contain the contractile protein *actin* as well as two other proteins- *troponin* and *tropomyosin* (Vander et al., 1994). A typical myofibril will contain about 450 myosin filaments in the centre of a sarcomere and 900 actin filaments at either end of the sarcomere. There are additional filaments present in skeletal muscle that probably assist in maintaining the structural integrity of the muscle (Baechle, 1994).

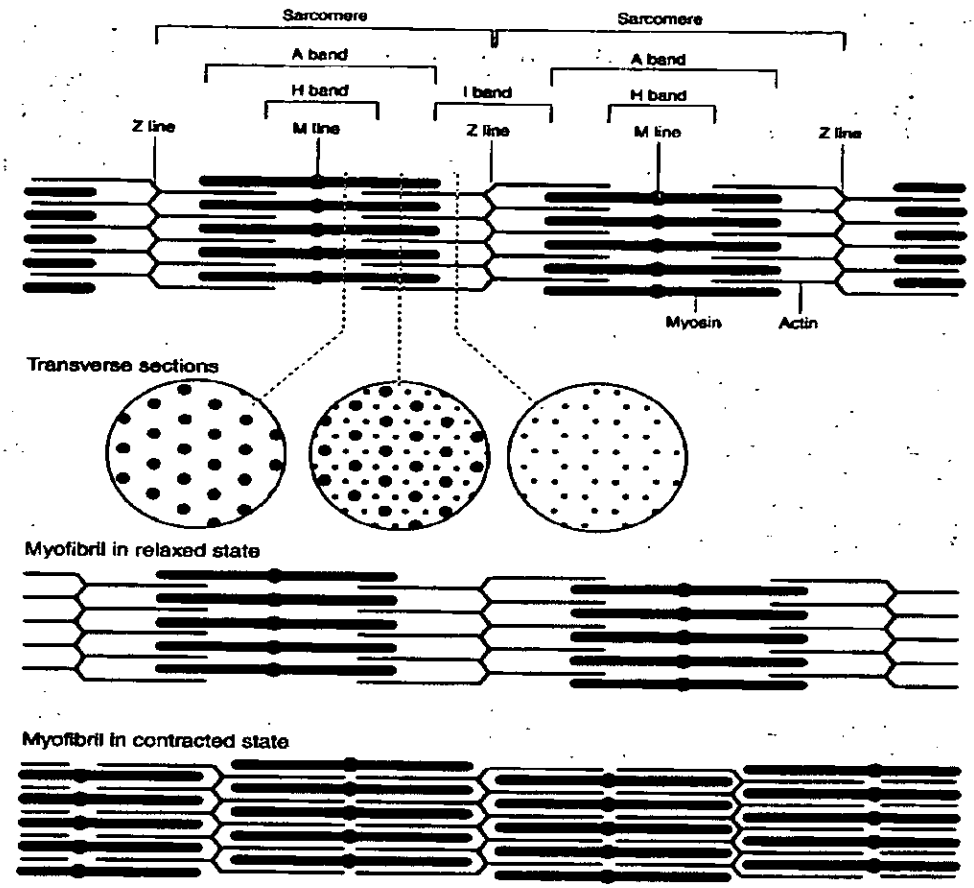


Figure 1.4 The arrangement of myofilaments in the sarcomere (source: Burkitt, Young, and Heath, 1993)

Regulation of the skeletal muscle contraction occurs through four proteins located within the actin thin filaments: tropomyosin and Troponins C, I and T. These proteins position is influenced by the changes in cytosolic Ca^{2+} concentration, which in turn control actin- myosin interactions. Tropomyosin (TM) is a helical molecule (about 40 nm long), where its molecules are arranged together head to tail, forming a continuous chain along each thin filament. In addition, each TM has seven binding sites and binds with seven respective actin monomers within the thin filament. The complex calcium binding subunit that is associated with TM is troponin (TN). Troponin consists of three subunits TN-C, TN-I and TN-T, which with a similar mechanism to calmodulin of the myosin light chains, control the position of TM on the surface of an actin filament. In the absence of Ca^{2+} myosin can bind to a thin filament, but the sliding of

myosin across the thin filament is prevented by the TM-TN complex. This is believed to be the first of the two positions that the TM and TN molecules can occupy on the thin filament under the control of Ca^{2+} and is called the "off position". On the other hand in the "on position" situation binding of Ca^{2+} ions to TN-C triggers a slight movement of TM towards the centre of the actin filament. This in turn exposes the myosin- binding sites on actin. (Lodish et al., 2000).Finally, as it has been demonstrated, the globular head regions are the ones, which contain binding sites for actin with the use of ATP (hydrolysis) (Zubay et al., 1995). It is now established that an intracellular calcium concentration larger than 10^{-6} M stop the TM-TN inhibition and contraction occurs (Lodish et al., 2000).

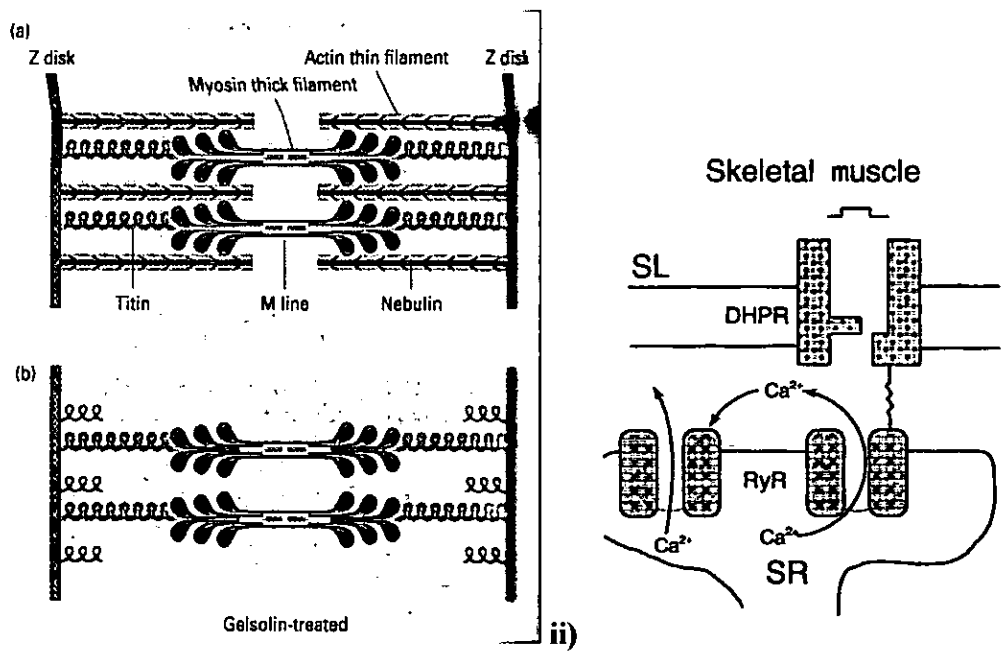


Figure 1.5 i) a) Skeletal muscle; structural organization of the thick and thin filaments b) The role of titin in the structure and function of the skeletal muscle. (Lodish et al., 2000). ii) The role of DHPR and Ryanodine receptors in Ca^{2+} influx for activation of the SR via a signal transduction mechanism (Wasserstrom, 1998).

The thick filaments are located in the middle of each sarcomere, where the *A band* (wide, dark band) is formed by their orderly parallel arrangement. Each sarcomere contains two sets of thin filaments, one at each end. The end of the sarcomere is the *Z*

line meaning that two successive Z lines define its limits. One end of each thin filament in a set is bound (like an anchor) to a network of interconnecting proteins (Z line), whereas the other end overlaps a portion of the thick filaments. Titin or connectin connects the ends of the myosin thick filaments to the Z discs and extends the thick filament along to the H zone. This set of proteins organize thin and thick filaments in their three-dimentional arrys and give muscle much of its elasticity (Lodish et al., 2000). The light band, known as the *I band* (figure 1.4), lies between the ends of the A bands of two adjacent sarcomeres and it is bisected by the Z line. It contains those portions of the thin filaments that do not overlap the thick filaments. In addition, two other bands are present in the A band region of each sarcomere. A relatively light band located in the centre of the A band, is known as the *H zone*, which corresponds to the space between the ends of the two sets of thin filaments in each sarcomere. As a result, only thick filaments, specifically their central parts, are found in the H zone. The narrow, dark band in the centre of the H zone is known as the *M line* and corresponds to the proteins that link together the central region of the thick filaments. Thin filaments are anchored to the Z line, while thick filaments are linked together by the M line and to the Z line by the titin filaments as mentioned, meaning that neither thick nor thin filaments are free-floating. Moreover, each thick filament is surrounded by a hexagonal array of six thin filaments, and each thin filament is surrounded by a triangular arrangement of three thick filaments. Altogether there are twice as many thin as thick filaments in the region of filament overlap (Vander et al., 1994).

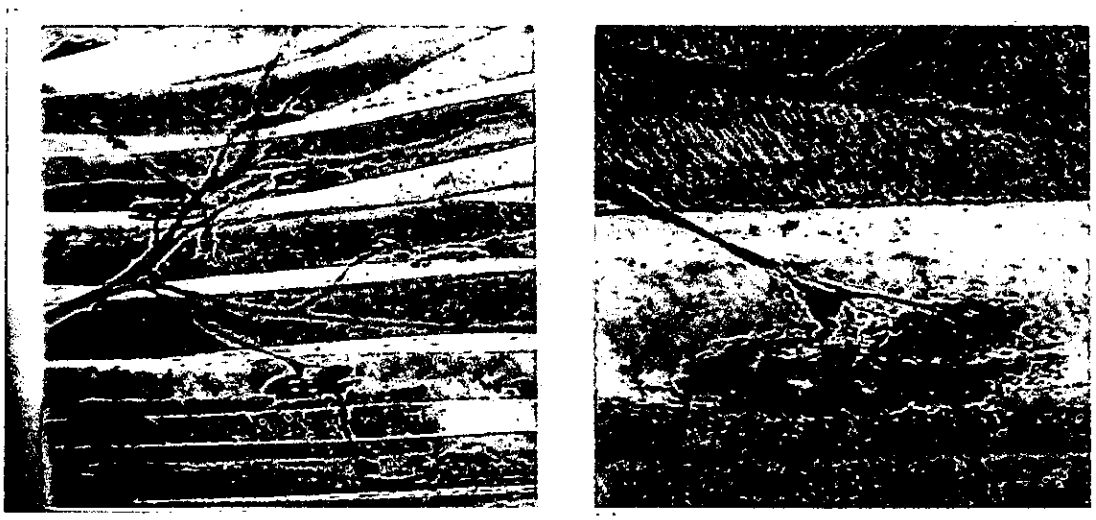
Each myofibril is surrounded by a system of tubes called *sarcoplasmic reticulum (SR)*, which terminates in the Z lines (see figure 1.2). Calcium ions are stored in the SR, in formations called vesicles, which play a very important role in muscle

contraction (Baechle, 1994). The electrical potentials that reach the skeletal muscle cells can rapidly be converted to a rise in cytosolic Ca^{2+}, which initiates contraction by a mechanism described later. The major anatomic features of this signalling pathway are the T (Transverse) tubules, which are located next to the SR, forming structures called Triads. The depolarization signal reaches the triad through this system and stimulates the SR causing calcium release through its membranes (Lodish et al., 2000). More specifically, in the intramembrane particle thought, the Ca^{2+} channels are organized into groups of four called tetrads. These tetrads are closely associated with the cytoplasmic domains of the four sub-units of ryanodine receptor (RyR). These are called "foot processes" and are organized like there is a tetrad opposite of every foot process, resulting in a tetrad for every two RyRs. These proteins are thought to be arranged tightly together, possibly with a physical connection between RyRs and Ca^{2+} channels. The mechanism, which will be explained later on, involves the activation of the Sarcolemmal Ca^{2+} channels (DHP receptors), which in turn activate the RyRs via a direct or mechanical transduction signal without requesting the influx of Ca^{2+} for activation of SR release (Figure 15_{ii}) (Wasserstrom, 1998).

Finally, the space between adjacent thick and thin filaments is bridged by projections known as *cross bridges*. These are portions of myosin molecules that extend from the surface of the thick filaments toward the thin filaments (figure 1.5). During muscle contraction, these cross bridges make contact with the thin filaments and exert force on them. Thus, the cross bridges are the force-generating structures in muscle cells.

1.2 Innervation

The nerve cells whose axons innervate skeletal muscle cells and cell bodies are located in either the brainstem or the spinal cord, are called motor neurons. These are myelinated axons and very large in diameter giving them the ability to propagate action potentials at very high velocities. When the axon of the motorneuron reaches the muscle it divides into many branches and each branch forms a single junction with a muscle fiber. This means that one motor neuron controls many muscle fibers but every single fiber is innervated by only one motor neuron. A motor unit is the complex formed by the motor neuron and the muscle fiber it innervates. The muscle fibers of the motor unit even though they are located in the same muscle they do not lay one next to an other, instead they are scattered throughout the muscle. In addition, an action potential will cause contraction of all the muscle fibers connected to the motor neuron, which is carrying the action potential (Vander et al., 1994).



a. b.

Figure 1.6 Motor end plates at low magnification in micrograph **a**, the terminal part of the axon of a motor neuron is seen dividing into several branches, each terminating as a motor end plate on a different skeletal fiber near to its mid-point. Micrograph **b** shows the lowermost of these motor end plates at higher magnification. The axonal branch is seen to lose its myelin sheath and divides to form a cluster of small bulbous swellings (terminal boutons) on the muscle fiber surface (as cited in Burkitt, Young, and Heath, 1993).

Moreover Salmons and Sreter (1976) suggested that the changes, which follow cross reinnervation of mammalian fast-to-slow twitch muscles, may reflect a capacity of skeletal muscle to respond adaptively to different functional requirements. This interpretation is supported by experiments in which long-term electrical stimulation was used both to reproduce and to oppose the effects of cross reinnervation (Salmons and Sreter, 1976).

The innervation of the lower leg in the animal used for experimentation (adult Sprague Dawley rats of either sex) is illustrated in Figures 187, 188 (Appendix 1).

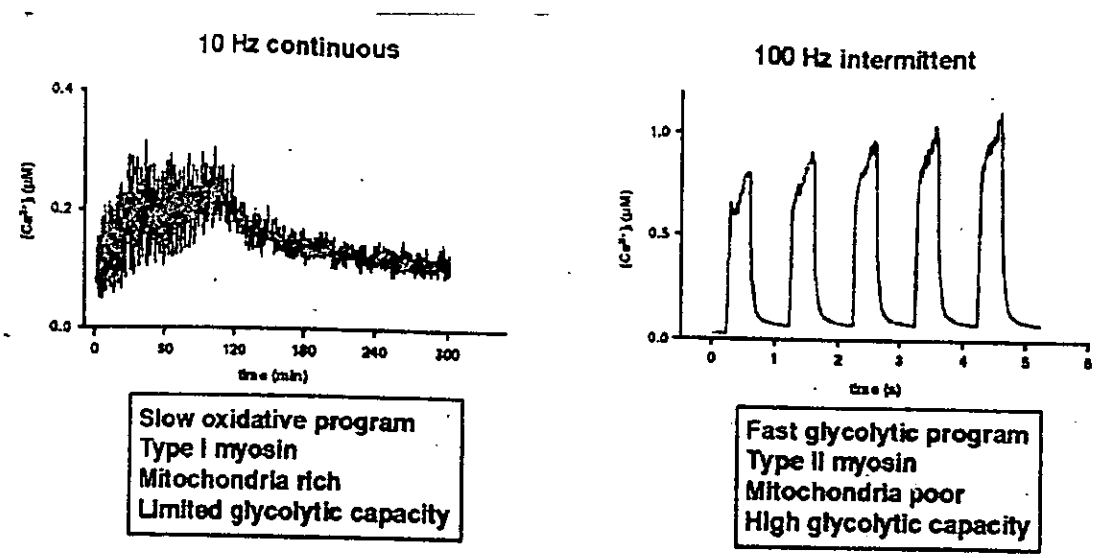


Figure 1.7. Continuous stimulation at 10 Hz (left panel) leads to sustained elevations of $[Ca^{2+}]$ in a range from 100 to 300 nM that are characteristic of slow fibers. Intermittent nerve stimulation at higher frequencies (100 Hz) produces large but transient oscillations in $[Ca^{2+}]$ that reach as high as 1000 nM, but are maintained at resting levels around 50 nM in a manner characteristic of fast fibers (as cited in Olson and Williams, 2000).

Finally, different patterns of motor neurons firing lead to distinctive waveforms of oscillations in intracellular calcium and determine skeletal muscle fiber type. Varying the frequency of neural stimulation will transform one fiber type into another causing muscle fiber differentiation (Figure 1.7) (Olson and Williams, 2000).

1.3 Muscle function

1.3.1 The neuromuscular junction

"The skeletal muscle fibers are innervated by large myelinated nerve fibers that originate in the large motorneurons of the anterior horns of the spinal cord" (as cited in Guyton, 1991). The *motor end plate* is defined as the region of the muscle fiber plasma membrane that lies directly under the terminal portion of the axon. Moreover, the junction of an axon terminal with the motor end plate is known as a *neuromuscular junction* (Figure 1.8).

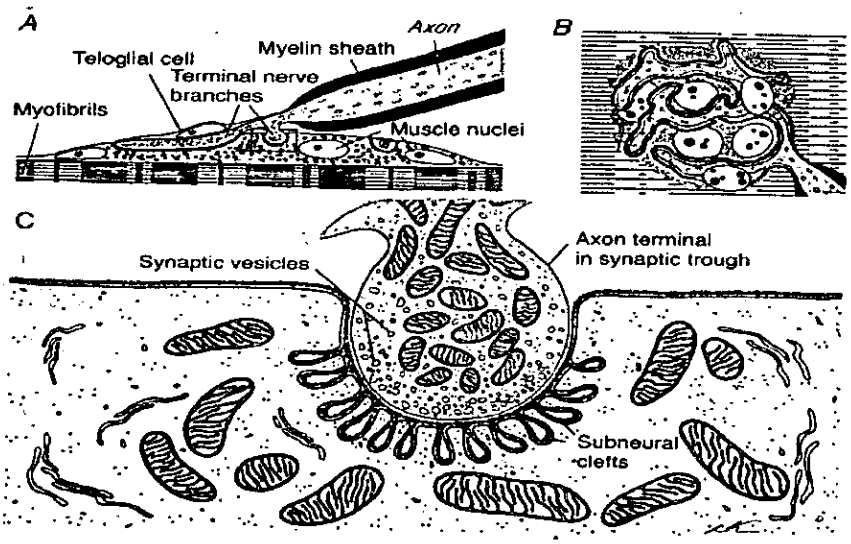


Figure 1.8. Different views of the neuromuscular junction and motor end plate (From Guyton, 1991).

Like the synaptic junctions (between two neurons), the axon terminals of a motor neuron have membrane bound vesicles, which contain the chemical transmitter *acetylcholine* (ACh) (Vander et al., 1994).

About 300 vesicles of acetylcholine are released to the synaptic trough when the neuromuscular junction is 'stimulated' by a nerve impulse (Guyton, 1991). The

synaptic trough with the nerval membrane are identified above and the muscle membrane with its subneural cleft below. The process is triggered by the action potential, which causes the calcium channels, located in the end of the neural membrane, to release large quantities of calcium, which in turn diffuses to the interior of the terminal. The calcium ions attract the acetylcholine vesicles through the *dence bars* to the synaptic trough where they release their acetylcholine by the process of exocytosis (Guyton, 1991).

The acetylcholine diffuses across the cleft and binds to specific nicotinic receptors, which in turn cause the movement of sodium and potassium ions though them. This causes a local depolarisation of the motor end plate called end-plate potential (EPP) (this is similar to the EPSP:excitatory postsynaptic potential, at a synapse). In addition, the motor end plate contains the enzyme *acetylcholinesterase* at its surface, which breaks down ACh. Finally, most neuromuscular junctions are located in the middle of a muscle fiber, and action potentials created can go in both directions toward the ends of the fiber (Vander et al., 1994).

1.3.2 Excitation- contraction coupling

Excitation- contraction coupling refers to the sequence of events by which an action potential in the plasma membrane of a muscle fiber leads to cross- bridge activity. The contractile activity of muscles, as previously explained, is influenced by neurotransmitters released by autonomic nerve endings (Vander et al., 1994). In addition, it has been established that L-type Ca^{2+} channels activation can in turn activate the release of Ca^{2+} from the Sarcoplasmic Reticulum (SR) stores; finally resulting in muscle contraction (Wasserstrom, 1998). This process occurs in response to the activation of the dihydropyridine (DHP) receptors located in the t-tubules

(Fig.1.5). When these sarcolemmal chanels are activated, as result of the action potential, they trigger the opening of the Ca^{2+} channels on the SR membrane and the release of Ca^{2+} in the cytosol. This "link" between the DHP receptors and the RyR receptors (the sarcolemal event and the SR event) is not well known but it is thought to rely on a mechanical process. This hypothesis was based on the fact that this process, that probably involves the "foot process" between the DHP receptors and the RyR, can occur in the absence of Ca^{2+} influx, which means that excitation contraction coupling is a voltage dependent process (Wassertrom, 1998).

1.3.3 Muscle contraction

Many people have linked the term contraction with shortening when this is not necessarily the case. Contraction refers to the turning or the force generating sites in a muscle fiber. In order for shortening to occur, the opposing force must be smaller than the force produced by the muscle fibers (Vender et al., 1994). First an action potential travels along a motor nerve towards its endings on the neuromuscular junction. The release of Ach as described before causes the opening of several acetylcholine-gated protein channels, on the motor end plate. This results on the influx of large quantities of sodium ions to flow to the interior of the muscle fiber membrane, which in turn initiates an action potential in the muscle fiber. The depolarisation caused by the action potential, travels deeply within the muscle fiber and causes calcium ion release from the SR. The calcium ions are the ones who trigger the attractive forces between the actin and myosin filaments, causing them to slide together. The calcium ions then return to the SR, after fraction of a second, where they are stored till a new action potential stimulates the SR, to cause a new contraction (Guyton, 1991).

"It has been estimated that about 30% of the SR Ca^{2+} is released during contractions of the perfused heart (Chen et al., 1996). Whether there is a decrease in SR Ca^{2+} content in fatigued muscle is controversial. It has been demonstrated that a decrease in the force of muscle contraction in electrical stimulation protocols correlates with decreased Ca^{2+} release from the SR (Fitts and Balog, 1996; Westerblad and Allen, 1991) and a decrease in the SR Ca^{2+} content has been reported (Schneider, Simon, and Szucs, 1987). However, a decrease in the SR Ca^{2+} with fatigue has not been a universal finding (Gonzalez-Serratos et al., 1978)" (as cited in Halseth et al., 2000).

The basic mechanism of contraction is illustrated in *figure 1.4*. It shows the relaxed (above) and the contracted state of the sarcomere (below). The thick filaments, which are composed mainly of the protein myosin, are maintained in register by their attachment to a disc-like zone represented by the M line. Similarly the thin filaments, which are composed mainly of the protein *actin*, are attached to a disc-like zone represented by the Z line. The widely accepted sliding filament theory proposes that under the influence of energy released from ATP, the thick and thin filaments slide over one another, thus causing shortening of the sarcomere (Burkitt, Young, and Heath, 1993). The energy for this process comes from the hydrolysis of the adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and phosphate (Pi) (as shown below).

$$ATP \Longleftrightarrow ADP + Pi$$

This reaction is catalysed by an enzyme called myosin ATPase, which allows the contraction process to continue or for relaxation to occur (Baechle, 1994).

1.4 Skeletal muscle plasticity

Skeletal muscle adapts with a great specificity to different types of exercise. Muscle plasticity [Plasticotita (Greek): the ability the change, reshape] refers to this ability of the muscle tissue to adapt to various stimuli depending on the contractile force, speed and duration. There is now strong evidence suggesting there is a link between the intracellular calcium increases (associated with contractile signals and stress) and a specifically activated signal transduction network (Chin et al., 1998). This network has been shown to consist of several pathways inducing muscle fiber differentiation or hypertrophy. There has been suggested that these signal transduction pathways interact with each other. This interaction is called 'cross-talk' and has been shown to link many pathways especially in the better-researched heart (Ruwhof and Van der Laarse, 2000). These pathways include the calcineurin pathway (Chin et al., 1998), the myogenic regulatory factor (MyoD) pathway (Cox, Quinn, and McDermott, 1999; Chin et al., 1998), the calmodulin-dependent protein kinase (CaMK) pathway (Cruzalegui and Bading, 2000), the mitogen-activated protein kinase (MAPK) pathway (Treisman, 1996), the nuclear factor-κB (NF-κB) pathway (Timmerman et al., 1996), as well as insulin signaling and developmental pathways (Musaro et al., 1999; Olson and Williams, 2000). These pathways and especially the calcineurin pathway will be explained in more detail next. Finally, they are thought to activate a variety of transcription factors, which in turn activate specific muscle genes. It might though require a combination of mechanisms or transcription factors in order to activate a certain muscle gene.

1.5 The role of Calcineurin

1.5.1 Introduction to the role of Calcineurin

Calcineurin is a serine- theonine specific Ca^{2+}-calmodulin- activated protein phosphatase that has been identified in many organisms from yeast to humans. This enzyme is targeted by cyclosporin A and FK506, two structurally unrelated immunosuppressive drugs. Both drugs form complexes with abundant intracellular binding proteins, cyclosporin A with cyclophilin A and FK506 with FKBP 12, which bind to and inhibit calcineurin (Hemenway and Heitman, 1999). In addition, calcineurin is known to play an important part in the regulation of the transcription factor family of NFAT proteins during T cell activation, and in mediating responses of microorganisms to caution stress (Hemenway and Heitman, 1999). Moreover, evidence was presented showing that calcineurin is a key component in T cell signalling pathways, just after the demonstration that calcineurin is the target of CsA and FK506 (Liu et al., 1992; Clipstone and Crabtree, 1992). Finally, there is direct evidence that calcineurin is involved in muscle growth and can induce muscle hypertrophy and fiber type changes (Chin et. al., 1998; Olson and Williams, 2000; Mayne et. al., 1996).

1.5.2 Structure of Calcineurin

Calcineurin was initially identified like a bovine brain cAMP phosphodiesterase inhibitor with a 'protein-like' activity (Wang and Desai, 1976; Wang and Desai, 1977). This protein was later termed as a Ca^{2+}- dependent activator protein, regulator protein and was used for the purification of other proteins with the use of a calmodulin by affinity chromatography. Klee and Krinks (1978) managed to purify

calcineurin while trying to purify cAMP phosphodiesterase by affinity chromatography. Klee et al. (1979), named this protein 'Calcineurin' as they identified that it was rich in bovine brain tissue, and bounded to Ca^{2+} even in the absence of calmodulin (Hemenway and Heitman, 1999). Calcineurin is not a large protein, but it does offer great opportunities to observe the many ways such a small protein can exert broad influence. Calcineurin is a heterodimer, with a 59-kDa calcineurin A (CnA= catalytic) subunit and a 19-kDa calcineurin B (CnB= regulatory) subunit (Cohen and Cohen, 1989; Ensembl: http://www.ensembl.org).

Primary Structure

At least 2 genes encoding isoforms of CnA have been identified from complimentary DNA cloning of the major catalytic subunit of Calcineurin in mammalian brain. The a and b genes are localized on human chromosomes 4 and 10 respectively. In addition two more isoforms of the protein have been identified in chromosomes 8, and 19 as presented in the international databases (Ensembl: http://www.ensembl.org ; NCBI: http://www.ncbi.nlm.nih.gov ; UCSC http://genome.cse.ucsc.edu) (Figure 1.9).

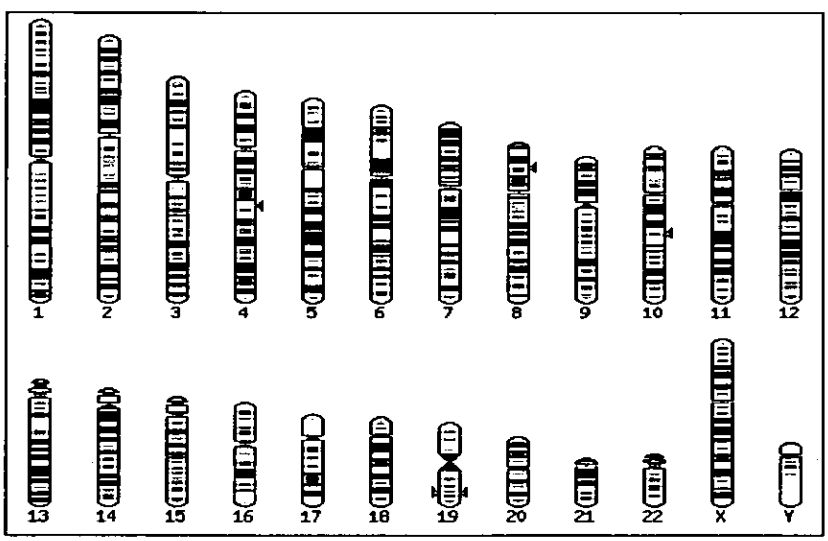


Figure 1.9 The location of the genes for Calcineurin A (CnA) subunit isoforms in the human genome map (as cited in Ensembl: http://www.ensembl.org).

Zhuo and co-workers (1994) have identified a major structural difference between two first isoforms, which is thought to play a role in regulation (Zhuo et al., 1994). Only 1 gene for Calcineurin B (CnB), located on human chromosome 2 has been found (Navia, 1996; Ensembl: http://www.ensembl.org). Below is a figure from work by Kawamura & Su (1995), which illustrates the primary sequence structure of the binding site of the subunits of the heterodimer on CnA (Figure 1.10)

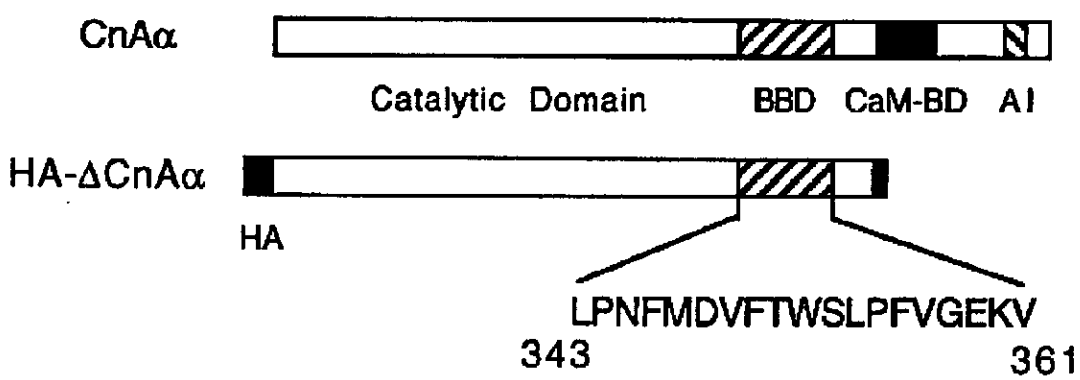


Figure 1.10 The primary sequence structure of the binding site of the subunits of the heterodimer on CnA (as cited in Kawamura and Su, 1995).

Secondary/Supersecondary Structure

Various domains have been identified on the Calcineurin subunits. The core structure is composed of a δ-strands, surrounded by α-helices and β-sheets (Goldberg et al., 1995).

The mechanism of calcineurin and the other protein phosphatases depends on the divalent metal coordinating site. The metal ions activate water molecules to catalyse hydrolysis of the phosphate in a single-step reaction.

The CnB-binding site is a long 22-residue α-helix (sometimes called the b-binding helix: BBH). CnB has 2 EF-hand Ca^{2+}-coordinating domains. Each domain coordinates 2 Ca^{2+} atoms. The EF-hand motif consists of α-helices joined by a β-loop.

In this way, CnB is very similar to CaM, except without the long linking α-helix (Griffith et al., 1995). The structure below illustrates the CnB subunit again with the EF-hands and with four Ca^{2+} ions bound to it (Figure 1.11).

Figure 1.11 The CnB subunit again with the myristoyl residues and EF-hands with four Ca^{2+} ions complexed (as cited in Http://attila.stevens-tech.edu/chebio/mjipping/structword.htm#references).

Despite the structural similarities of CnB and calmodulin, reconstitution of the holoenzyme from purified subunits demonstrates that CnB cannot substitute for the activity of calmodulin nor can calmodulin functionally substitute CnB (Merat et al., 1985). The importance of the structures mentioned will be discussed in more detail in the section of calcineurin regulation.

1.5.3 Regulation of Calcineurin

It is well known that protein phosphorylation controls many cellular events meaning regulation requires many levels. The Calcineurin pathway involves many different regulatory mechanisms. These include site localization, Ca^{2+}-activation, auto-inhibition (by auto-inhibitory domain - AID), CnB-activation, CaM-activation. In

addition, there are a number of recent studies elucidating the great complexity of the regulation to which Calcineurin is subject (Klee, Ren, and Wang, 1998).

Site localization

One regulatory mechanism is that kinases and phosphatases are maintained at discrete cellular locations through their interaction with anchoring proteins. Enzymes may be positioned in close proximity to specific substrates, which then can be efficiently modified in response to the appropriate signals (Http://attila.stevens-tech.edu/chebio/mjipping/structword.htm#references). Politino & King suggested in 1990 that it might function in membrane anchoring, from studies with phospholipid interactions, while Griffith has noted that other EF-hand superfamily proteins use Ca^{2+} as a switch (Griffith et al., 1995).

Auto-inhibition

There is an autoinhibitory domain (AID) at the carboxyl terminal of the CnA subunit. It lies over the substrate-binding channel of the catalytic domain. When Calcineurin is auto-inhibiting, the CaM-binding domain, which is an amphipthic α-helix at the carboxyl terminus of the CnA, lies under the CnB-binding helix, linked at one end to the AID. This places the AID close to the active site where it could inhibit the binding of substrates and inhibitors (Stoddard and Flick, 1996).

Calcium activation

Upon the addition of Ca^{2+}, both CaM and CnB are activated (even without CaM, CnB confers some activation on CnA). The activation apparently disrupts the interaction

between CaM and the CnB-binding helix on CnA, moving the AID away from its inhibitory position (Barford, 1996).

Calmodulin (CaM) binding

CaM and CnB apparently activate CnA by different but complimentary mechanisms. In the Kissinger structure, CaM binds with the two domains on opposite sides of the helix but is very difficult to define the link to the CnA molecule (Http://attila.stevens-tech.edu/chebio/mjipping/structword.htm#references).

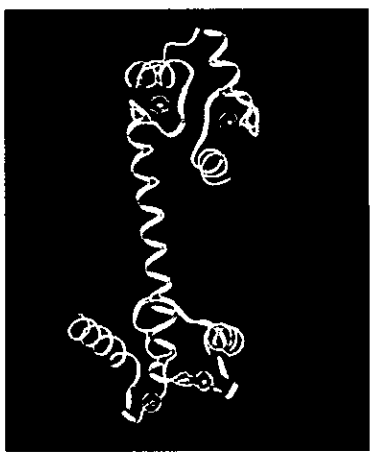


Figure 1.12 Calmodulin ribbon structure (as cited in Http://attila.stevens-tech.edu/chebio/mjipping/structword.htm#references).

CnB-binding

The CnB-binding site on CnA is a long 22-residue α-helix. In 1995, Watanabe, et al., identified that CnB binds to CnA very differently from how CaM binds, even though they are very similar. The Kissinger structure of the human Calcineurin structure has an additional CnA amino-terminal sequence that assists in CnB-binding (Kissinger et al., 1995). While there seems to be some conformational change in CnA upon the binding of CnB, it is not clear how the information is transmitted to the active site on the CnA subunit.

1.6 Transcription factors of the NFAT family: Regulation and function

1.6.1 Introduction to the role of NFAT

Transcription factors play a major role in tissue development and maintenance by regulating the expression of genes required for cell function. In skeletal muscle cells, the best-studied transcription factors are the myocyte enhancer factor 2 (MEF2), the nuclear factor of activated T cells (NFAT) family and the muscle regulatory factor (MRF), which are among other things involved in muscle differentiation.

Proteins that comprise the family of transcription factors known as nuclear factor of activated T cells play an essential role in inducible gene expression during immune responses (Rao, Luo and Hogan, 1997). Although first identified in T cells, these proteins have also been detected in many other tissues and cell types, like skeletal muscle. Calcium mobilization as well as receptor stimulation result in activation of many intracellular enzymes including, the pathway that involves the calcium and calmodulin dependent phosphatase calcineurin. As mentioned before, this is a very important upstream regulator of NFAT proteins (Weiss and Littman, 1994; Cantrell, 1996). Different cell types when stimulated have been shown to elicit transcription of activation-associated genes, many of which are potential targets of NFAT; the genes encode transcription factors, signalling proteins, cytokines, cell surface receptors, and other effector proteins (Crabtree, 1989; Kelly and Siebenlist, 1995; Cockerill et al., 1995; Rao, Luo and Hogan, 1997).

Calcineurin controls the translocation of NFAT proteins from the cytoplasm to the nucleus of activated cells by interacting with an N-terminal regulatory domain conversed in the NFAT family (Rao, Luo and Hogan, 1997). Rao, Luo and Hogan (1997), suggest that the DNA- binding domains of NFAT proteins resemble those of

Rel- family proteins, and Rel and NFAT proteins show some overlap in their ability to bind to certain regulatory elements in cytokine genes. In addition, NFAT is also notable for its ability to bind cooperatively with transcription factors of the AP-1 (Fos/Jun) family (Rao, Luo and Hogan, 1997).

1.6.2 Structure and distribution of NFAT proteins

Isolation and Nomenclature

The studies from several laboratories indicated that NFAT is a mixture of proteins belonging to a novel family of transcription factors [reviewed in Crabtree and Clipstone, 1994; Jain, Loh, and Rao, 1995; Serfling, Avots, and Neuman, 1995; see Figure 1.13 and Table 1.1].

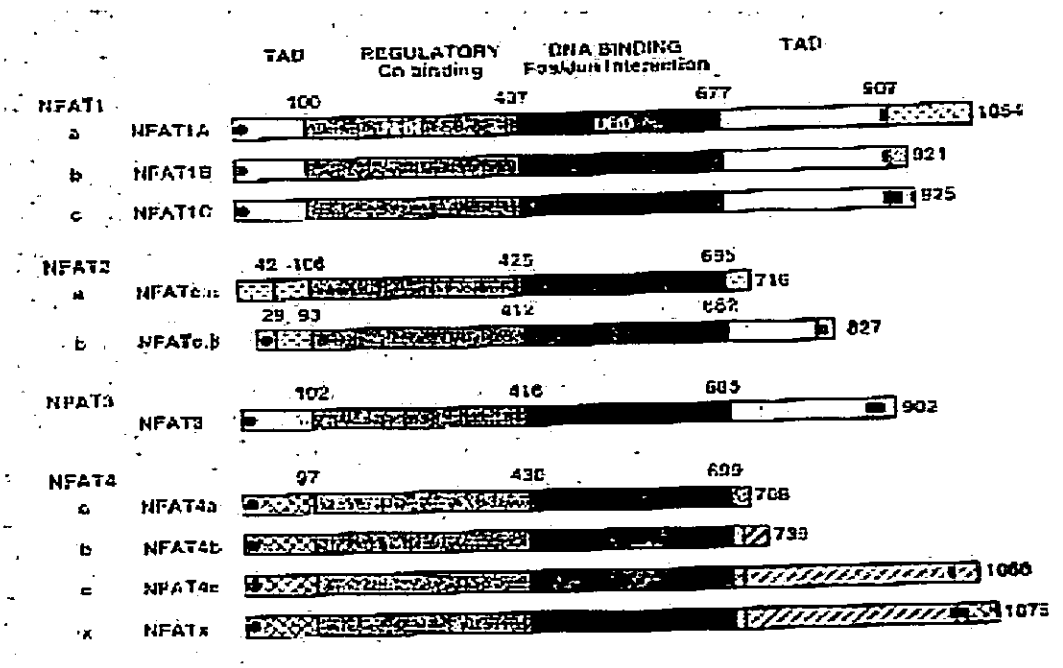


Figure 1.13 Schematic alignment of NFAT proteins predicted from their cDNAs. For nomenclature, see table 1.1. The region of highest homology within NFAT proteins is the DNA-binding domain (DBD), which shows similarity to the Rel homology region of Rel-family transcription factors. A second region of homology is the NFAT homology region (NHR), which binds to calcineurin (Cn) and is found only in NFAT family proteins. The N- and C-terminal transactivation domains (TADs) are indicated. The acidic/hydrophobic motifs in the N-terminal TADs *are* schematised as a *dot*. The C-terminal motif present is indicated as a *thick bar*. For protein isoforms, identical shading patterns

represent identical sequences. The boundary of each region is labelled above the sequences with numbering referring to position in the human protein. (as cited in Rao, Luo and Hogan, 1997; For more information: Ensembl: http://www.ensembl.org ; NCBI: http://www.ncbi.nlm.nih.gov ; UCSC http://genome.cse.ucsc.edu).

Table 1.1 Proposed nomenclature for NFAT family proteins and chromosomal location of the genes (as cited in Rao, Luo and Hogan, 1997).

Proposed	***Nomenclature***	***Chromosomal location***	***Chromosomal location***
	Current	***Human***	***Mouse***
NFAT1		20q13.1-13.31 (Li et al., 1995; Luo et al., 1996b)	2[a] (Li et al., 1995; Luo et al., 1996b)
NFAT1a	NFATp (McCaffrey et al., 1993), NFAc2(Li et al., 1995), NFAT1A(Luo et al., 1996a)		
NFAT1b	NFAT1b (Luo et al., 1996a)		
NFAT1c	NFAT1c (Luo et al., 1996a)		
NFAT2		18q23-qter[b] (Li et al., 1995	18E4 (Li et al., 1995)
NFAT2a	NFATc (Northrop et al., 1994), NFATc1 (Li et al., 1995), NFATc.α (Park, Takeuchi, and Sharma, 1996)		
NFAT2b	NFATc.β (Hoey et al., 1995)		
NFAT3			
NFAT3	NFAT3 (Hoey et al., 1995)		
NFAT4		16q21-22 (Masuda et al., 1995)	8D (Ho et al., 1995)
NFAT4x	NFATx (Masuda et al., 1995), NFATc3 (Ho et al., 1995)		
NFAT4a, b, c	NFATa,b,c (Hoey et al., 1995)		

[a]The Nfat locus maps between *Ada* and *Gnas* (expressed as genetic distance in centiMorgans): centromere-*Ada*-3.1-(*Nfat1, Cebpb*)-4.8- *Gnas* (184)
[b]The human NFAT2 gene is closely linked to STS marker D18S497 (183)
NOTE: $NFAT_p$ = preexisting / $NFAT_c$ = cytoplasmic
NOTE: **For more information**: Ensembl: http://www.ensembl.org ; NCBI: http://www.ncbi.nlm.nih.gov ; UCSC http://genome.cse.ucsc.edu).

It must be noted that NFAT is used as a general abbreviation, which stands for the NFAT family proteins as well as NFAT containing complexes. The first member of

the family (NFATp, later renamed NFAT1) was purified from cytoplasmic extracts of a murine IL-2 promoter (Jain et al., 1993; McCaffrey et al., 1993) and cloned from murine (Ar-5) and human (Jurkat) T cell cDNA libraries (McCaffrey et al., 1993; Luo et al., 1996; Rao, Luo and Hogan, 1997). A Unifying nomenclature for NFAT proteins is proposed in Figure 1.18 and Table 1, which also list the chromosomal location of the NFAT genes (For more information: Ensembl:http://www.ensembl.org ; NCBI:http://www.ncbi.nlm.nih.gov ; UCSC:http://genome.cse.ucsc.edu). Rao, Luo and Hogan (1997) have suggested the present complicated nomenclature from the isolation, in different laboratories, of multiple isoforms and species variants of the same protein; and from the fact that the names NFATp (preexisting) and NFATc (Cytoplasmic), which were originally used to describe NFAT DNA binding activity, were carried over to the recombinant proteins (Rao, Luo and Hogan, 1997).

Sequence Homologies, Functional Domains

Members of the NFAT family are divided according to primary structure homology into four subgroups: NFAT1 (1A, 1B, and 1C), NFAT2 (NFATc.a and NFATc.b), NFAT3, and NFAT4 (4a, 4b, 4c, and NFATx). NFAT proteins vary in size from NFAT4a (708 amino acid residues) to NFATx (1075 amino acid residues) (Rao, Luo and Hogan, 1997). Two major regions of homology exist among these subgroups: the DNA-binding domain (DBD) and the NFAT homology region (NHR). The DBD is positioned within amino acid residues ~400 and ~700 in the known isoforms of NFAT and shows some similarity to the DBD of Rel-family transcription factors (Figure 1.14) (Rao, Luo and Hogan, 1997).

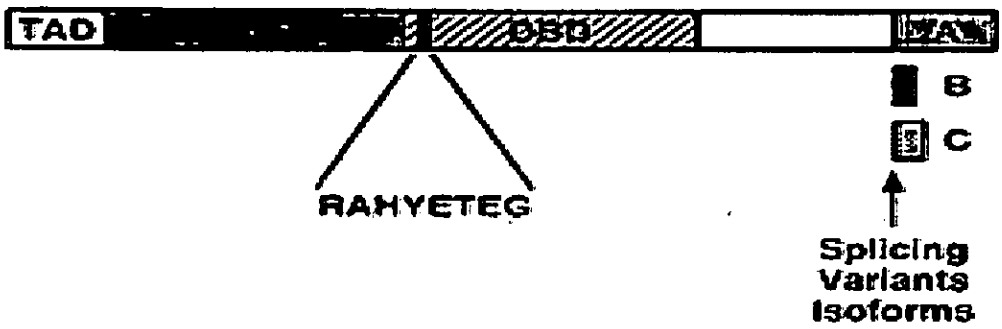


Figure 1.14: Schematic diagram of the primary structure of the NFAT1 protein, as deduced from analysis of cDNA clones. The region of highest homology within NFAT proteins is the DNA-binding domain (DBD), which shows similarity to the Rel homology region of Rel-family transcription factors, and encodes the amino acids that contact DNA. Other regions such as transactivation domain (TAD), NFAT homology region (NHR), and splicing variants isoforms are indicated (Source: Memorias do Instituto Oswaldo Cruz-on line: http://iris.dbbm.fiocruz.br/www-mem/index.html).

The NHR is about 300 amino acid residues in length and is located N-terminal to the DBD. Although the DBD sequences of different isoforms of NFAT proteins have distinct affinities, they bind to a consensus sequence of T(A)GGAAANT(A/C) (Chin et al., 1998; Rao, Luo and Hogan, 1997; Fiering et al., 1990). This site is present in many genes involved in immune responses, including interleukin-2 (IL-2), IL-4, IL-5, granular macrophage colony-stimulating factor (GM-CSF), IFN-g, and IL-13 (Rao, Luo and Hogan, 1997). In many cases, for DNA binding and transactivation, NFAT proteins cooperate with AP-1, which binds c-Jun/fos dimers. Complexes formed from cooperative binding of NFAT proteins, AP-1, and DNA are significantly more stable than complexes of NFAT and DNA alone (Hoey et al., 1995; Ho et al., 1995).

In resting cells, NFAT proteins are phosphorylated and reside in the cytoplasm. Stimuli that increase calcium mobilization result in the activation of NFAT proteins. The rapid dephosphorylation and translocation of NFAT proteins to the nucleus characterize activation; upon translocation, the dephosphorylated proteins show higher affinity for DNA. CsA or FK506 can block each step in the activation of

NFAT proteins (Chin et al., 1998; Hemenway and Heitman, 1999; Rusnak and Mertz, 2000). Therefore, NFAT activation follows precisely the activation of calcineurin by increased free calcium levels in the cytoplasm (Ruff et al., 1995; Loh et al., 1996). Researchers from diverse fields are interested in the NFAT family of transcription factors, which are potential targets for newer and safer immunosuppressive drugs (Rao, Luo and Hogan, 1997). In addition, the activation of NFAT proteins involves various cellular signal transduction pathways, including calcium mobilization and MAP kinase pathways linked to T-cell receptors and Ras (Rao, Luo and Hogan, 1997).

Tissue Distribution, potential Target Genes

Despite their name, NFAT proteins are expressed not only in T cells, but also in other classes of immune and non-immune cells. At the protein level, NFAT1 and NFAT2 are expressed in peripheral T cells and T cell lines, and NFAT1 is also expressed in B cells, mast cells, NK cells, monocytes and macrophages (Ho et al. 1994, Aramburu et al. 1995, Ruff & Leach 1995, Wang et al. 1995, Weiss et al. 1996). Moreover, NFAT1 is expressed in a neuronal cell line and in the nervous system (Ho et al. 1994), and an endothelial cell line (Cockerill et al. 1995, Wang et al. 1995). NFAT1 and NFAT2 mRNAs are expressed in peripheral lymphoid tissue (spleen), and NFAT2 mRNA is upregulated in activated T cells and NK cells (Northrop et al. 1994, Aramburu et al. 1995, Hoey et al. 1995, Masuda et al. 1995, Park et al. 1996). NFAT4 mRNA is expressed at high levels in the thymus (Hoey et al. 1995, Ho et al. 1995, Masuda et al. 1995), and NFAT3 is expressed at low levels in lymphoid tissues (Hoey et al. 1995).

The transcript sizes and reported sites of expression of NFAT mRNAs are listed in Table 1.2, following, where the information were taken from its complete version in

Rao, Luo and Hogan, 1997 publication (all the tissues are listed were NFAT family members have been identified by different laboratories as well as the techniques used). Furthermore, it is indicated that a correlation with protein isoforms has only been done in the case of NFAT2a and NFAT2b. Likely, after analysing NFAT1 it was shown that protein and mRNA expression do not always correlate, although NFAT1 mRNA has been variably detected in brain, heart and skeletal muscle, NFAT1 protein expression has not been detected in circulating lymphocytes or resident mast cells (Wang, McCaffrey, and Rao, 1995; Ruff and Leach, 1995). As illustrated in Table 1.2, mRNA of NFAT1, NFAT2a, NFAT2b, and NFAT4 have been identified in muscle, while just NFAT1 and NFAT3 were found in the heart (Rao, Luo, and Hogan, 1997).

Table 1.2 Tissue distributions of NFAT family members

		Tissue distribution	Tissue distribution
Member	mRNA size	mRNA	Protein
NFAT1	8 kb (h,m)	Thymus, spleen, **heart**, brain (Northrop et al., 1994), **muscle** (Hoey et al., 1995)	Thymus, spleen and many others but not present in cardiac or skeletal muscle
NFAT2a (NFATc.α)	2.7 kb (h)	Thymus, spleen, **muscle** (Hoey et al., 1995; Masuda et al., 1995)	T cell, mast cells, not detected in NK cell, not determined in other tissues
NFAT2b (NFATc.β)	4.5 kb (h)	Thymus, colon, spleen, **muscle** (Hoey et al., 1995; Masuda et al., 1995)	Not determined
NFAT3	3 kb (h)	Placenta, kidney,	Not determined
	4.5 kb (h)	**Heart**, colon,	Not determined
	4.7 kb (h)	Low in brain, spleen, thymus Hoey et al., 1995)	Not determined
NFAT4	7.0 kb (h)	Thymus, spleen, kidney, **muscle** (Hoey et al., 1995; Masuda et al., 1995; Ho et al., 1995)	Not determined

Abbreviations: h, human; m, mouse

(Source: Rao, Luo, and Hogan, 1997)

1.6.3 Regulation of NFAT activation

NFAT transcription factors are cytosolic proteins constitutively expressed in resting cells (Rao et al. 1997). NFAT proteins are activated by stimulation of receptors coupled to calcium mobilization, such as the antigen receptors on T and B cells (Yaseen et al. 1993, Choi et al. 1994, Crabtree & Clipstone 1994, Venkataraman et al. 1994, Jain et al. 1995, Serfling et al. 1995, Loh et al. 1996b), Fce receptors on mast cells and basophils (Hutchinson & McCloskey 1995, Prieschl et al. 1995, Weiss et al. 1996), the Fcg receptors on macrophages and NK cells (Aramburu et al. 1995), and receptors coupled to heterotrimeric G proteins (Desai et al. 1990, Wu et al. 1995, Boss et al. 1996). Three different steps of activation have been defined for NFAT proteins: dephosphorylation, nuclear translocation, and DNA binding. In resting cells, NFAT proteins are phosphorylated and cytoplasmic, and show a low affinity for DNA (Shaw et al. 1995). Stimuli that trigger calcium mobilization result in rapid dephosphorylation of NFAT proteins and their translocation to the nucleus, and dephosphorylated proteins show increased affinity for DNA (Chin et al., 1998; Shaw et al. 1995, Loh et al. 1996a,b). Receptor stimulation and calcium mobilization result in activation of the calmodulin-dependent phosphatase calcineurin (Chin et al., 1998; Weiss & Littman 1994). In the nucleus, NFAT forms complexes with the AP-1 family members, the MEF2 family as well as other transcription factors, leading to transcriptional activation (Chin et al., 1998; Karen et. al., 1998). The calcineurin inhibitors CsA or FK506 can block each step of NFAT activation, suggesting that calcineurin is a major upstream regulator of NFAT proteins, and that dephosphorylation is the initial step of NFAT activation (Chin et al., 1998; Hemenway and Heitman, 1999; Rusnak and Mertz, 2000).

In lymphocytes, the general role of NFAT proteins in the induction of early gene expression in response to antigen receptor engagement is well defined (Rao et al., 1997). On the other hand, the involvement of NFAT in gene expression has been shown in other tissues like skeletal muscle, where NFAT proteins are expressed at all three stages of myogenesis: myoblasts, nascent and mature myotubes (Karen et. al., 1998). Moreover, different developmental stages of muscle may differ to the degree to which either intracellular calcium or AP-1 can be elevated. In addition, it has been shown that NFAT 4/x/c3 is the only isoform in high-density myoblasts as well as in the heart that undergoes nuclear translocation in response to an intracellular calcium stimulus (Ruwhof and Van der Laarse, 2000). Karen and co-workers (1998) have suggested that in lymphocytes each isoform undergoes nuclear translocation with the same kinetics in response to a given stimulus. In contrast, in skeletal muscle cells each isoform undergoes nuclear translocation only at specific stages of myogenesis. The ability of individual NFAT isoforms to undergo calcium-induced nuclear translocation at only specific stages of myogenesis suggests that individual NFAT isoforms may regulate distinct subsets of genes necessary for muscle cell physiology (Karen et. al., 1998) (see Table 1.3).

Table 1.3. Each NFAT isoform undergoes nuclear traslocation at specific stages of muscle differentiation (as cited in Karen et al., 1998).

	Stage of myogenesis	Stage of myogenesis	Stage of myogenesis
NFAT isoform	Myoblasts[a]	Myotubes (nascent)[b]	Myotubes (mature)[c]
C	(-)[d]	(+)[e] Nuclear selectivity	(+) Nuclear selectivity
P	(-)	(+) All nuclei	(-)
4/x/c3	(+)	(-)	(-)

[a] 80-90% confluent.
[b] 24 h in FM (fusion medium).
[c] 75-80 h in FM.
[d] (-) indicates lack of calcium- dependent nuclear translocation.
[e] (+) indicates occurrence of calcium- dependent nuclear translocation.

This fact also suggests specificity in the activation of NFAT beyond just that amplitude and the duration of changes in intracellular calcium (Dolmetsch et. al., 1997). This specificity may occur in the level of NFAT dephosphorylation by calcineurin (Chow et al., 1997), and possibly individual isoforms of NFAT exist in different subcellular compartments that differ in accessibility to calcium (Thomas et al., 1996) or downstream effectors.

1.6.4 DNA binding and transactivation

The nerves innervating slow muscles are characterised by a tonic motor activity, which achieves sustained $[Ca^{2+}]_i$ levels and activates the calcineurin pathway. Calcineurin dephosphorylates NFAT, which in turn binds to DNA in conjunction with other transcription factors like MEF2. Chin et al. (1998) suggest that the binding sites, where NFAT and other transcription factors bind DNA, are clustered in promoter-enhancer regions controlling transcription of genes encoding proteins of the slow fiber program (Chin et al., 1998) (Figure 1.5).

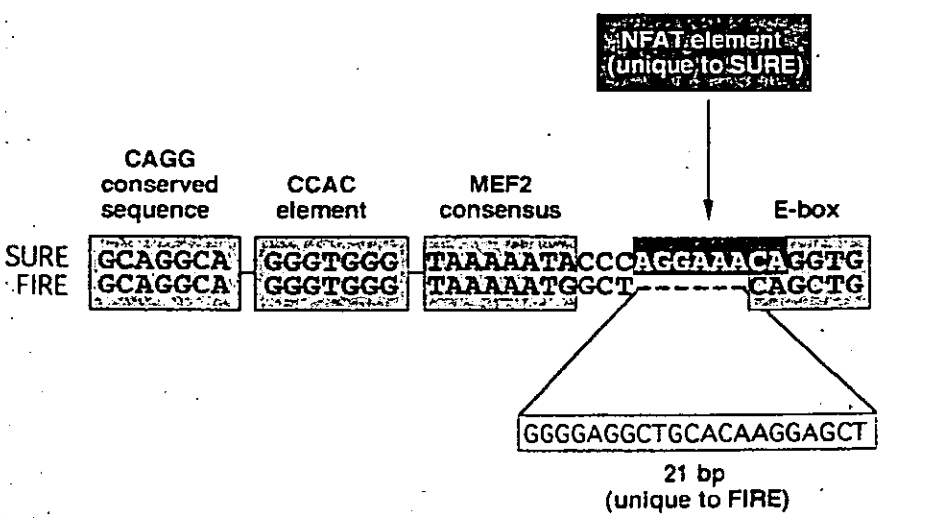


Figure 1.15 NFAT consensus binding sequences, which are present in transcriptional control regions, which Chin et al. (1998) have shown to direct transcription selectivity in slow-oxidative myofibers. Conserved sequence blocks [CAGG, CCAC, MEF2, and E box] common in rat skeletal muscle genes are presented (as cited in Chin et al., 1998).
SURE: Slow Upstream Regulatory Element; FIRE: Fast Intronic Regulatory Element

In addition, it has been shown that a continuous active calcineurin selectively transactivates promoters from two genes that are expressed preferably in slow genes (Chin et al., 1998). The DNA-binding domains of NFAT proteins are likely to be similar in three-dimensional structure to the DNA-binding domains of Rel-family proteins, and to utilize corresponding loops to make some of their contacts with DNA (Jain et. al., 1995; Nolan, 1994; Chityl and Verdine, 1996). The minimal DNA-binding domain (~190 amino acids) of NFAT1 (Jain et. al., 1995) corresponds almost exactly to the N-terminal specificity domain of NF-KB p50, which makes the majority of base-specific contacts with DNA (Müller et. al., 1995; Ghosh et. al., 1995). NFAT proteins are monomeric in solution and when bound to DNA, whereas Rel proteins are dimeric (Hoey et. al., 1995). NFAT proteins can also bind to certain κB-like sites (Chityl and Verdine, 1996). The monomeric nature of NFAT proteins explains why the minimal DNA binding fragment of NFAT1, which lacks the region corresponding to the C-terminal dimerization domain of Rel proteins, can bind independently to DNA (Jain et. al., 1995). In addition, it has been shown that all four of the NFAT family proteins are able to bind to IL-2 promoter NFAT site, after binding the AP-1 proteins (Hoey et. al., 1995; Masuda et. al., 1995; Park, Takeuchi, and Sharma, 1996).

Luo and colleagues, in their 1996 publication suggest that both the N- and C-terminal regions of NFAT contain transcriptional activation domains. Moreover, NFAT proteins have been shown to bind to sites that are, or resemble, binding sites of the Rel family proteins. In addition, sites in which the GGAAA core sequence is preceded by a T rather than an A appear to bind NFAT proteins more strongly (Rao, Luo, and Hogan, 1997). The general DNA binding sequence of the NFAT family proteins as derives for all the findings so far is T(A)GGAAAA(N)T(A/C)N (where N= A/T/G/C)

(Chin et al., 1998; Rao, Luo, and Hogan, 1997). Finally, an important feature of NFAT- dependent promoters and enhancers is the presence of multiple NFAT binding sites. Each of these regulatory regions contains three to five sites for NFAT binding, within a total length of 200 to 300 base pairs (Rao, Luo, and Hogan, 1997). It is so hypothesised that the implication is that higher-order, synergic interactions among NFAT- containing complexes are required for effective transcription (Rao, Luo, and Hogan, 1997).

Finally, Chin and co-workers (1998) in their study have suggested that DNA binding of NFAT is insufficient to cause signal transduction via the calcineurin pathway. NFAT transcription factors require collaboration with MEF2 and/or other transcription factors in order to cause fast-to-slow differentiation.

1.7 Inhibition of Calcineurin by Cyclosporin A (and FK506)

A number of natural products have been isolated that are potent inhibitors of calcineurin as well as the other serine /threonine protein phosphatases. The best-known inhibitors are the immunosuppressant drugs Cyclosporin A (CsA) and FK506 (Figure 1.16), which inhibit calcineurin when complexed with their respective cytoplasmic receptors cyclophilin and FKBP (Figure 1.17).

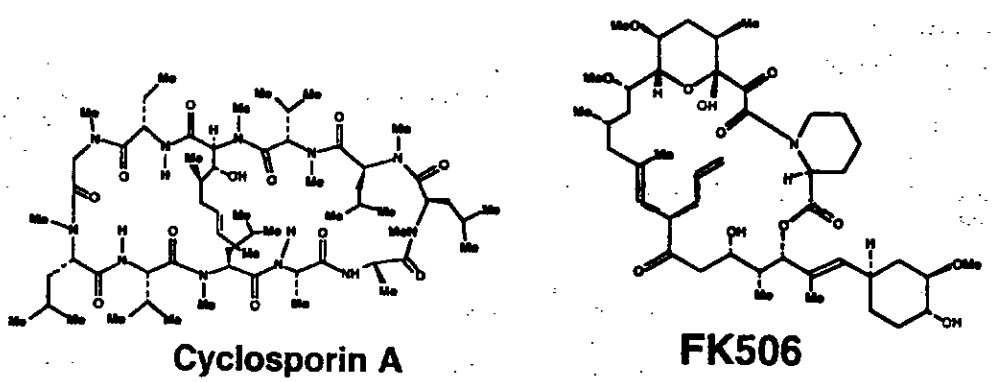


Figure 1.16. Natural product inhibitors of calcineurin, the immunossupressant drugs CsA and FK506 (as cited in Rusnak and Mertz, 2000).

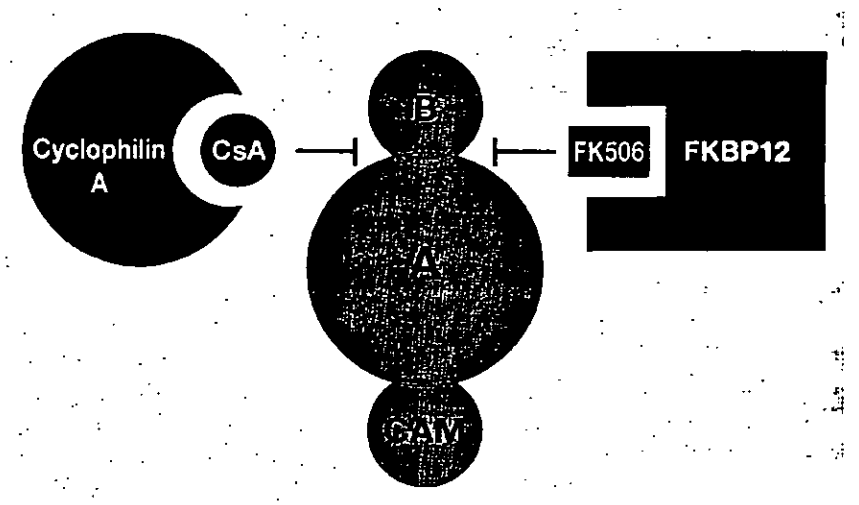


Figure 1.17. Immunophilin- immunosuppressant complexes inhibit calcineurin. The natural products CsA and FK506 associate with highly conserved intracellular binding proteins, the immunophilins cyclophilin A and FKBP12, to form the active protein- drug complexes that bind to and inhibit the protein phosphatase calcineurin. Calcineurin is a heterodimer composed of catalytic (A), regulatory (B), and calmodulin (CAM) subunits. The cyclophilin A-CsA and FKBP12- FK506 complexes bind to a groove between the calcineurin A and B subunits (as cited in Hemenway and Heitman, 1999)

Moreover, cyclophilin A and FKBP12 have similar structural features, while Cyclosporin A (CsA) and FK506 are very different compounds when it comes to structure (Figure 1.16). In each case, drug binding occurs within the hydrophobic catalytic site (Hemenway and Heitman, 1999). Despite these dissimilarities between the immunophilins and the immunosuppressants, the resulting complexes cyclophilin A-CsA and FKBP12-FK506 may share some common features (Hemenway and Heitman, 1999). The FKBP12-FK506 complex neither blocks nor induces an observable conformation change in the active site. Kissinger et al. (1995) have presented evidence that FKBP12- FK506 inhibits calcineurin in a classical non-competitive fashion. This has been shown to be the case for cyclophilin A-CsA inhibition as well (Etzkorn et al., 1994).

In understanding the regulation mechanism for Calcineurin, the cyclophilin-cyclosporin A(CyP-CsA) -binding site is very important. It appears to be the same binding site for binding of FKBP12-FK506 (Liu et al., 1992) and the site for binding

of AKAP79 (Crabtree, 2000). All three exhibit classical non-competitive inhibition, suggesting a common mechanism. The suggested Calcineurin-binding domain on AKAP79 is has high sequence similarity to residues 32-47 of FKBP12. It has also been found that a 22-residue peptide encompassing this FKBP12 sequence inhibits Calcineurin by other than steric hindrance (Kawamura, 1995).

Wei and Lee (1997) recently showed that mutagenesis of the L7 loop connecting β-strands 12 and 13 has significant effects on activity. Both modification of the L7 loop and truncation of the C-terminus lead to the activation of Calcineurin. They also determined that the effects of mutation of the L7 loop are separable from the effects of deletion of the AID. It is thought that CnA exists in a conformationally restrained state that is not completely relaxed by CnB and CaM. It has double inhibition to ensure that it only acts when needed (Wei and Lee, 1997).

In recent years, five different classes of calcineurin inhibitors have been discovered; each of these (Table 1.4) inhibits calcineurin by binding to the protein inhibiting its ability to dephosphorylate substrates, like NFAT (in skeletal muscle and other tissues), preventing their nuclear localization.

Table 1.4. Cellular inhibitors of calcineurin function (as cited in Crabtree, 2000)

Protein	Function	Kd nM or Ki nM	References
AKAP79	Scaffolding protein, Inhibits NFAT function	100-200	51
Cain/ cabin	Implicated in T cell activation and exocytosis in neurons Inhibits NFAT function	440	46,47
CHP	Similar to CnB, Prevents nuclear translocation of NFATc1	4000	49
DSCR1 (MCIP1)	May be involved in Down's Syndrome. Prevents nuclear translocation of	70 nM**	43,52,53

	NFATc proteins		
DSCR2 (MCIP2)	N.D.	N.D.	52,53
ZAK4 (DSCR3)	Prevents nuclear translocation of NFATc1	N.D.	43,54
rcn1p/CBP1	Related to DSCR1, mutants are action sensitivity and defective for crzy/tcn function.	6,500* (peptide) 7,000 (protein**)	44,45
A23SL	Blocks NFATc translocation; viral encoded	N.D.	50

*This measurement was made with a synthetic peptide derived from rcn1p/CBP1 and the Ki for the full-length protein might be much lower. **Kyle Cunningham, personal communication with Crabtree, G.R. Stanford University, California.

DSCR1 gene and its relatives (DSCR2 and ZAK14) are thought to be the most important calcineurin inhibitors of the above (Rothermel et al., 2000). DSCR1 is the Down's syndrome Critical Region 1 gene and is located on chromosome 21. As it is well known a trisomy, which causes the Syndrome, results in the over expression DSCR1, which in turn effects the development of the brain, immune system, heart and skeleton. Moreover, the CHP proteins seem to compete with CnB for binding to the A protein, while Cabin is a non-competitive inhibitor of calcineurin phosphatase activity. The A238L protein encoded by a virus (African Swine Fever virus) binds tightly to calcineurin and blocks NFATc translocation. The final inhibitor is AKAP79 protein, was the first calcineurin inhibitor found, bind both calcineurin and PKA and may anchor calcineurin at specific sites that allow the protein to engage the proper substrates when activated.

1.8 Muscle fibre differentiation induced by Calcineurin- the role of calcium

Calcium signalling plays a very important role in heart and skeletal muscle hypertrophy in response to mechanical load and a variety of intrinsic and extrinsic stimuli. These calcium alterations have been shown to cause long-term changes in muscle gene expression, apart from hypertrophy, but the exact mechanism is yet to be clarified. As mentioned before calcineurin is though to be the key molecule involved in the control of gene expression (Chin et al., 1998). As shown in 1996 by Mayne and co-workers, calcineurin controls the contractile and metabolic properties of a skeletal muscle by activating the Ca^{2+}-dependent slow muscle fibre gene program (Figure1.18; as cited in Chin and co-workers in1998).

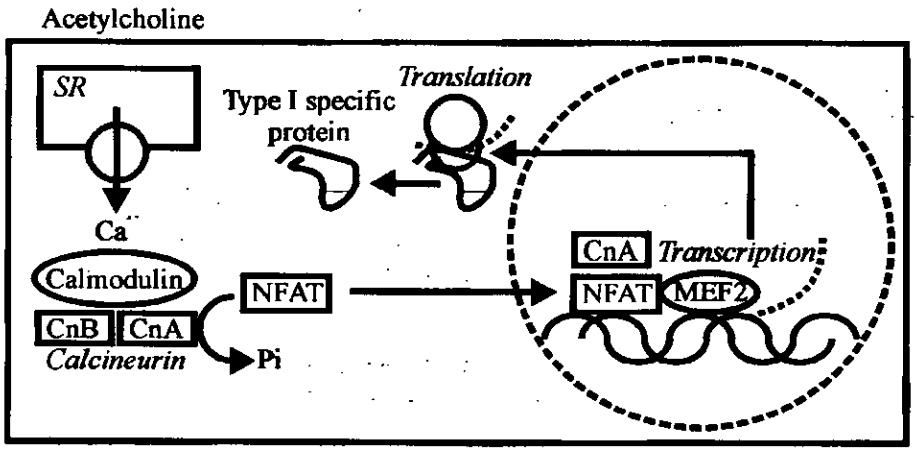


Figure 1.18. Calcineurin dependent gene regulation in skeletal muscle. CnB, CnA Calcineurin B and A. NFAT Nuclear factor of activated T-cells. MEF2 Myocyte enhancer factor 2. SR Sarcoplasmic reticulum (Chin et al., 1998).

Fibre-type specific programs of gene expression can be detected at early stages of myogenic development in the embryo (DiMario et al., 1993; Ontell et al., 1993; Stockdale, 1997). On the other hand, they are thought to remain plastic in adults, and are shown to be controlled by contractile load, hormonal shifts and systemic diseases

(Holloszy and Coyle, 1984; Massie et al., 1998; Ianuzzo et al., Sabbah et al., 1993; Williams and Neufer, 1996).

Skeletal myofibers are known for their specialized characteristics when it comes to size, metabolic activity and contractile function. The slow-twitch (Type I) muscles have as their main function the maintenance of posture, balance and opposition to gravity forces. They are also rich in mitochondria and enzymes of oxidative metabolism. Moreover, slow myofibers have been shown to express specialized forms of myosin and other sarcomeric proteins that convert chemical energy into contractile work (Olson and Williams, 2000; Chin et al., 1998). On the other hand, Fast- twitch muscles function concentrates in powerful bursts of contractile work, hence generation of movement. Fast myofibers express different sarcomeric proteins, form those of the slow-fibers, and are known to generate force rapidly. They are enriched in enzymes of glycolysis and are not energy efficient (Type IIb). Type IIa fibers are Fast fibers of a different subset that resemble type I fibers with respect to high expression of proteins of oxidative metabolism (such as myoglobin and mitochondrial enzymes of oxidative phosphorylation) (Olson and Williams, 2000).

An increase in the intracellular calcium ion concentration has been shown to control a wide range of cell function like motility, adhesion, gene expression and proliferation (Ghosh and Greeberg, 1995; Berridge, 1993). Chin et al., in 1998, have shown that activation of calcineurin in skeletal myocytes selectively up-regulates slow-fibre-specific gene promoters, while inhibition of this pathway by CsA promotes slow to fast fiber transformation.

Studies have shown that the calcineurin-NFAT pathway is not sensitive to transient, high-amplitude oscillations in $[Ca^{2+}]i$. On the other hand, calcineurin-NFAT pathway responds perfectly to sustained, low-amplitude elevations of $[Ca^{2+}]i$ (Timmerman et

al., 1996; Dolmetsch et al., 1997). This suggests that calcineurin pathway has the ability to distinguish and discriminate between different patterns in the amplitude and duration of changes in $[Ca^{2+}]i$, and influence in proportion fibre type specific gene expression (Figure 1.19).

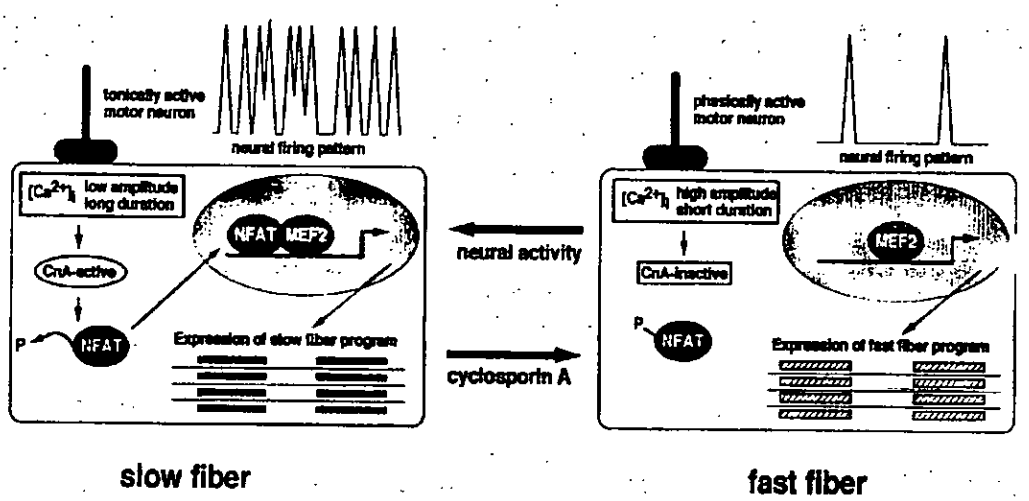


Figure 1.19. Model for a calcineurin-dependent pathway linking specific patterns of motor nerve activity to distinct programs of gene expression that establish phenotypic differences between slow and fast myofibers. MRF2 is shown to represent the requirement for collaboration between activated NFAT proteins and muscle-restricted transcription factors in slow-fiber-specific gene transcription, but other proteins (not shown) also are likely to participate (as cited in Chin et al., 1998).

Tonic motor nerve activity at 10-15 Hz is a characteristic of slow- twitch fibers (Hennig and Lomo, 1985). Chin and Allen (1996) have provided information suggesting that this nerve activity leads to a sustained elevation of $[Ca^{2+}]i$ within a concentration range between 100 and 300 nM (Chin and Allen 1996). This pattern is thought to activate the Calcineurin pathway (Chin et al., 1998). On the other hand, Westerblad and Allen (1991) have shown that in fast myofibers intracellular calcium resting levels are maintained at 50 nM (Westerblad and Allen 1991). A high amplitude (1 μM) of calcium has been suggested to be insufficient duration to evoke calcineurin-stimulated signalling (Chin et al., 1998).

Moreover, it has been shown that at single fiber level, as well as in skeletal muscle tissue, chronic stimulation can alter mitochondrial composition, cause myosin heavy chain changes towards the slow phenotype (Wehrle, Dűsterhőft, and Pette, 1994), and enzyme metabolism changes (Chi et al., 1986; Henriksson et al., 1986). After the change to a slow phenotype, caused by chronic stimulation, all the fibers resemble slow fibers in terms of histochemical classification, fatigue and contraction time patterns (Mayne et al., 1996; Jarvis et al., 1996; Dirk, 1984).

Calcium evoked changes in cultured muscle cell line (L_6 myotubes)

Motor neuron activity is known to cause sarcolemmal depolarisation and transient increases in cytosolic Ca^{2+}, which in turn leads to muscle contraction through actin and myosin interactions. The cytosolic Ca^{2+} concentration varies from <100 nM in resting muscle to about 1-2 μM in contracting muscle fibers (Allen, Lee, and Westerblad, 1989; Westerblad and Allen, 1991). The primary source of calcium in the muscle cells is the sarcoplasmic reticulum (SR) (Robert et al., 1998; Vander et al., 1994). It is now well known that after each contraction calcium goes back to the SR, through an active re-uptake mechanism, which involves a Ca^{2+}- ATPase pump located in the SR membrane. The role of Ca^{2+} in mediating contraction has been well studied, in contrast with its role in skeletal muscle gene regulation (Huang et al., 1994; Shin et al., 1997). Ginty (1997) presented data implying that cytosolic calcium fluctuations are involved in the alteration of gene expression in different cell types through several mechanisms (Ginty, 1997).

Calcium is additionally thought to play a crucial role in development and cell differentiation in the neuromuscular system. The process of myoblast fusion during skeletal myogenesis is calcium regulated (Seigneurin-Venin et al., 1996). In addition,

both dihydropyridine receptor and ryanodine receptor are already present on muscle precursors, at the perfusional stage, before they are required for excitation-contraction coupling (Seigneurin-Venin et al., 1996). Moreover, it is thought that the gap junction formation (Rash and Fambrough, 1972; Balogh, Naus, and Merrifield, 1993) is likely to be involved in the synchronization and metabolic coupling of myoblasts during the fusion process (Mege et al., 1994). Likely, transmembrane Ca^{2+} flux, controls the conversion to the multinucleate state, since fusion is stimulated by the Ca^{2+} ionophore A23187 (David, See and Higginbotham, 1981) and inhibited by D600 (Entwistle et al., 1988), an L-type Ca^{2+} channel antagonist. The L-type Ca^{2+} channel is a complex of five subunits ($\alpha 1$, $\alpha 2$, β, γ, δ) of which the $\alpha 1$ subunit contains the binding sites for dihydropyridine (DHP-receptor, DHPR) and other Ca^{2+} channel modulating drugs (Seigneurin-Venin et al., 1996).

In adult skeletal muscle, the DHPR is mainly localized in the transverse tubular system (T-tubules). There it mediates the long-lasting Ca^{2+} current and also serves as the voltage sensor for excitation-contraction coupling (Fosset et al., 1983; Romey et al., 1989; Catterall, 1991). Other findings suggest that at these junctions mentioned, the DHPR associates with the Ca^{2+} release channel of the sarcoplasmic reticulum (ryanodine receptor, RyR) to form an electron-dense structure called the foot of the triad (Franzini-Amstrong, 1970; Marty et al., 1994). However, Seigneurin-Venin et al. (1996) in their study mention that in addition to being present in adult muscle, both the DHPR and the RyR are expressed throughout myotube fusion and consequently before any excitation-contraction coupling takes place (Fosset et al., 1983; Airey, Baring, and Sutko, 1991; Kyselovic et al., 1994; Seigneurin-Venin et al., 1996). Differentiation of skeletal muscle cells is known to be associated with glycosylation events as shown in Sato and Miyagi publication, in 1996. As mentioned before, the

L6 cells are produced from myoblasts (satellite cells). These were obtained from the skeletal muscle of a rat, which was one day old at sacrifice (Yaffe and Fachs, 1967). A reduction of the fetal bovine serum (FBS) in the α-MEM used for culturing, from 10% to 2% caused the cells to undergo differentiation and fuse into myotubes (Sato and Miyagi, 1996). The maturation of the myotubes can be detected by the multinuclei cell formations, by activating factors such as MyoD1 and myogenin (Specific for skeletal muscle) (Sato and Miyagi, 1996), and by specific proteins expressed in skeletal muscle, such as actin and myosin heavy chain, and Creatine kinase (Friday, Horley, and Parlath, 2000; Incerpi et al., 1995; Seigneurin-Venin et al., 1996).

There is strong evidence suggesting that the muscle regulatory factor family (Myf-5, Myo-D, myogenin, MRF4) of transcription factors is involved in regulating myogenesis. In addition, it has been shown that they are regulated by phosphorylation and could therefore be potential targets for phosphatase activity of calcineurin (Friday, Horley, and Parlath, 2000).

The first part of this study involves the investigation of the role of Ca^{2+} in skeletal muscle gene regulation. This type of research has limitations when it comes to manipulating intramuscular Ca^{2+} concentrations in vivo independently of muscle contraction. For this reason, the second part of the study was performed on a cultured muscle cell line, L6 myotubes (Osawa, Printz, and Granner, 1993). Apart from the 'advantage' this tissue model has; there is strong evidence suggesting that, when these L6 cells are at the myotube state, they resemble with mature skeletal muscles, since they have similar morphological, metabolic, and biochemical characteristics (Yaffe and Fachs, 1967; Klip et al., 1984).

1.9 Other transcription factors and calcium induced pathways involved in muscle fibre plasticity

There is evidence that Myocyte enhancer factor 2 (MEF2) is associated with calcineurin dependent gene regulation (Chin et al., 1998), and interacts with myogenic regulatory factors (MRF's: MyoD, Myf-5, myogenin, MRF4) causing an amplification of muscle protein expression (Molkentin et al., 1995; Cox et al., 2000). In addition, MEF2 is also though to interact with several mitogen-activated protein kinases (MAPK; Bennett and Tonks 1997, Zetser et al., 1999, Cox 2000). Other interactions of the MEF2 proteins have been reported, like with the class II histone deacetylases HDAC4 and HDACS, resulting in repression of MEF2-dependent genes. Calcium/calmodulin-dependent protein kinase (CaMK) signalling, stimulates myogenesis, prevents formation of MEF2-HDAC complexes, and induces nuclear export of HDAC4 and HDAC5 by phosphorylation of these transcriptional repressors (McKinsey et al., 2000).

In 1997, Dolmetsch and co-workers have shown that the amplitude and the duration of calcium signals in B lymphocytes controls differential activation of the pro-inflammatory transcriptional regulators NF-kB, c-Jun, N-terminal kinase (JNK) and NFAT. Moreover, NF-kB and JNK are selectively activated by a large transient $[Ca^{2+}]_i$ raise, whereas a low, sustained Ca2+ plateau activates NFAT. Differences in the Ca^{2+} sensitivities and kinetic behaviour of all three above pathways cause this differential activation.

Murgia et al., (2000) illustrated that the slow myosin heavy chain protein is induced by a constituvely active Ras in regenerating muscle. Ras is a mitogen- activated protein (MAP) kinase kinase, which phosphorylates the extracellular signal regulated kinase (ERK1,2). ERKs, in turn, translocate into the nucleus where they activate

transcription factors (Cruzalegui and Bading, 2000), like NFATn (Crabtree, 1999). Moreover, an increased ERK activity in regenerating and stimulated muscle, illustrated by Murgia and co-workers in 2000 publication, suggests a physiological role of Ras/ERK signalling. In 2000, Crabtree suggested that Ca^{2+} signalling becomes dependent on coincident Ras/PKC signalling and vice versa. In nearly all cell types studied, activation of Rac, Ras or PKC must accompany a Ca^{2+} signal for activation of NFAT- dependent transcription (Figure 1.20).

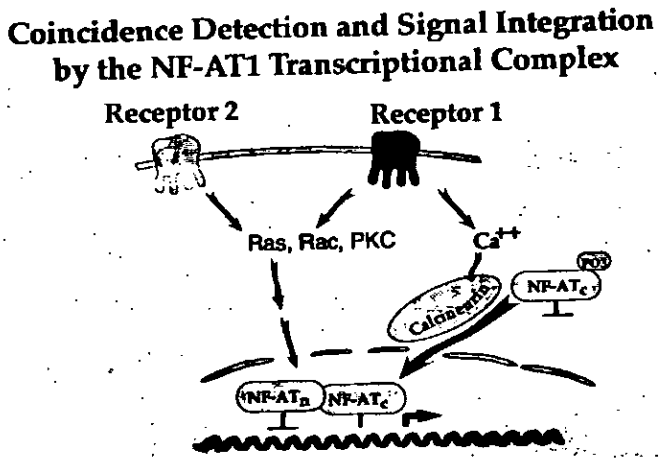


Figure 1.20. Ras or Rac or PKC must be coincident with Ca^{2+} / calcineurin signalling to activate NFAT and in turn genes. More that one receptor can activate the above pathways, like in T cells (as cited in Crabtree, 2000).

In addition the movement of NFATc3 into the nucleus is enhanced (rather than blocked) by the actions of either JNK kinase (Chow et al., 1997) or the combination of MEKK1 and CK1 (Zhu et al., 1998). NFAT is also a substrate for glycogen synthase kinase (GSK-3) but this has only been reported in lymphoid cells (Beals et al., 1997). GSK-3 is thought to be a potential export kinase for NFAT family of proteins. In order to maintain NFAT in the nucleus, the persistent activation of calcineurin via the CRAC (Capacitance Regulated Activation Channels) is required (Crabtree, 2000).

It has been shown that blockade of calcineurin with CsA prevents insulin like growth factor (IGF-1) induced hypertrophy, suggesting that this type of hypertrophy is mediated by Calcineurin (Dunn et al., 1999; Semsarian et al., 1999; Musaro et al., 1999), which seems to be the main pathway involved (Figure 1.26) (Olson and Williams, 2000; Dunn, Burns, and Michel, 1999).

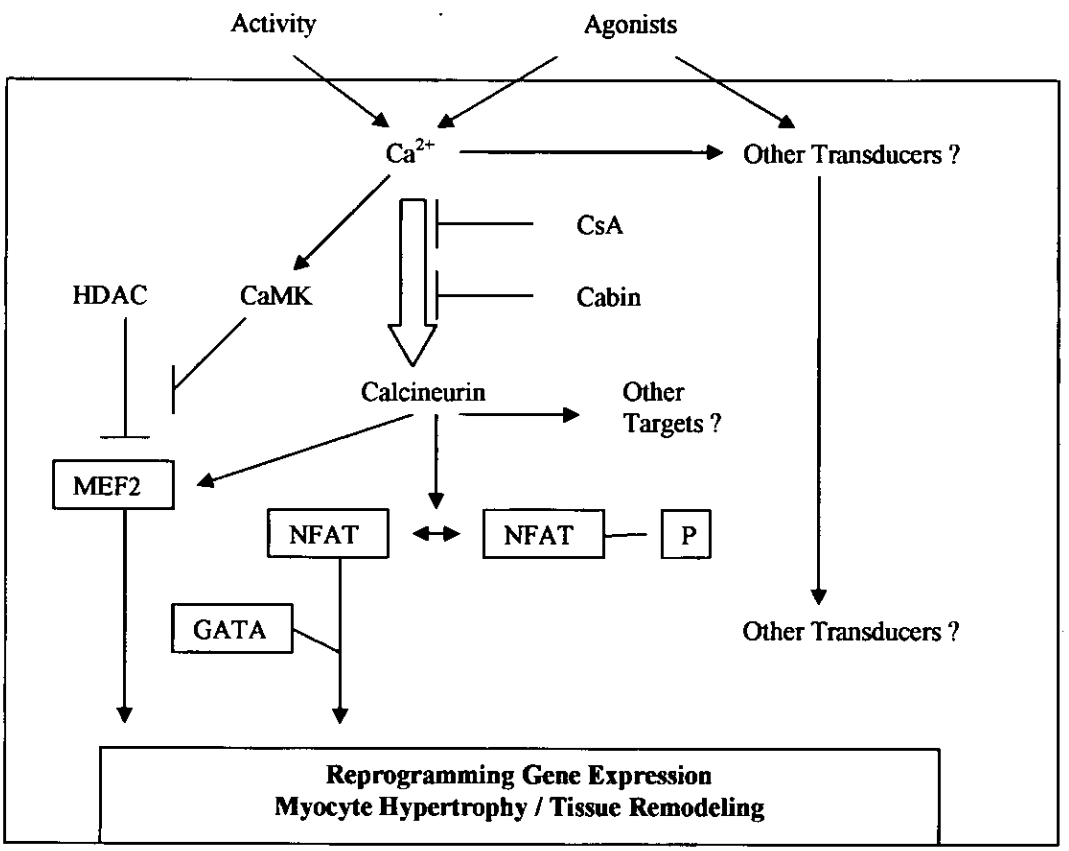


Figure 1.21. Ca^{2+} Signalling Pathways Implicated in Myocyte Remodelling and Hypertrophy (as cited in Olson and Williams, 2000 **b**)

Both calcineurin and its activity-linked upstream signalling elements are crucial for muscle adaptation to overload (Dunn, Chin, and Michel, 2000). In addition, IGF's are acting by stimulating expression of L-type Ca^{2+} channels, which in turn increase near-membrane Ca^{2+} concentrations (Olson and Williams, 2000).

Both IGF and Calcineurin induce expression of GATA2 in skeletal muscle, which associates with calcineurin and NFATc1 (Olson and Williams, 2000).

Myostatin, a member of the TGF-β superfamily expressed specifically in skeletal muscle, appears to act as an inhibitor of skeletal muscle hypertrophy, since strains of livestock or transgenic mice bearing mutations in the myostatin gene exhibit massive increases in muscle size (McPherron, Lawier, and Lee, 1997).

The pathways mentioned are though to be 'interacting' with the calcineurin pathway, by either affecting the pathway itself or via a different routes regulating gene expression in skeletal muscle. As a result, they have to be taken into consideration, since calcineurin might be one of the most important gene regulation pathways but definitely not the only one.

1.10 Scope of the study

The main objective of this study was to investigate the role of calcineurin in calcium induced muscle fiber differentiation both in rat skeletal muscle and in L_6 rat muscle cell line. There are four specific aims:

1. To investigate the histological and metabolic changes, after 3 weeks of 10Hz chronic stimulation, in rat skeletal muscle.
2. To investigate the differences in the calcineurin content between fast-twitch and slow-twitch skeletal muscle fibers.
3. To investigate the inhibitory effect of cyclosporin A on the calcineurin pathway under the effect of chronic stimulation in rat skeletal muscle.
4. To determine the mechanism of action of calcineurin, through NFAT phosphorylation and nuclear translocation, in L_6 rat skeletal muscle myocytes.

METHODS

2.1 Induction of a fast- oxidative phenotype by chronic muscle stimulation (*in vivo study*) and investigation of the effect of cyclosporin A : histochemical and metabolic studies

2.1.1 Muscle fibre determination

2.1.2 Calcineurin and NFAT localization and translocation

2.1.3 Investigation of changes on enzyme activities of four metabolic pathways

2.2 The effect of increased intracellular Ca^{2+} in NFAT translocation on L_6 Muscle cell cultures (*in vitro study*)

2.2.1 L_6 muscle cell culture

2.2.2 Increase intracellular Ca^{2+} of L_6 myocytes using Calcium Ionophore 4-Bromo-A23187

2.2.3 Fura-2 $[Ca^{2+}]_i$ measurements in L6 myocytes grown on cut glass coverslips

2.2.4 Effects of increased intracellular Ca^{2+} in NFAT localization and translocation

2.3 Statistical analysis

METHODS

2.1 Induction of a fast- oxidative phenotype by chronic muscle stimulation (*in vivo study*) and investigation of the effect of cyclosporin A : histochemical and metabolic studies

Ten Adult Sprague Dawley rats of either sex were divided to groups and all of them received 10 Hz stimulation to the left limb. The right limb was used as a control for every treatment. In addition, all animals with the code name "Green" received vehicle injections (no cyclosporin), while the ones labelled "Black" and "Red" received cyclosporin injection throughout the whole period of stimulation (see Table 2.1). The naming based on colour identifies different animal groups that have been used. These names are for lab organization only and these groups have no special features, have not received any special treatment and were randomly used. The chronic stimulation, termination and sample collection procedures were performed in collaboration with Dr. S.Salmons, Dr. J.Jarvis and Dr. H.Sutherland at the *Department of Human anatomy and Cell biology, University of Liverpool, Liverpool L69 3 BX, United Kingdom.*

Table 2.1 Animal code name and stimulation pattern relation

Animal code name	*Amount of stimulation (Days)*
Green1	17
Green 2	18
Green 3	19
Green 4	19

Green 5	20
Black 1	18
Black 2	18
Red 1	19
Red 2	19
Red 3	20

Chronic stimulation

All 10 animals were prepared for aseptic surgery with a preanesthetic medication of diazepam (Valium, 5 mg/kg; Roche Products) and atropine sulfate (Bimeda, 3 mg/kg) delivered subcutaneously, followed after 20 min by an intramuscular injection of Hypnorm (Janssen Pharmaceuticals; fluanisone, 10 mg/ml, and fentanyl citrate, 0.315 mg/ml, 0.3 ml/kg). Miniature electrical stimulators (see Jarvis and Salmons, 1991) were implanted into these animals under strictly aseptic conditions. The electrodes were arranged to stimulate the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles via the common peroneal nerve. Further details of the procedure may be found in the companion papers (Jarvis et al., 1996 and Mayne et al., 1996). In all cases, the nerve was subjected to a continuous train of pulses at a frequency of 10 Hz. Stimulation was confined to muscles in the left hindlimb; the right was used as a non-stimulated control. This pattern was used since no evidence was found relealing differences of either systemic or contractile effects in a variety of previous experiments (Henriksson et al., 1986) (also see discussions: Eisenberg and Salmons, 1981; Salmons and Henriksson, 1981).

Anaesthesia procedures

Animals were anesthetized by intravenous injection of urethane (50 mg/kg; Sigma Chemical) followed immediately by pentobarbital sodium (Sagatal, 30 mg/kg to effect; May and Baker). Deep anaesthesia was maintained by supplementary intravenous injections of pentobarbital sodium.

Sample collection

The method of "freeze- nibbling" (Mayne, Jarvis, and Salmons, 1991) was used for the purpose of measuring metabolite levels. This consisted of clamping small samples from the surface of the exposed muscle with forceps that had been cooled in liquid nitrogen. A small cut under the forceps freed the frozen tissue with minimal bleeding. This sampling procedure has been shown to have no significant effect on the subsequent mechanical performance of the muscle (Myane, Jarvis, and Salmons, 1991). Biopsies were stored at -77°C, pending analysis.

After the physiological measurements had been completed, further samples for biochemical analysis were obtained from the whole TA and EDL muscles by freeze-clumping the distal section in brass- jawed tongs cooled to the temperature of liquid nitrogen. Tissues were then wrapped in cool aluminium foil and stored at -77°C in hermetically sealed bags, pending analysis.

The procedure mentioned above was entirely performed in the Department of Human anatomy and Cell biology, University of Liverpool, by licensed personnel. The samples were kindly provided to the Muscle research group of the Biological Sciences department at the of the University of Central Lancashire pending further analysis.

For the histological and histochemical analysis, a transverse slice through the widest part of the muscle was mounted on a cork disk, frozen in melting isopentane above liquid nitrogen, and stored in the -80°C. The muscle samples were held upside down just long enough for the fibres to hang down straight before placed in the isopentane. The samples were left in the liquid nitrogen once they had been frozen for further analysis. The ideal thickness for the ATPase and NBT staining is though to be 7-10 μm muscle sections in the microtome at - 22 °C, while for immunohistochemical techniques 5 μm. The sections were mounted on glass gelatin coated slides.

2.1.1 Muscle fibre determination

Skeletal muscle is made of muscle cells (fibers) having different contraction speeds. Muscles that are active most of the time are typically slow contracting while muscles that have bursts of activity are typically fast contracting. These differences in contraction speed are the result of different myosin isoforms that have different ATPase activities. Mammalian muscles may be separated into type I or type II, as the major fiber types, using the calcium dependent adenosine triphosphatase (ATPase) reaction. The differences in alkaline and acid stabilities of myosin ATPase are the basis for this stain. A method to determine muscle fiber type (fast or slow myosin ATPase) is to cut a section then treat it with acid. This acid kills the activity of the fast myosin ATPase activity. This is followed by washing and adding ATP which is hydrolyzed to ADP + Pi. The amount of Pi is detected by a chemical reaction that makes a dense precipitate with the Pi. Thus, the slow fibers stain black and the fast fibers have no stain. Using mATPase histochemistry, four major fiber types, three fast and one slow, are currently delineated in skeletal muscle of small mammals. One of the problems with this method is the species variation in acid liability of myofibrillar

ATPase. However, Tunell and Hart in 1977 report a rapid and reproducible method, which we used after slight modifications, that enables three (or more) fiber types to be determined from a single slide, making this diagnostic procedure applicable for routine use. In this method the sections are treated with pre-incubation medium at pH 7.25, resulting in slow fibers staining white, while fast fibers stain from light dark to black (Tunell and Hart, 1977; Brooke and Kaiser, 1970; Pette and Staron, 1997).

The fist method used in this study, for the muscle fibre identifications was the ATPase staining technique (Modified protocol of the original: Tunell and Hart, 1977).

Reaction	Enzyme
ATP $\leftrightarrow$ ADP + P_i	Ca,Mg-ATPase

Figure 2.1 The enzyme catalysing the ATP hydrolysis (Zubay, Parson and Vance, 1995).

The sections were treated with the pre-incubation medium (pH 7.25) of the following composition Glycine (100 nM), Calcium chloride (1%) and formaldehyde (3.7%) for 5 min, following a 90 minutes incubation period with the incubation medium [ATP (2.8 mM), Sodium barbital (100 mM) and $CaCl_2$ (2%)]. After treating the sections with 1 % calcium chloride solution and 2 % cobalt chloride solution, 2 % ammonium sulphide solution was applied to give the reaction the black and white staining. This myofibrillar ATPase method clearly demonstrates three or even four fibre types in each section.

Table 2.2 ATPase results interpretation.

Stain	Type I fibers	Type IIa fibers	Type IIx fibers	Type IIb fibers
ATPase	**0**	**1**	**2**	**2**

Legend: **0** no staining; **1** light stain; **2** heavy stain.

In order to verify the results of the ATPase staining NADT-TR (slight modification of the: Novikoff, Shin, and Drucker, 1961) staining was performed to all the muscle sections of the samples of Table 2.1. This stain stains for mitochondrial enzymes such as succinate dehydrogenase. The sections were stained for 20 to 30 minutes with the Stock NADH-TR solution [TRIS base (40mM), TRIS HCL (160mM) and Nitro blue tetrazolium (1mg ml^{-1})] with an addition of 1 mg of NADH per tube on the day of the experiment. Sometimes, three fibre types (or four) can be determined. The type I and IIa fibres stain dark, while Type IIx and IIb stain light. However, normal results show the following:

Table 2.3 NADH-TR results interpretation.

Stain	Type I fibers	Type IIa fibers	Type IIx fibers	Type IIb fibers
NADH-TR stain	**2**	**2**	**1**	**0-1**

Legend: **0** no staining; **1** light stain; **2** heavy stain.

2.1.2 Calcineurin and NFAT localization and translocation

1. Immunofluorescence studies: Calcineurin A

Additional sections (thickness 5 μm) reacted with Calcineurin A antibody (1:100 dilution in PBS) (anti-rat CnA raised in mouse, monoclonal obtained by Santa Cruz), which has been shown to be specific for Calcineurin A (CnA). A secondary antibody (1:100 dilution in PBS) anti-mouse, raised in goat, was then bound to the primary antibody. This was visualised with the FITC conjugated (fluorescein-5-isothiocyanate with Excitation: 490 and Emission: 520) of the secondary, which is fluorescent green. The sections were fixed with in 4 % paraformaldehyde for 30 min and goat serum

(1:10 dilution in PBS) was used as blocking serum, in order to avoid non-specific binding. The sections were also treated with 1 % triton X-100 for 1 minute in the permeabilisation step before the blocking serum addition, while PBS washes of the following composition NaCl (120mM), KCl (2.7mM), NaH_2PO_4 (10mM) and KH_2PO_4 (1.5 mM), took place in the intervals. Moreover, 1.5 % of blocking serum was added to the primary antibody as Santa Cruz recommend.

2. Immunofluorescence studies: NFAT

The exact same procedure was used for the NFAT staining. This time NFATc1 anti-rat, raised in goat (provided by Santa Cruz), was the primary antibody used (1:100 dilution in PBS), while the secondary was an anti-goat IgG raised in rabbit (1:100 dilution in PBS). This was visualised with the Cy3 conjugated secondary, which is fluorescent red. In addition, rabbit serum (1:10 dilution) was used as the blocking serum, and1.5 % of that blocking serum was added to the primary antibody as Santa Cruz recommend.

3. Nuclear identification

The investigation of NFAT translocation to the nucleus was performed with the use of a DNA stain, DAPI (4', 6-diamidine-2'-phenylindole, dihydrochloride, optional, stains blue) stain (Excitation: 372; Emission: 456). Dilute DAPI stock solution (1:2000 with distilled water) was applied to the same sections used for CnA or NFAT staining, for 3 minutes. This produced a fluorescent blue stain, which did not effect the other staining.

4. Microscopy

The microscopy set up used constitutes a fluorescent microscope with a special camera linked to a computer. The software package chosen was SPOT version 3.2.4 for windows. Diagnostic Instruments, Inc. 1997-2001.

5. Western blots

A small piece of muscle (30 mg) was homogenised (on ice) in 0.6 ml of homogenisation buffer [Tris HCl (50mM), Triton X-100 (0.1%), EDTA (1mM), EGTA (1mM), NaF (50mM), B-glycerolphosphate (10mM), Sodium pyrophosphate (5mM), 2-mercaptoethanol (0.1%) and Okadaic acid (100 nM)]. The samples were then rotated for 60 minutes at 4 °C and centrifuged at 13,000g and 4 °C for 10 minutes. The supernatant was measured for protein concentration using Bradford assay. Additions of 20 µl per lane were used and the protein was adjusted to 1 mg/ml (or more if the bands are not clear enough) by diluting in SDS sample buffer of the following composition Tris-HCl 0.5M, glycerol 27mM, SDS (stock) 10%, bromophenol blue (stock) 0.5% in Deionized water. Moreover, for biotinylated protein standards BIORAD recommended diluting 1:4 with SDS buffer, heating to 95 °C for 5 minutes and loading 10 µl per well for mini-gels. The samples were run on 10 % SDS-PAGE gel (200 V constant voltage) for approx 40 min. Moreover, both the gel and the nitrocellulose membrane were equilibrated in transfer buffer [Tris-base 25mM, glycine 192mM and methanol 20% in deionised water] for 30 minutes. The gel sandwich was prepared and then the transfer took place for about 1 hour at 100 V (constant voltage) and 350 mA. Following the completion of transfer, the membrane was incubated in 30 ml of blocking buffer of the following composition Tris buffer saline, 0.1% Tween-20 and 5% w/v non-fat dry milk powder with gentle agitation for

1 hour at room temp. After incubating in blocking buffer the membrane was washed 3 times for 5 minutes in 30 ml of wash buffer [Tris buffer saline and 0.1% Tween-20] again with gentle agitation. In addition, the membrane was incubated with the primary antibody [SIGMA monoclonal Anti-calcineurin (α- subunit)] overnight at 4 °C with gentle agitation (for monoclonal antibody in 25 ml of blocking buffer/ for polyclonal antibody substitute 5% non-fat milk powder with 5% BSA). For the calcineurin blot anti-CnA at a 1 in 1000 dilution was used. The membrane was again washed in 30 ml wash buffer with gentle agitation, 3 times for 5 minutes. The secondary antibody was applied at this state to incubate the membrane for 1 hour at room temp in 30 ml of blocking buffer with gentle agitation [secondary: (anti-mouse IgG HRP conjugate) at a 1 in 1000 concentration and streptavidin-HRP conjugate (to detect biotinylated protein standards) at a concentration of 1 in 200]. The first method used to reveal the "bands" was by washing the membrane with DAB, which was applied for 15 min to allow colour development. Finally, excess colour development was prevented by immersing the membrane in deionised water for 10 mins. The second method, which had far better results, was the Phototope HRP Western Blot Detection System. This method uses LumiGLO Reagent and peroxide. The membrane was incubated with lumiglo for 1 min. Lumiglo is a light producing substance, which is attached to the secondary antibody. The produced light was captured by a film (KODAK scientific imaging film: X-OMAT $25^{13 \times 18\ cm}$), which in turn was developed.

2.1.3 Investigation of changes on enzyme activities of four metabolic pathways

1. Extraction

The first step for the investigation of enzyme activities is the extraction of enzymes from the muscle samples tested (Modified protocol from: Cheng et al., 1997). The

muscle samples were freeze-dried for about 4 h and were cut to small pieces with scissors. About 1 mg of tissue was the homogenised with ice cold phosphate buffer to yield a 1:49 (w/v) dilution [$KHPO_4$ (100mM), β-mercaptoethanol (20mM), EDTA (2mM) and 0.02% BSA at pH 7.5]. The samples were then sonicated in order to disrupt all the membranes and then centrifuged for 2 min at 10,000 revolutions min^{-1} in a minifuge (Eppendorf: Centrifuge 5415R). The supernatant was than stored at –80 °C pending analysis.

2. Measurements of enzyme activities for the following enzymes:

Creatine Kinase Assay (CK), Spectrophotometer

All the enzyme activities were temperature (37 °C) and pH (pH 7) controlled based on the protocol of Passoneau and Lowry (1993). In contrast to a metabolite assay, the enzyme under investigation is a "bottle neck" in the pathway. For this reason, a lot of substrate was added, at least ten times the concentrations of the K_m Michaelis constant. Under these conditions, the reaction rate is maximal and independent of the substrate concentration.

Table 2.4 The reactions involved in Ceatine Kinase assay and the enzymes, which catalyse them (Passoneau and Lowry, 1993).

Reaction	*Enzyme*
P-creatine + ADP ↔ ATP + creatine	Creatine kinase
Glucose + ATP ↔ glucose-6-phosphate + ADP	Hexokinase
Glucose-6-phosphate + $NADP^+$ ↔ 6-P-gluconolactone + NADPH + H^+	Glucose-6-phosphate dehydrogenase

For this assay, 0.01 ml of extract (2-4 μg skeletal muscle) was used, 1 ml of creatine kinase reagent with the following composition [Imidazole base (100mM), Acetic acid (1N), ADP (1mM), Mg acatate (10mM), Dithiothreitol (5mM), AMP (20mM), glucose (2mM), $NADP^+$ (0.1mM) and 0.02% Bovine serum albumin (BSA)], 2.8 U ml^{-1} hexokinase dissoved in water, 0.18 U ml^{-1} glucose-6-phosphate dehydrogenase from yeast dissolved in water as well, and 0.01 ml of 2.5 M P-creatine solution.

In all enzyme measurements the room temperature was recorded and each determination was repeated twice. In addition, a 1 ml cuvette filled with distilled water was inserted into the spectrophotometer in order to zero the experiment (before every measurement). For this enzyme measurement 0.01 ml (10 μl) of extract and 1 ml of the creatine kinase reagent were added to a cuvette and the absorbance was read once stability was achieved (Figure 2.2). For all the assays, recordings of the absorbance for every single minute were obtained, until the reaction started to slow down. For the analysis: 100 μM of NADPH yield an absorbance of 0.627 in a 1 cm light path (ε = 6270).

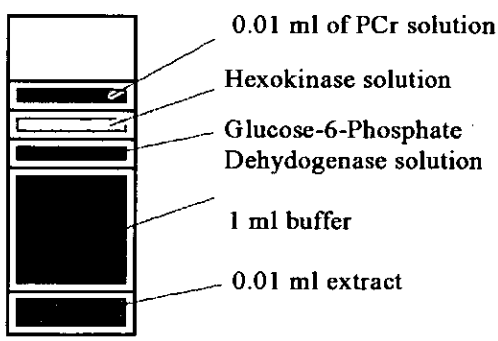


Figure 2.2 The extract and the buffer were added to the cuvette following glucose 6-Phoshate dehydrogenase and hexokinase solutions. Finally, 0.01 ml (10 μl) of 2.5 M phosphocreatine (PCr) was added and the cuvette was 'mixed' before any measurements were obtained.

Hexokinase Assay (HEX), Spectrophotometer

For this assay, 0.01ml of extract, 0.98 ml of hexokinase reagent [Tris HCl (50mM), Tris base (50mM), $MgCl_2$ (5mM), ATP (5mM), 0.5% Triton-X and 0.05% BSA], and 0.01 ml of glucose solution (200 mmol l^{-1}) were required. The reactions involved in this enzyme measurement are illustrated in the table 2.5.

Table 2.5 The reactions involved in Hexokinase assay and the enzymes, which catalyse them (Passoneau and Lowry, 1993).

Reaction	*Enzyme*
Glucose + ATP ↔ glucose-6-phosphate + ADP	Hexokinase (measured)
Glucose-6-phosphate + NADP $^{+}$↔ 6-P-gluconolactone + NADPH + H^{+}	Glucose-6-phosphate dehydrogenase (added)

For this enzyme measurement 10 µl of extract and 1 ml of the lactate dehydrogenase reagent were added to a cuvette and the absorbance was read once stability was achieved. Moreover, 10 µl of 200 mmol l^{-1} glucose was added to the cuvette, before the timer was started.

Lactate Dehydrogenase (LDH) Assay, Spectrophotometer

This assay was designed to obtain measurements of the rate of β-hydroxyacyl CoA dehydrogenase. The relative chemical reaction is illustrated in Table 2.6.

Table 2.6 The reaction in Lactate dehydrogenase assay and the enzyme involved. (Passoneau and Lowry, 1993).

Reaction	*Enzyme*
Pyruvate + NADH + H^{+} ↔ lactate + NAD^{+}	Lactate dehydrogenase

This assay requires 0.01ml of extract and 1 ml of lactate dehydrogenase reagent of the following composition Imidazole-HCl (50mM), Imidazole-base (50mM) and Sodium pyruvate (1mM). For this enzyme measurement 1 ml of the lactate dehydrogenase reagent was added to a cuvette and the absorbance was read once stability was achieved and following 0.01 ml (10 µl) of extract.

Malate Dehydrogenase (MDH), Assay Spectrophotometer

Measurements were obtained of the rate of β- hydroxyacyl CoA dehydrogenase, which requires 0.01ml of extract and 1 ml of malate dehydrogenase reagent of the following composition 2-amino-methyl-1,2-propanediol (50mM), glutamic acid; sodium salt (10mM), malic acid; monosodium salt (10mM). Malate dehydrogenase reagent (1ml) was added to a cuvette followed by 0.01 ml (10 µl) of extract before any measurements were obtained. The relevant chemical rections-taking place in the cuvette are shown in table 2.7.

Table 2.7 The reactions involved in malate dehydrogenase assay and the enzymes, which catalyse them (Passoneau and Lowry, 1993).

Reaction	*Enzyme*
Malate + NAD^+ ↔ Oxaloacetate + NADH	Malate dehydrogenase
Oxaloacetate + glutamate ↔ aspartate + α- ketoglutarate	Aspartate aminotransferase (or glutamic-oxalaetic transaminase, GOT)[1)]

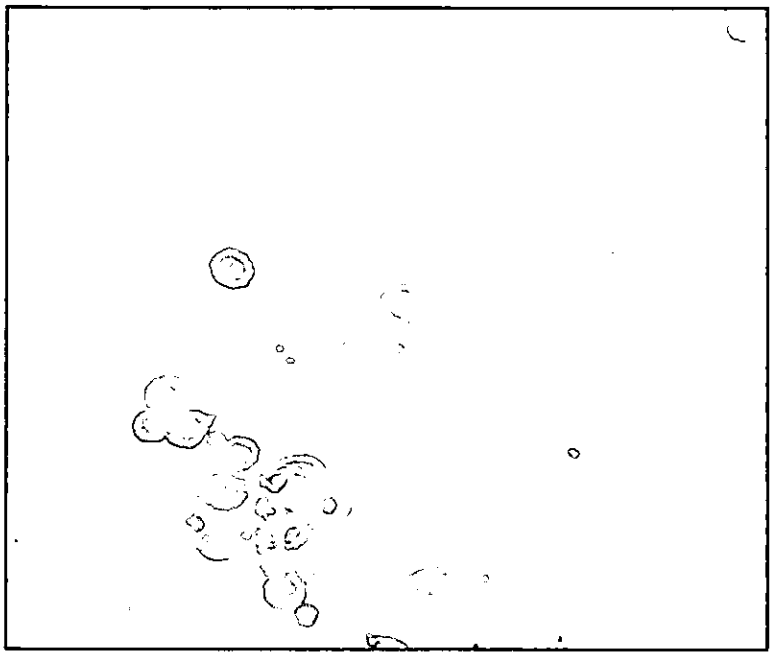
[1)]Oxaloacetate is removed with aspartate aminotransferase because of the unfavourable equilibrium of the malate dehydrogenase reaction

2.2 The effect of increased intracellular Ca^{2+} in NFAT translocation on L_6 Muscle cell cultures (*in vitro study*)

2.2.1 L_6 muscle cell cultures

Monolayers of L_6 cell cultures were prepared by a previously established method (Hundal et al., 1992). The L_6 cells are preserved (long term) frozen in liquid nitrogen (-192 °C), while the cryoprotectant DMSO added, protects the cell structures. The cells which were to be cultured, were removed from the low temperature environment to 37 °C, while the cryoprotectant DMSO was removed before subsequent use. Standard centrifugation (600 rpm) and supernatant removal techniques applied here. The cells were grown in α Minimal Essential Medium (MEM) to the differentiated stage of myotubes, in an atmosphere of 5% CO_2 and 5% air at 37 °C. Once the cell cultures reached 30% - 50% confluence they were sub-divided using trypsinisation and sub-culturing techniques. Most of the cells were cryopreserved in liquid nitrogen using freezing mixture and Mr. Frosty technology. This is an alternative to the temperature reduction schedule using liquid nitrogen vapour. This approach uses the thermal characteristics of a special freezing liquid (2-propanol or isopropyl alcohol) to gradual cool the cells to a point where they may be transferred to liquid nitrogen. After trypsinisation, trypsin was removed by centrifugation and re-suspension in fresh a-MEM. The cells were then sub-cultured before they were transferred the same way to coverslips.

1.



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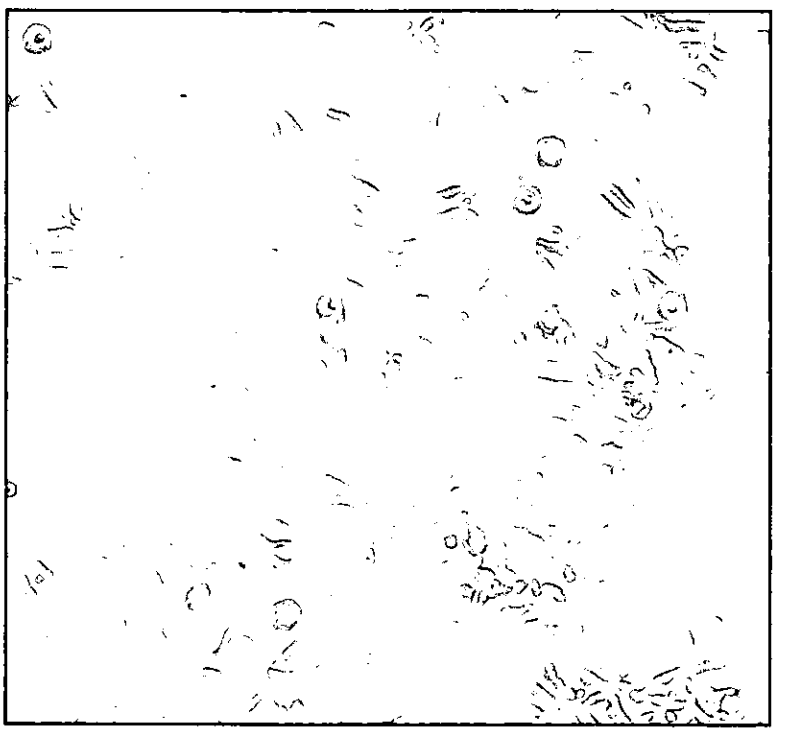
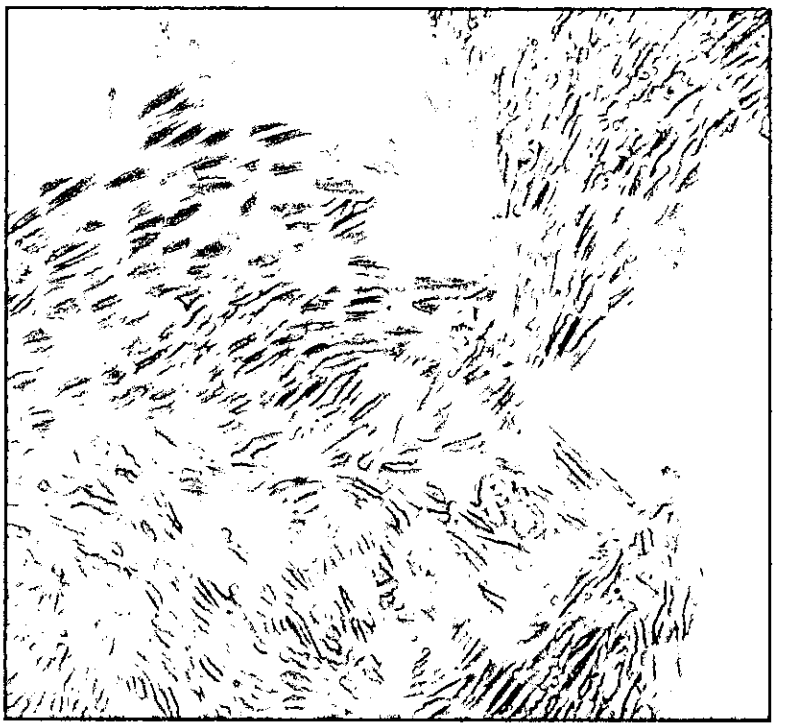


3. 

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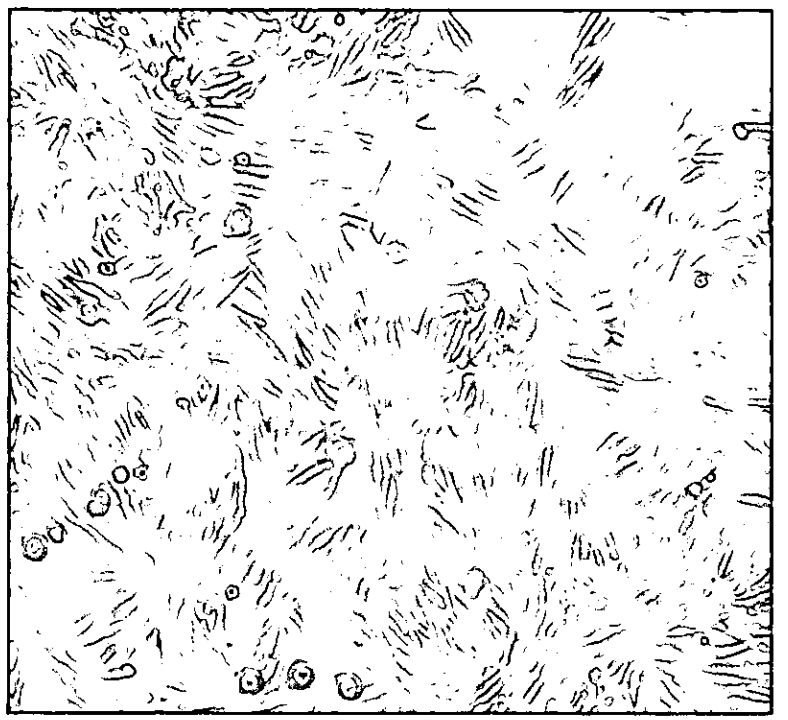

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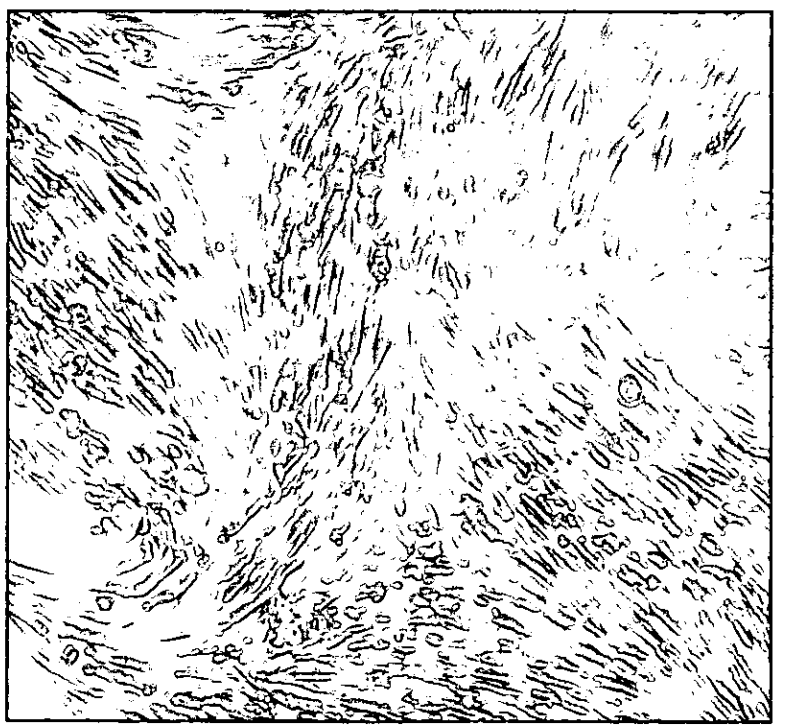


6.

Figure 2.3. L6 muscle cell line photographs in different stages of cell growth and confluency (in the flask): **1.** 10% **2.** 50% **3.** 70% **4.** 80% **5.** 90% **6.** 95%.

Standard glass coverslips (size 25 mm x 60 mm) were purchased from SIGMA Ltd. They were cut to quartz cuvette size using a diamond-tipped glass-cutter. They were

stored in absolute ethanol and were flamed before use. Both un-coated and 0.1 % gelatin-coated coverslips could be used for the culture of L6 myocytes and subsequent free Ca^{2+} determinations. Preliminary experiments indicated that there was no difference in the morphology or behaviour of the cells in either situation.

Two flamed coverslips were placed in the bottom of standard 6 cm plastic culture dishes and covered with $5cm^3$ of complete culture medium (α MEM containing 8 % FCS). The cells were re-suspended in approximately 10 cm^3 of complete medium and approximately $1cm^3$ of cell-containing medium was gently 'dribbled' over the cut coverslips. It was inevitable that some of the cells would attach to the plastic dish rather than the coverslip however this is not important since once the cells had been allowed to attach the coverslips were moved to fresh Petri dishes.

Once the desired number of dishes had been set up the dishes were moved to the CO_2 incubator for a few hours or overnight to allow the cells to fully attach to the substrate. Once this had happened fresh dishes were set up using 5 cm^3 of complete medium. Using alcohol-dipped and flamed forceps the coverslips were then carefully moved to the new containers and replaced in the CO_2 incubator.

The degree of confluence of the culture was monitored daily and if necessary the cells were fed at required intervals. Once the cells had reached 70-80 % confluency the coverslips were placed in fresh Petri dishes containing differentiation medium (α MEM containing 2 % FCS) (Sato and Miyagi, 1996; Incepri et al., 1995). Myotube formation occurred and the cells were kept in the incubation for 24 hours until further analysis took place.

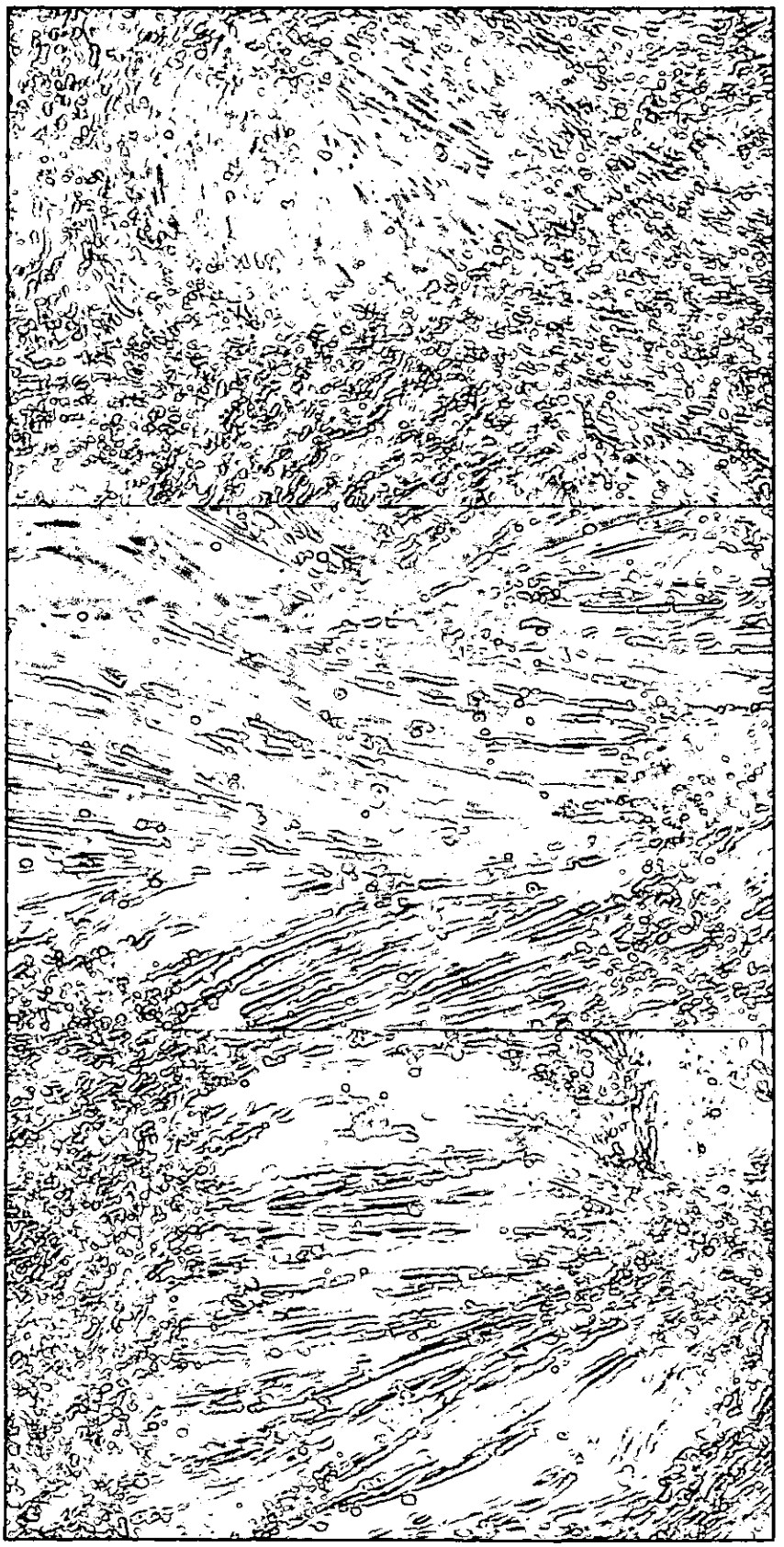


Figure 2.4. L6 muscle cell line grown in coverslips (photographs) in different stages of cell differentiation (myotube formation). In picture **1** the myotubes are in early stages, while complete myotube formation is observed in the other photographs (**2 and 3**).

The myotube formation was verified by an Eosin / Hematoxylin stain which revealed multi-nuclei myotubes. For this staining the cell were incubated with Hematoxylin for 10 minutes (in 25 °C) and after a wash with Eosin for 2 minutes. The following figure (Figure 2.5) illustrates pictures of L6 myotudes (multi-nuclei structures).

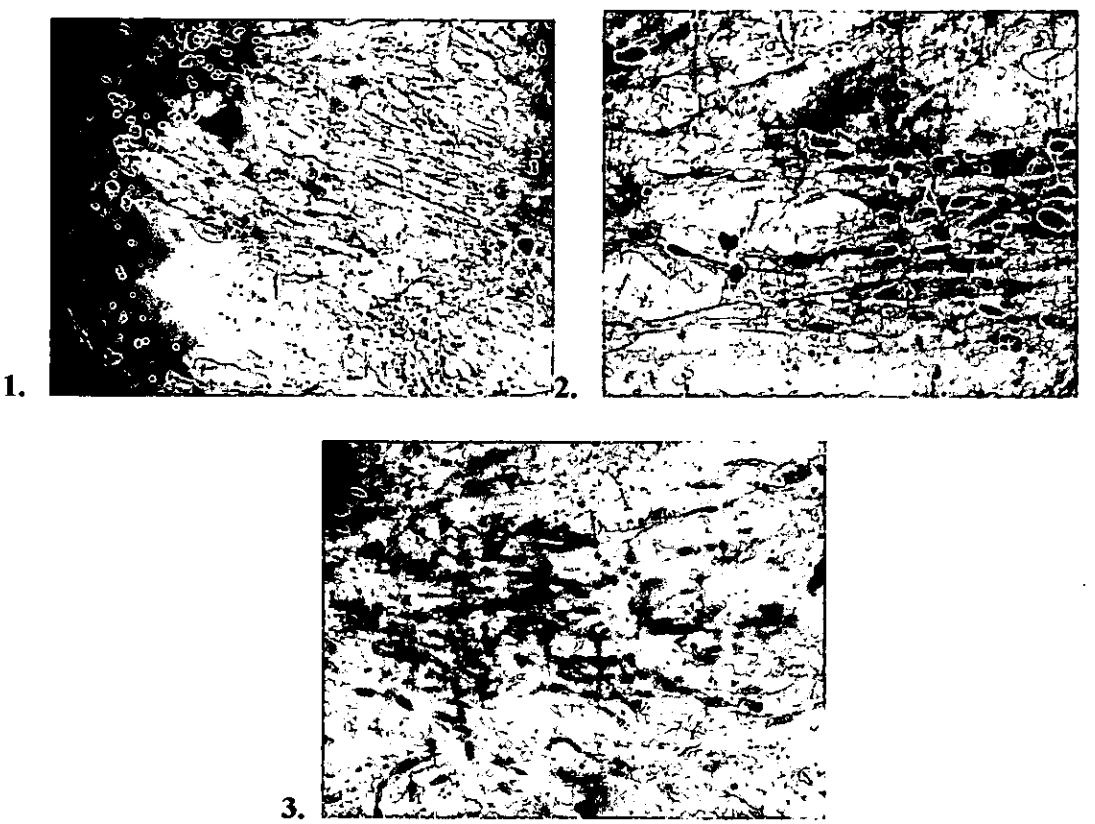


Figure 2.5. Eosin/Hematoxylin stain in L6 cells cultured in coverslips. Myotube formation is observed within 3 days after reducing the serum (FBS) in the α-MEM from 8% to 2% (pictures **1-3**).

2.2.2 Increase intracellular Ca^{2+} of L6 myocytes using Calcium Ionophore 4-Bromo-A23187

Intracellular calcium changes were investigated in L6 myocytes at the state of myotubes cultured in coverslips. The calcium measurements were performed using

the antibiotic A23187, which is only suitable as its halogenated analogue 4-bromo-A23187, as native A23187 exibits autofluorescence at the upper fura-2 wavelength (Mason, 1993). Addition of the ionophore causes a rapid, sustained rise in intracellular calcium, which reaches maximum within tens of seconds. This whole process was measured for about 16 min (960 sec), in order to allow the calcium to equilibrate (reach a steady plateau phase). A range of 4-bromo-A23187 concentrations was tested in order to investigate the cell line's response to that range and finally achieve the 'optimal' intracellular calcium increase: 50 nM, 100 nM, 200 nM, 400 nM, 500 nM, 1000 nM, 2000 nM, 4000 nM, 8000 nM (Meiβner et al., 2001; Halseth et al., 2000; Dolmetsch et al., 1997).

2.2.3 Fura-2 $[Ca^{2+}]_i$ measurements in L6 myocytes grown on cut glass coverslips

A modified protocol from Jung, Pfeiffer and Deitmer (2000) was used in order to investigate free intracellular calcium changes in the L_6 cells. The L_6 myotubes cultured in coverslips were removed from the culture medium and washed three times in 10 ml of washing medium (step 2 in figure 2.6). The coverslips were then placed in 5 ml Fura-2 loading medium [Fura 2 AM (acetoxylmethyl ester) purchased from Molecular Probe] containing 8 μM of Fura-2 and left for 50 min (step 3 in figure 2.6), in the dark. After removing them from the Fura-2 solution they were washed in 10 ml of washing solution (step 4 in figure 2.6). In addition, the coverslips were placed in a further 10 ml of washing solution and left for 30 minutes to liberate free Fura 2 inside the cells (step 5 in figure 2.6). Fura-2 fluorescence was then measured in cell populations with a Perkin- Elmer fluorescence spectrophotometer, as shown in step 6 in Figure 2.6. This instrument is equipped with a rotating filter wheel, which was used

to alternate 340 and 380 nm, excitation. Fura-2 displays emission peak at 510 nm, but two calcium-dependent absorption maxima, one at 340 nm, which increases with increasing ionised calcium and a second at 380 nm, which similarly decreases with arise in ionised calcium. A coverslip was mounted vertically on a 30 deg angle to the light path in a cuvette, which contained continuously stared washing medium. The software package used to obtain the measurements was FC WinLab 3.0 (1999: The Perkin-Elmer corporation), which automatically measures and subtracts background fluorescence.

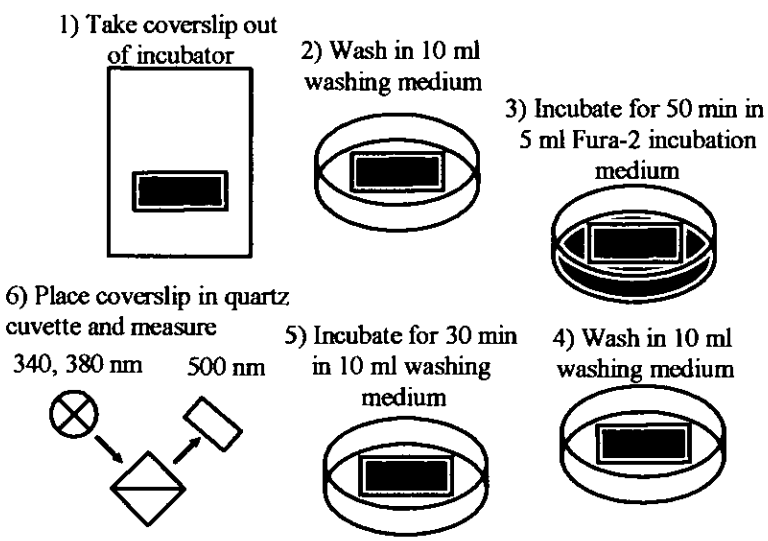


Figure 2.6. Schematical figure showing the steps involved in $[Ca^{2+}]_i$ measurements in L6 myocytes. Steps 3-6 should be carried out in the dark.

2.2.4 Effects of increased intracellular Ca^{2+} in NFAT localization and translocation

The final concentration, which elicited intracellular calcium increase to 'optimal level' was found to be 10^{-6}M of 4-bromo-A23187. In order to investigate NFAT

translocation immunohistochemical techniques were used. The L_6 cells were cultured for 24 h 10^{-6}M concentration before they were fixed with 100% methanol at -20 °C for 10 min.

Fixed L6 myotubes cultured in glass coverslips were incubated overnight at 4 °C with NFATc1 anti-rat, raised in goat (Santa Cruz) (1:100 dilution in 1% BSA in TBS). A secondary antibody (1:100 dilution in TBS for 30-45 minutes in room temperature in the dark) anti-goat IgG raised in rabbit, was then bound to the primary antibody. This was visualised with the Cy3 conjugated of the secondary, which is fluorescent red. Moreover, rabbit serum (1:10 dilution in TBS, 0.02% Sodium azide NaN_3 and 1% BSA) was used as blocking serum, in order to avoid unspecific binding. The sections were also treated with 1 % triton X-100 for 1 minute in the permeabilization step before the blocking serum addition, while TBS washes took place in the intervals. Control slides were used in order to investigate unspecific binding of the secondary antibody.

The investigation of NFAT translocation to the nucleus was performed with the use of a DNA stain, DAPI (4', 6-diamidine-2'-phenylindole, dihydrochloride, optional, stains blue) stain. Dilute DAPI stock solution (1:2000 with distilled water) was applied to the same sections used for CnA or NFAT staining, for 3 minutes. This produced a fluorescent blue stain, which did not effect the other staining.

2.3 Statistical analysis

Analysis of variance (ANOVA) was performed and statistical significance of differences was evaluated using students T test. The level of significance was defined as $P<0.05$.

RESULTS

3.1. Induction of a fast- oxidative phenotype by chronic muscle stimulation and investigation of the effect of cyclosporin A : histochemical and metabolic studies

3.1.1 The effect of chronic stimulation on muscle fiber differentiation

3.1.2 Calcineurin content of different muscle fiber types

3.1.3 The effect of chronic stimulation on enzyme activities of four metabolic pathways

3.2. Investigation of the effect of increased intracellular Ca^{2+} in NFAT translocation on L_6 Muscle cell cultures

3.2.1 The effect of Br-A89123 Ionophore in intracellular calcium concentration of L6 myotubes

3.2.2 The effect of increased intracellular calcium in NFAT localization and translocation

RESULTS

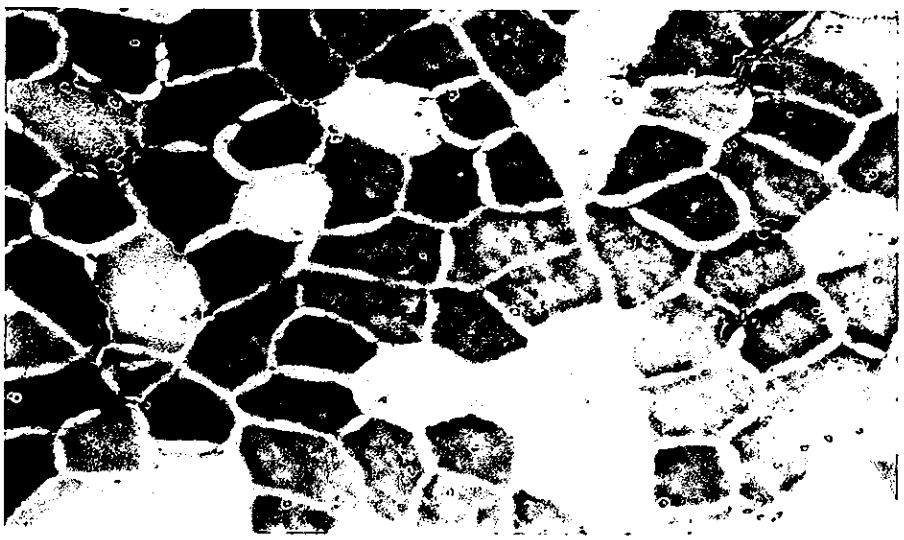
3.1. Induction of a fast- oxidative phenotype by chronic muscle stimulation and investigation of the effect of cyclosporin A : histochemical and metabolic studies

3.1.1 The effect of chronic stimulation on muscle fiber differentiation:

The sections were stained histochemically for the demonstration of myofibrillar ATPase, which revealed three (in some cases four: Figure 3.3) distinct fiber type populations in the rat muscles tested (TA: Tibialis Anterior EDL: Extensor digitorum longus Sol: Soleus) (Figures 3.1-3.4). In limb muscles of adult rodents, four distinct fiber types have been recognized, as described by Parry (2001), the slow type I and fast types IIa, IIx and IIb that express myosin heavy chains $MyHC_{I}$, $MyHC_{IIA}$, $MyHC_{IIX}$, $MyHC_{IIB}$, respectively (Parry, 2001). In large muscles such as tibialis anterior and gastrocnemius, there is a heterogeneous distribution of fiber types such that the superficial regions are composed of largely or exclusively types IIb and IIx (about 80:20 %, respectively, in mouse), with types I and IIa limited to deeper regions (Parry, 2001). That is why all the results compared are from the whole muscle, while the pictures compared are from same region of the muscle. Five samples (10 μm thickness sections) of each group shown in Table 2.1(see Methods section) were tested.

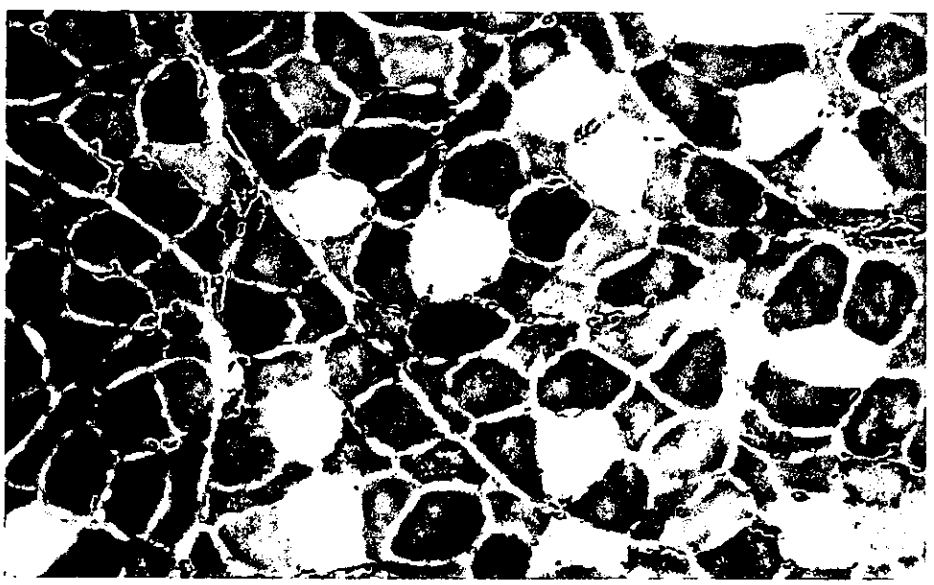
Purely histochemical evidence must, however, be interpreted with caution, since fibers that appear to stain homogeneously may still contain a mixture of myosin isoforms (Mayne et al., 1996). The ATPase results illustrate a shift towards a slower phenotype. It seems that 20 days of 10 Hz stimulation was enough to *initiate* the change to slow phenotype but not to cause a complete change in the muscle fibers.

In both control (vehicle only) and control samples, which recieved cyclosporin treatment muscles, all four types of muscle fibers are visible (Figures 3.1 and 3.3), while in stimulated muscle the number of type IIb fiber is starting to reduce because they change to IIx and IIa (Figures 3.2 and 3.4). As the results illustrate in Figures 3.1-3.4, cyclosporin A had no blocking effect in the muscle fiber differentiation, caused by the stimulation. Moreover, after stimulation the fibers appear smaller and have a more round appearance (Figures 3.2, 3.4, 3.6 and 3.7). During this 3 week period of 10 Hz continuous stimulation, the proportion of type I fibers did not show a rise but instead a slight reduction between the control and the stimulated muscles, which illustrates that a total transition towards a slow phenotype had failed to occur. These section photographs are representative samples of the results obtained in this study (n=7). As the tables in figures 3.1 to 3.4 illustrate, there is big increase (10 %-45%) in the type IIa fibers and a reduction in type I (about 10-35 %) and type IIb fibers (up to 10%). This illustrates that with chronic stimulation we get a mixture of myosin heavy chain with an amazing in crease mostly in the MHC_{IIa}. The results illustrate that chronic stimulation at 10Hz for 3 weeks in rat skeletal muscle causes the formation of an oxidative-glycolytic phenotype. In addition, figures 3.5-3.8 following, illustrate a marked increased in mitochondrial content in all fiber types. Based on this staining all fibers are shown to be of the type I (slow-twitch), illustrating incredible adaptation.



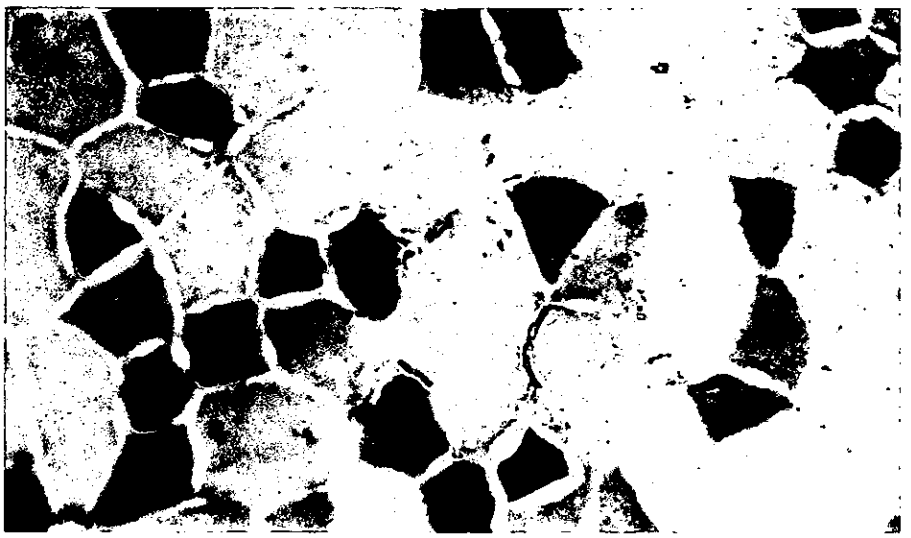
Type I	**23.6 %**
Type IIa	**58.8 %**
Type IIb	**17.6 %**

Figure 3.1. This picture illustrates a muscle section of Tibialis Anterior, which did not receive stimulation or Cyclosporin A treatment. The section was stained for ATPase activity, as described in methods, at pH 7.4 (no stimulation, vehicle, magnification x40) (n=7).



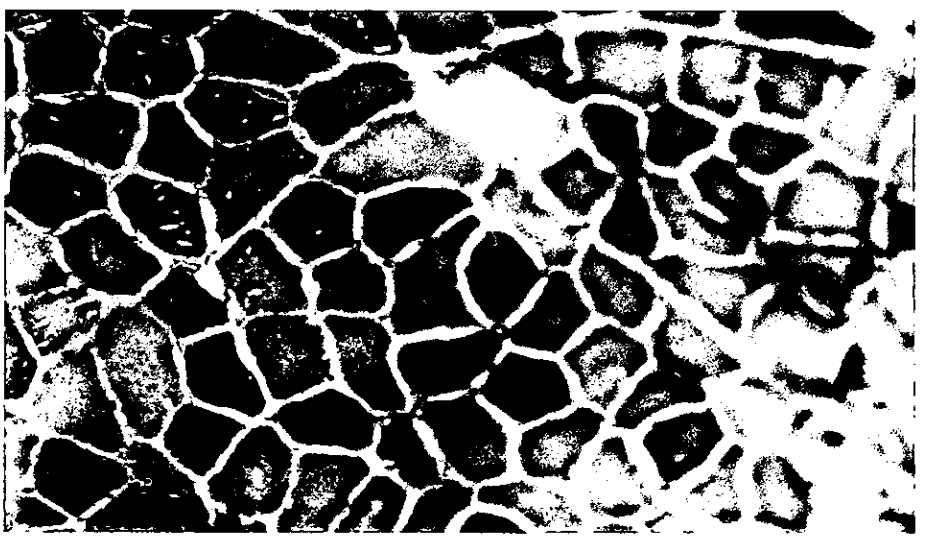
Type I	**12.4 %**
Type IIa	**70.8 %**
Type IIb	**16.8 %**

Figure 3.2. This picture illustrates a muscle section of Tibialis Anterior, which was chronically stimulated for 19 days and did not receive cyclosporin A treatment. The section was stained for ATPase activity, as described at the methods, at pH 7.4 (19 days of 10 Hz chronic stimulation, vehicle, magnification x40) (n=7).



Type I	**40.4 %**
Type IIa	**36.8 %**
Type IIb	**22.8 %**

Figure 3.3. This picture illustrates a muscle section of Tibialis Anterior, which bid not receive stimulation but received Cyclosporin A treatment for 19 days. The section was stained for ATPase activity, as described in methods, at pH 7.4 (no stimulation, cyclosporin: 19 days cyclosporin A treatment, magnification x40) (n=7).

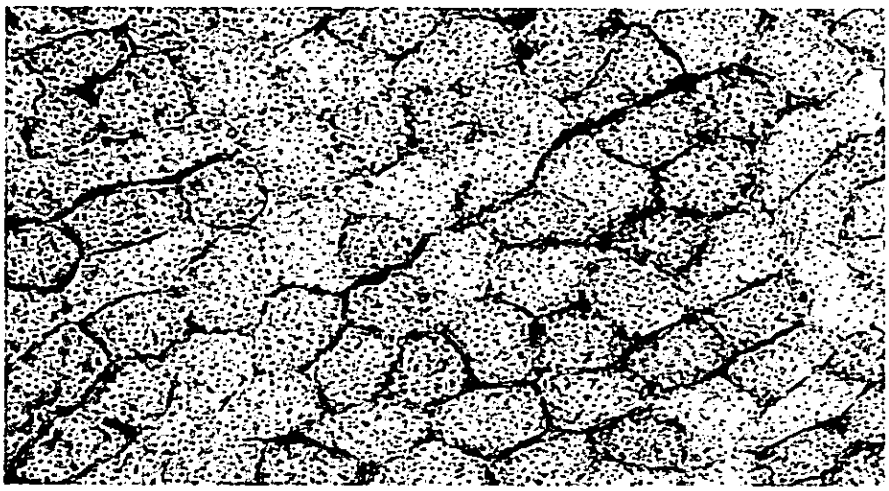


Type I	**4 %**
Type IIa	**80 %**
Type IIb	**16 %**

Figure 3.4. This picture illustrates a muscle section of Tibialis Anterior, which received both stimulation and Cyclosporin A treatment for 19 days. The section was stained for ATPase activity, as described in methods, at pH 7.4. (Stimulation and Cyclosporin: 19 days treatment, magnification x40) (n=7).

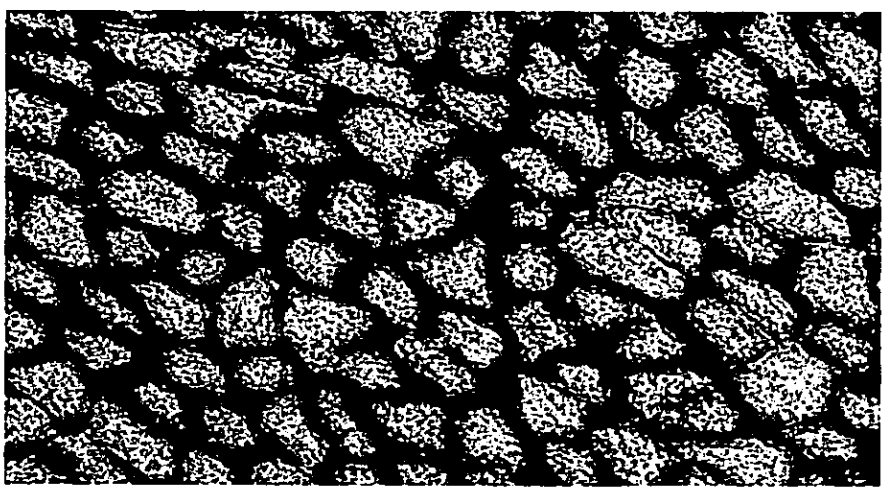
Armstrong and Phelps (1984), based on previous research (e.g.,Gollnick et al., 1983), suggest that histochemical techniques that differentiate among the different myosin ATPase isoenzymes (Brooke and Kaiser, 1970; Gollnick et al., 1983) may provide a more objective means of identifying different fiber types in the muscles, since the ATPase reaction intensity patterns for the fibers are discrete and are less influenced by contractile behaviour than are the metabolic enzymes. At this time, however, most physiological and biochemical studies on rat muscle have used a fiber classification system based on ATPase after alkaline preincubation and a mitochondrial oxidative enzyme similar to the one also used in this study. Although it has been proposed that there is a close correspondence between classifications based on the ATPase system and on metabolic properties (Spurway, 1981), others have demonstrated there is not a close correlation between fast fibers identified as IIa or IIb with a metabolic enzyme (e.g., NADH-TR) or as type IIa or IIb, respectively, with the myofibrillar ATPase technique following acid preincubation (Nemeth et al., 1979; Gollnick et al., 1983). Moreover, NADH-TR stain is a mitochondrial stain (NADH tetrazolium reductase activity), which has revealed that the mitochondrial content in all the fibers, which received stimulation, has risen substantially. All the fibers based in their mitocondrial content appear to be Type I and IIa. In comparison to the control situation there appears to have been a dramatic reduction in Type IIx and IIb fiber content. Furthermore, cyclosporin A does not seem to prevent the change towards slow phenotype. The pattern of coexpression of various Myosin Heavy chains ($MyHC_s$) has led to general acceptance of the following sequence of changes in fiber type, as cited in Parry (2001), which agrees with these results:

I ↔ I/IIa ↔ IIa ↔ IIa/IIx ↔ IIx ↔ IIx/IIb ↔ IIb



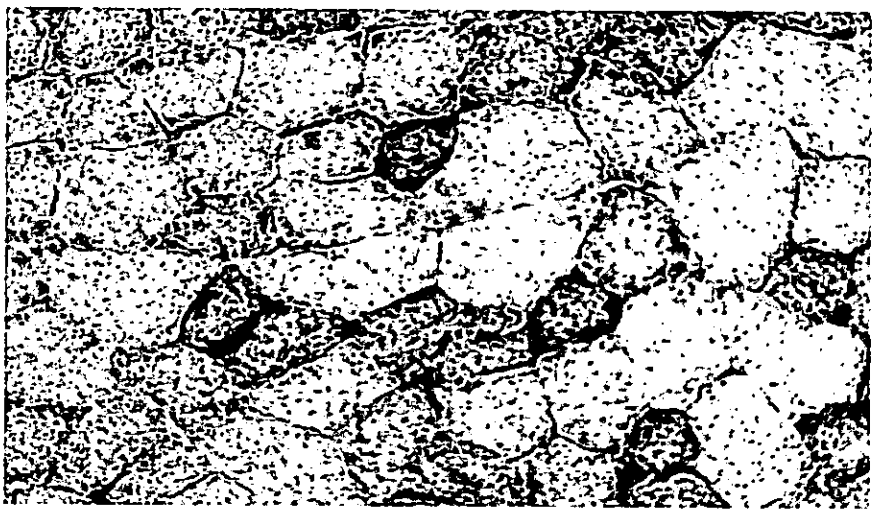
Type I	**48.8 %**
Type IIa	**37.5 %**
Type IIb	**13.5 %**

Figure 3.5. This picture illustrates a muscle section of Tibialis Anterior, which bid not receive stimulation or Cyclosporin A treatment. The section was stained with an NADH-TR stain illustrating mitochondrial content, as described in methods (no stimulation, vehicle, magnification x40) (n=7).



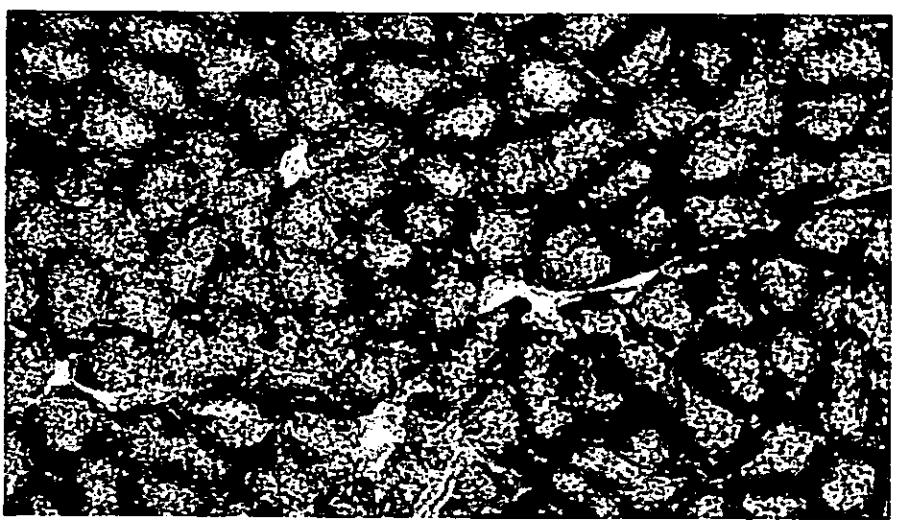
Type I	**100 %**
Type IIa	-
Type IIb	-

Figure 3.6. This picture illustrates a muscle section of Tibialis Anterior, which was chronically stimulated for 19 days and did not receive cyclosporin A treatment. The section was stained with an NADH-TR stain illustrating mitochondrial content, as described in methods (19 days of 10 Hz chronic stimulation, vehicle, magnification x40) (n=7).



Type I	**11.5 %**
Type IIa	**27.9 %**
Type IIb	**66.6 %**

Figure 3.7. This picture illustrates a muscle section of Tibialis Anterior, which bid not receive stimulation but received Cyclosporin A treatment for 19 days. The section was stained with an NADH-TR stain illustrating mitochondrial content, as described in methods (no stimulation, cyclosporin: 19 days cyclosporin A treatment, magnification x40) (n=7).



Type I	**100 %**
Type IIa	-
Type IIb	-

Figure 3.8. This picture illustrates a muscle section of Tibialis Anterior, which received both stimulation and Cyclosporin A treatment for 19 days. The section was stained with an NADH-TR stain illustrating mitochondrial content, as described in methods (Stimulation and Cyclosporin: 19 days treatment, magnification x40) (n=7).

3.1.2 Calcineurin content of different muscle fiber types:

1. Immunofluorescence studies

Immunofluorescence studies were performed in the muscles investigated, in order to identify differences in Calcineurin concentrations in different fiber types. The study identified differences in concentration between muscle fibers, which were demonstrated by differences in fluorescence intensity. This is a comparative test and the study was to identify differences in proteins rather than amounts.

The following photographs illustrate Calcineurin stain using FITC conjugate (Fluorescent green) in Soleus (SOL) and Tibialis Anterior (TA) muscles, control (no stimulation and no cyclosporin treatment) and stimulated (10 Hz chronic stimulation for 20 days and no cyclosporin treatment). FITC (fluorescein-5-isothiocyanate) is a conjugate used to identify proteins, which was bound to the secondary antibody. In addition, DAPI stain was applied to the same section in order to identify the location and the number of the nuclei. DAPI stain attaches specifically to double-stranded DNA (chromosome Q banding), and is a fluorescent blue stain (Mason, 1993).

The immunohistochemical stain has identified that there are clear differences in fluorescence among different muscle fibers. These results can be interpreted as differences in the Calcineurin protein content (figures 3.9-3.12). Because of the limitations of this technique no results were obtained in terms of which fiber type has a high or low protein content. The identification of the Calcineurin differences among fiber types was accomplished with the relation of the previous data with results obtained by Weststen blotting.

Figure 3.9. Immunohistochemical stain for Calcineurin in a Soleus muscle section. This is a control muscle and has not received stimulation. Clearly identifiable differences in protein concentration among fiber types (no stimulation, vehicle, magnification x 10) (n=5)

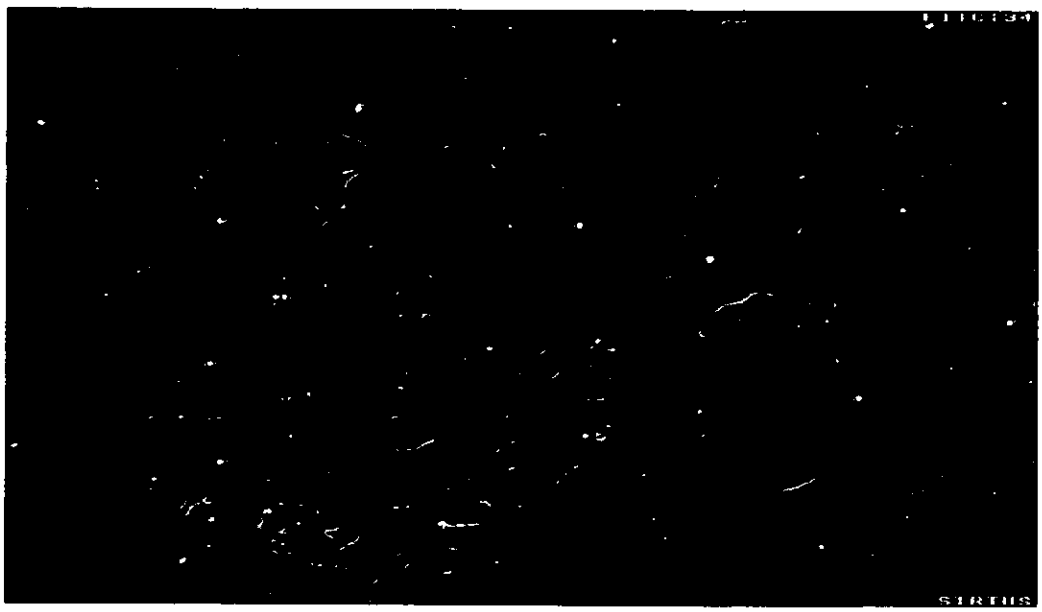


Figure 3.10. Immunohistochemical stain for Calcineurin in a Soleus muscle section. This is a control muscle which has not received stimulation (no stimulation, vehicle, magnification x 10) (n=5)

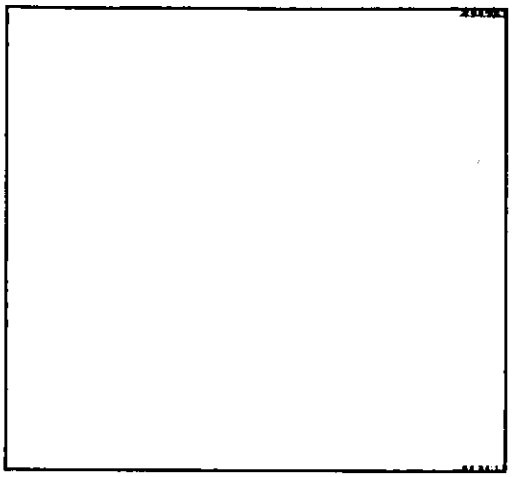


Figure 3.11. Immunohistochemical stain for Calcineurin in a Tibialis Anterior section. This is a control muscle and has not received stimulation. The protein is concentrated more in different fiber types than others. 'Hot spots' of the protein within selected fibers (no stimulation, vehicle, magnification x 25) (n=5)

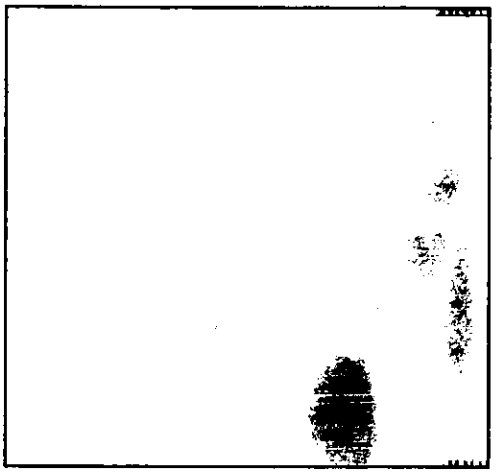
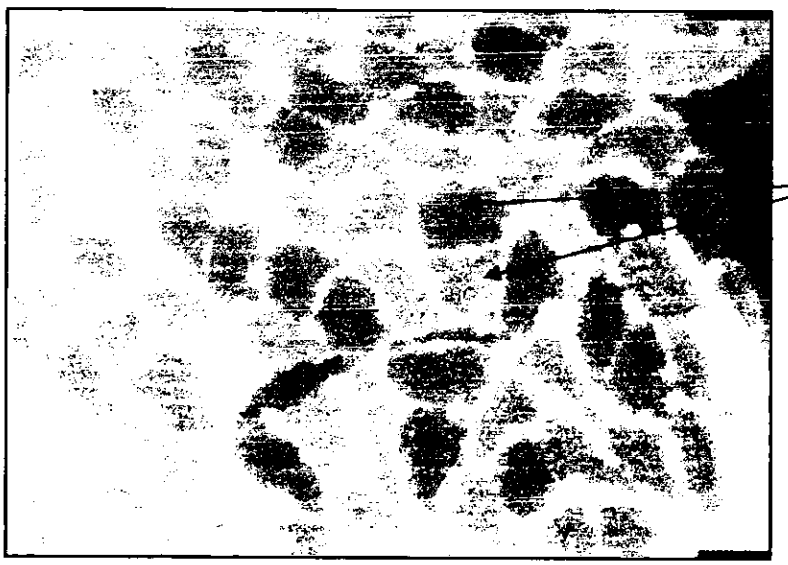


Figure 3.12. Immunohistochemical stain for Calcineurin in a Tibialis Anterior section. This muscle has received 10 Hz chronic stimulation for 20 days. Once again identifiable differences in protein concentration among fiber types (10 Hz chronic stimulation for 20 days, vehicle, magnification x 25) (n=5)



Differences in relative Calcineurin concentration among fiber types interpreted as differences in fluorescence intensity

Figure 3.13. Calcineurin stain in TA muscle and DAPI stain picture of the same section: overlayed photographs. Calcineurin stain is illustrated with Green fluorescence, while DAPI with blue. Different muscle fibers and the Nuclei (DNA stain) are illustrated. The results identified differences in Calcineurin concentration between fiber types (stimulation, Vehicle: G5= 20 days of stimulation) magnification x25 (n=6).

2. Western blots

The adaptive ability of the skeletal muscle was further explored in this study where 10 Hz of chronic stimulation was applied in Rat Tibialis Anterior (TA) and Extensor Digitorum Longus (EDL) for 3 weeks. Western Blots were performed in order to investigate the relative changes in Calcineurin concentration between the stimulated and control groups that did not receive any Cyclosporin treatment as shown in Table. 2.1 ($G_{R/L}$ 1-5). In addition, relative study has demonstrated that there is no difference between the left and right muscles of the lower leg in rat and that is why for all the experiment this pattern was repeated (right leg = control ; left leg = stimulated) (Salmons and Sreter, 1976)

The primary antibody used for this experiment is specific for the CnA domain of the Calcineurin molecule (as described in methods). The molecule was "broken down" (the molecular weight was identified through the molecular weight markers in the Western blots) when the samples were boiled at 95 °C (as described in methods), and the primary antibody bounded to the CnA subunit of the protein. The Calcineurin heterodimer is composed by the catalytic subunit CnA, which consists of a calmodulin binding domain and a autoinhibitory domain with a molecular weight of 59 KDa, while the regulatory subunit CnB has a molecular weight of 19 KDa (http://www.ensembl.org ; Olson and Williams, 2000).

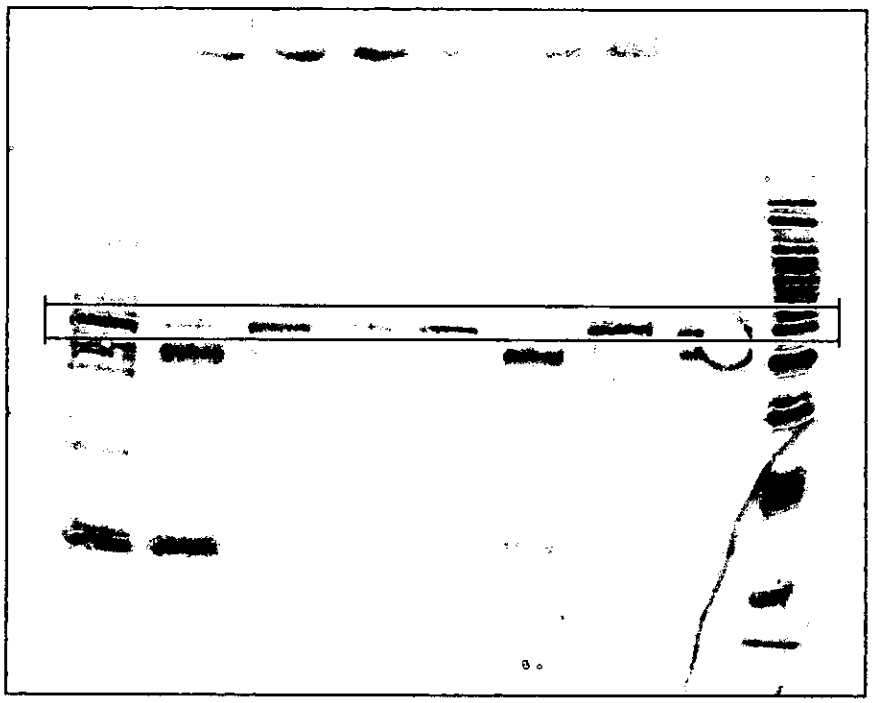
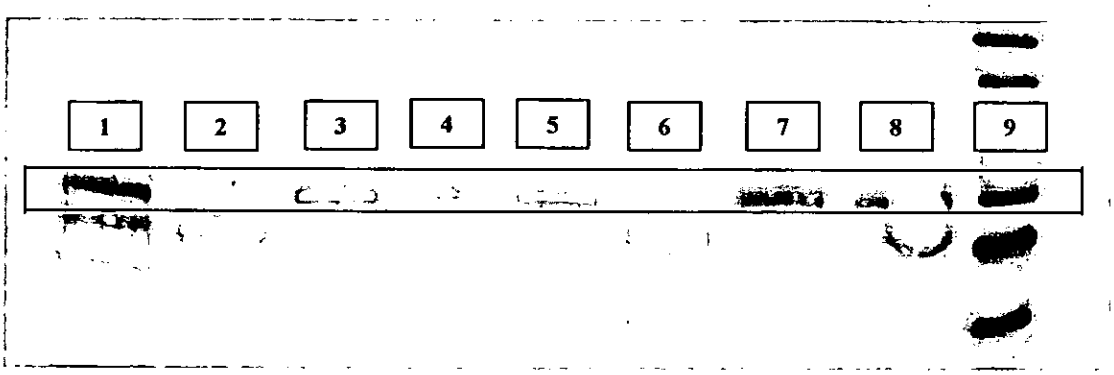


Figure 3.14. Western bolt for Calcineurin in EDL control and stimulated muscles. The Calcineurin bands are easily identified and verified with the molecular weight markers.

This technique is used to identify differences in Calcineurin concentrations between the controls (right muscle of the hind limb) and the samples that received 10 Hz chronic stimulation (left muscle of the hind limb). This is a comparative approach and the investigation of the exact concentrations was not attempted.

A.



B.

Line No:	1	2	3	4	5	6	7	8	9
Sample:	C EDL	S EDL	C EDL	S EDL	C EDL	S EDL	C EDL	S EDL	Mol. W. Markers
	G5[1)] right	G5 left	G4 right	G4 left	G2 right	G2 left	G1 right	G1 left	
Calcineurin:	17.45	2.05	8.3	3.81	5.67	1.99	31.40	11.17	

Figure 3.15. **A**: Western blot for Calcineurin. In lanes 1, 3, 5 and 7 EDL control muscles; lanes 2, 4, 6 and 8 EDL muscles stimulated at 10 Hz for 3 weeks; and lane 9 the molecular weight markers. **B**: Amount values obtained by densitometric scanning of the western blots (software used: UVP Laboratory imaging and analysis system). The amount in each band, are in relation to amount load in each lane. **[1) See methods for the meaning of the G1-G5 codes (Table 2.1).]**

The EDL muscle samples of the unstimulated limb have the highest amounts of calcineurin. In the EDL control muscles the fast fiber types are predominant (Armstrong and Phelps, 1984). The EDL samples that received 10 Hz stimulation and the predominant muscle type seems to be Type I or Type IIa, within 3 weeks the calcineurin concentration has reduced up to 8 times (G5 sample). In other samples like G4, calcineurin concentration has decreased to a half, while the general marked decreases are about a third compared to the controls. There is high variability between the data and an underpowered test because of the low sample size. However, this nonparametric test is significant at the 5% significance level indicating a positive outcome ($P = 0.050$).

3.1.3 The effect of chronic stimulation on enzyme activities of four metabolic pathways:

The investigation of the metabolic changes, which took place after chronic stimulation for 20 days (10 Hz, 24 h/day, for 3 weeks), were interpreted as changes in four enzyme activities belonging to different metabolic pathways. In addition, the assays for a given muscle were made on a few samples, from different animals all stimulated for 3 weeks (between 17 and 20 days). This caused some problems in terms of variation in the results. The skeletal muscles used were composed of a mixture of different fiber types and as mentioned before these are not necessarily distributed throughout the muscle. As a result, there was a difference in fiber content for every part of muscle tested in every repetition. On the other hand, there are differences among samples tested in regard to content of connective tissue, blood, and interstitial fluid. In order to keep this variation to a minimum freeze-dried muscle was used for testing and the same regions for a given muscle were used. Finally, in all the enzymes investigated, there are no significant changes in enzyme activities after 3 weeks of stimulation even though the changes occur towards the "expected" direction. These changes were expected based on the similar published experiments (Henriksson et al., 1986; Mayne et al., 1996; Chi et al., 1986; Meiβner et al., 2001). The best animal model for investigating changes in enzyme activities is rabbit, but this study was done on rat skeletal muscle. In this animal model the changes are very hard to show, because it requires a longer period of stimulation. If the following results were compared with similar studies done in rabbit it would be seen that for the same period of chronic stimulation the changes would be much higher and definitely significant (Henriksson et al., 1986; Mayne et al., 1996; Chi et al., 1986; Meiβner et al., 2001).

Enzymes of Glycolysis

The first enzyme examined in this study was *lactate dehydrogenase (LDH)*, which catalyses the reduction of pyruvte in glycolysis.

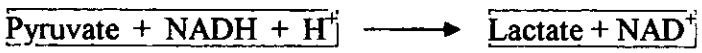


Figure 3.16. The chemical reaction in which Pyruvate is converted to Lactate. This reaction is catalysed by Lactate Dehydrogenase.

In most studies of endurance training both oxidative and glycolytic enzymes are effected (Henriksson et al., 1986; Mayne et al., 1996; Chi et al., 1986; Meiβner et al., 2001). The muscle examined was rat Tibialis Anterior (TA), which received 10 Hz (24 h/day) stimulation for 20 days. This experiment illustrates a progressive decline (Figure 3.17) in lactate dehydrogenase levels between control and stimulated muscles. Even though the reduction in activity is apparent, is not though statistically significant within the first 3 weeks. A marked decrease is only observed between the control muscles that received cyclosporin (as well as the control muscles that did not receive cyclosporin) and the stimulated samples that also received cyclosporin treatment. Even though muscles that received stimulation and Cyclosporin A follow the same decline in activity pattern with significant differences compared to controls ($p = 0.024$), no results can be obtained about the blocking ability of Cyclosporin A, since no significant decrease is observed between the control and stimulated muscles for this enzyme ($p = 0.254$).

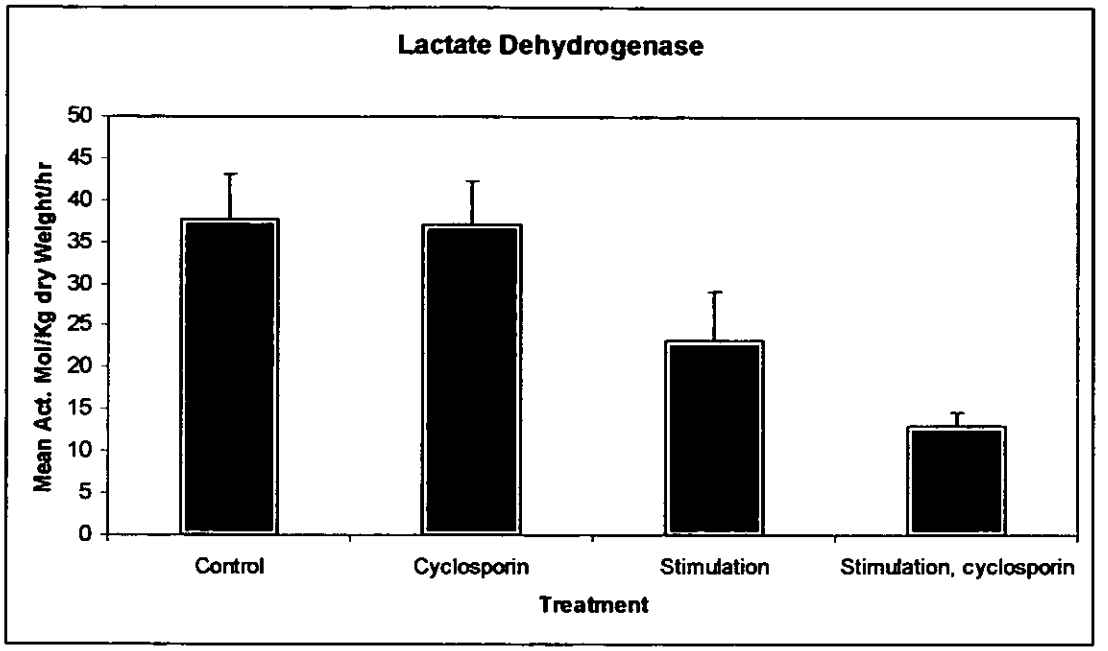


FIGURE 3.17. Changes in Mean activity Mol/Kg dry weight/hr for Lactate Dehydrogenase after 20 days of chronic stimulation at 10 Hz (n=6 for control and stimulation; and n=4 for control Cyclosporin and stimulation Cyclosporin) in Tibialis anterior muscles. Error bars ± SE are indicated.

Enzymes of High-Energy Phosphate Transfer

Creatine kinase (CK) is one of the high-energy phosphate transfer enzymes, which are involved in carbohydrate metabolism. The two major precursors to obtain energy are glucose from blood and stored glycogen (and under certain conditions lactate in blood). Carbohydrates are the only fuel that can be used anaerobically to yield ATP (by glycolysis) (Bullock et al., 1986).

Phosphocreatine + ADP ⟶ ATP + Creatine

Figure 3.18. The chemical reaction in which Phosphocreatine is converted to Creatine. This reaction is catalysed by Creatine Kinase.

Like lactate dehydrogenase levels, here is illustrated the progressive decline in Creatine kinase levels between control and stimulated muscles; and between control

cyclosporin and stimulated cyclosporin muscles. Again the reduction in activity is obvious, but it cannot be established statistically within the first 3 weeks of stimulation.

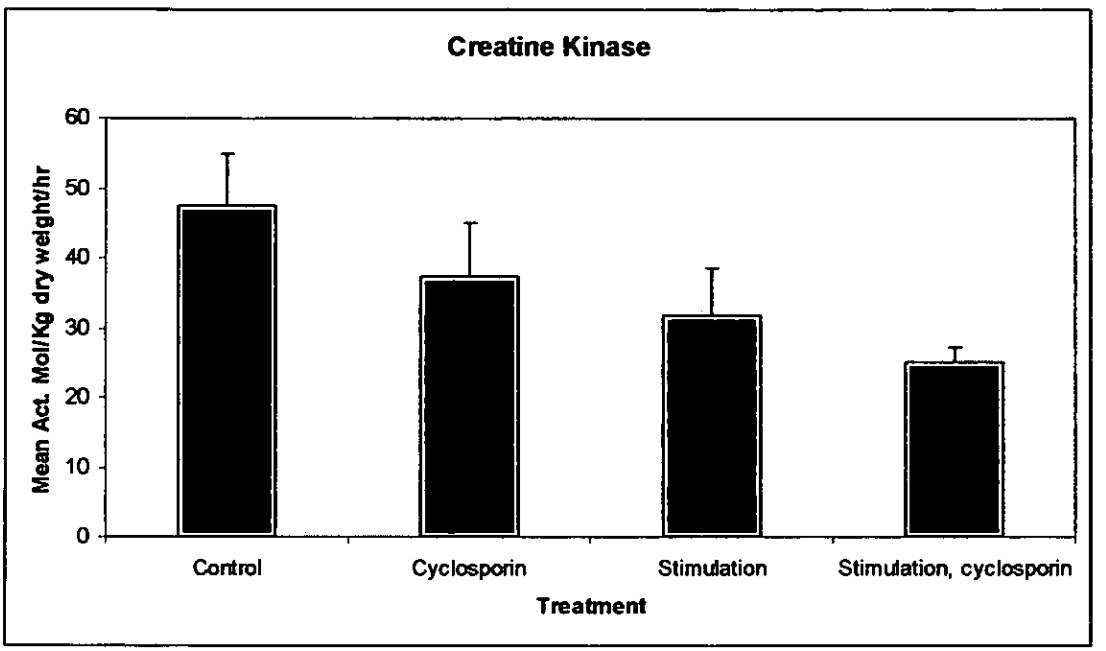


FIGURE 3.19. Changes in Mean activity Mol/Kg dry weight/hr for Creatine Kinase after 20 days of chronic stimulation at 10 Hz (n=6 for control and stimulation; and n=4 for control Cyclosporin and stimulation Cyclosporin) in Tibialis anterior muscles. Error bars ± SE are indicated.

Enzymes of TCA cycle (Tricarboxylic Acid cycle)

The final oxidation step of the TCA cycle involves the conversion (oxidation) of malate to oxaloacetate by *malate dehydrogenase* (MDH).

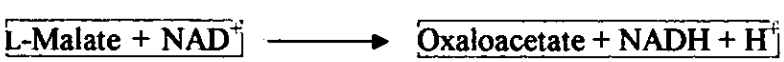


Figure 3.20. The chemical reaction in which Malate is converted to Oxaloacetate. This reaction is catalysed by Malate Dehydrogenase.

Malate Dehydrogenase was used as a marker for the oxidative activity changes within the muscle groups tested. To some extent MDH activities, varied in an increasing

fashion towards the stimulated muscles compared to controls. Moreover, no blocking effect was illustrated when cyclosporin was used in combination to stimulation, but the lack of significance in the recorded changes make conclusions impossible.

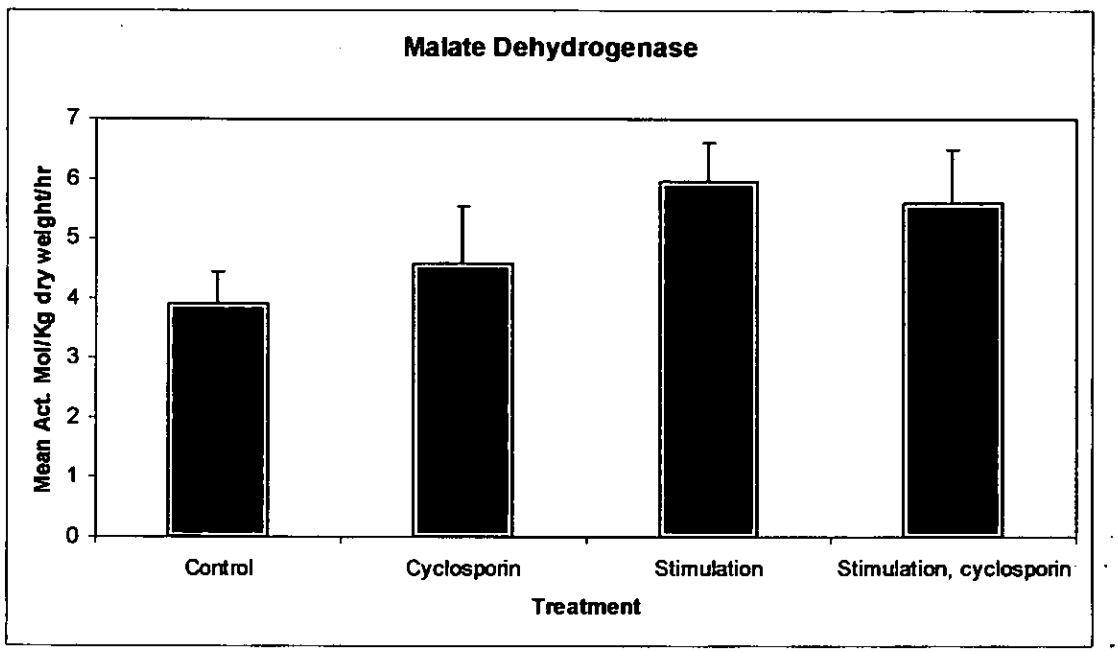


FIGURE 3.21. Changes in Mean activity Mol/Kg dry weight/hr for Malate Dehydrogenase after 20 days of chronic stimulation at 10 Hz (n=6 for control and stimulation; and n=4 for control Cyclosporin and stimulation Cyclosporin) in Tibialis anterior muscles. Error bars ± SE are indicated.

Enzymes of oxidative metabolism

The last enzyme investigated is *hexokinase (HEX)* an enzyme of the glycolytic pathway involved in glycogen metabolism. This enzyme catalyses phosphorylation at the oxygen attached to C-6 of glucose and the source of the phosphate group is ATP.

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-phoshate} + \text{ADP} + \text{H}^{+}$$

Figure 3.22. The chemical reaction in which glucose is converted to glucose-6-phospate with the use of ATP. This reaction is catalysed by Lactate Hexokinase.

Hexokinase is typically higher in slow- twitch than in fast-twitch muscles (Henriksson et al., 1986); in this case it was not significantly higher in stimulated samples

compared to control, since the stimulating period was not enough to cause significant changes. The only significant difference is between the control muscles that received cyclosporin A treatment and the muscles that were stimulated for 20 day with 10 Hz chronic stimulation but did not receive cyclosporin ($p = 0.023$). Even if no conclusions can be obtained from the measurements in enzyme activities, because of the lack of significance, these results are presented since they illustrate that 3 weeks with 10 Hz chronic stimulation are not enough to cause a marked change in rat skeletal muscle metabolic activities.

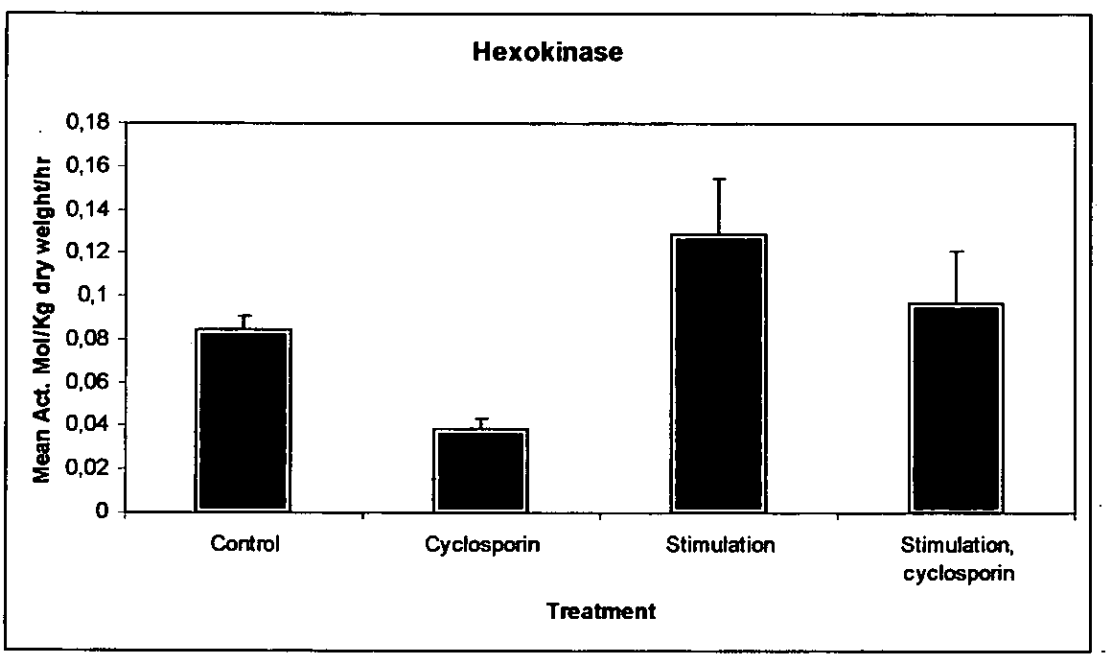


FIGURE 3.23. Changes in Mean activity Mol/Kg dry weight/hr for Hexokinase after 20 days of chronic stimulation at 10 Hz (n=6 for control and stimulation; and n=4 for control Cyclosporin and stimulation Cyclosporin) in Tibialis anterior muscles. Error bars ± SE are indicated.

3.2. Investigation of the effect of increased intracellular Ca^{2+} in NFAT translocation on L_6 Muscle cell cultures

3.2.1. The effect of Br-A23187 Ionophore in intracellular calcium concentration of L6 myotubes

The adult skeletal muscle has the ability to respond in changes of physiological demands. This is achieved by switching from glycolytic to oxidative phenotype and vice versa (Salmons and Sreter, 1976; Semsarian et al., 1999; Parry, 2001; Chin et al., 1998; Olson and Williams, 2000). An increased intracellular calcium concentration, caused by endurance training or chronic stimulation, would be expected to cause a change towards the oxidative phenotype in a given skeletal muscle. This high degree of plasticity in differentiated muscles is a remarkable illustration of adaptation.

A skeletal muscle L_6 cell culture, after being established and characterised, was used as a model, in order to investigate the underlying mechanisms that control muscle plasticity. This was illustrated by observing the NFAT translocation, within the Calcineurin pathway, as a result of increased intracellular calcium.

The effect of 4-bromo-calcium ionophore was investigated in the L_6 cell cultures. This type of 4-bromo-calcium ionophore was used because it causes the release of intracellular calcium from the SR, while it does not interact with the fluorescence of Fura-2. A range of concentrations between 5 $x10^{-9}$ – 8 $x10^{-6}$ M were tested as illustrated in Figure 3.25. The results are presented in Ratios (F340/F380) since FURA-2, which was used, displays a single peak at 500 nm, but two calcium-dependent absorption maxima, one at 340 which increases with increasing ionised calcium and a second at 380 nm which similarly decreases with a rise in ionised calcium (Mason, 1993). This fluorometric method is shown in Figure 3.24 below.

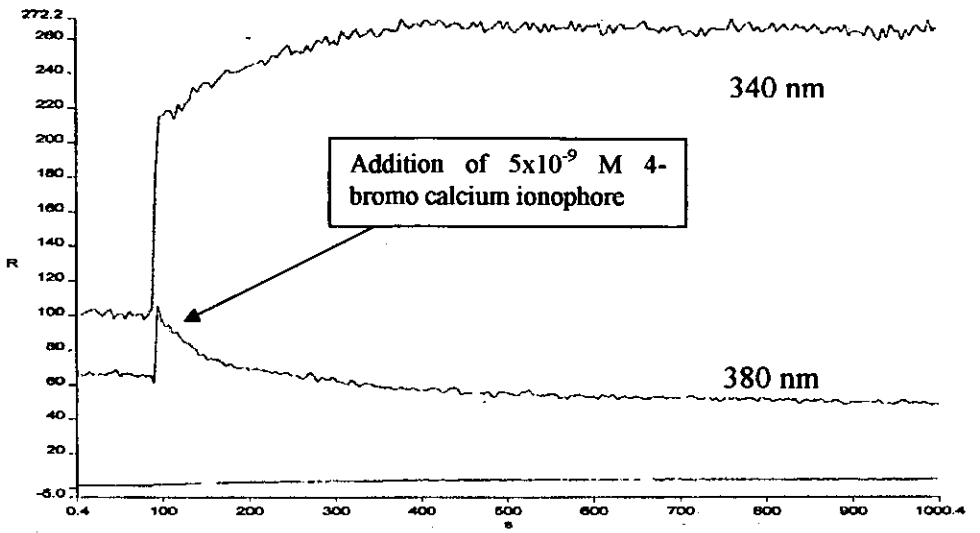


Figure 3.24. Dual-wavelength photon counting measurements of intracellular calcium in L_6 myotubes stimulated with 5 x 10^{-9} M 4-bromo-calcium ionophore and loaded with fura-2. Excitation wavelengths 340 and 380 nm, measurements in ratios v seconds.

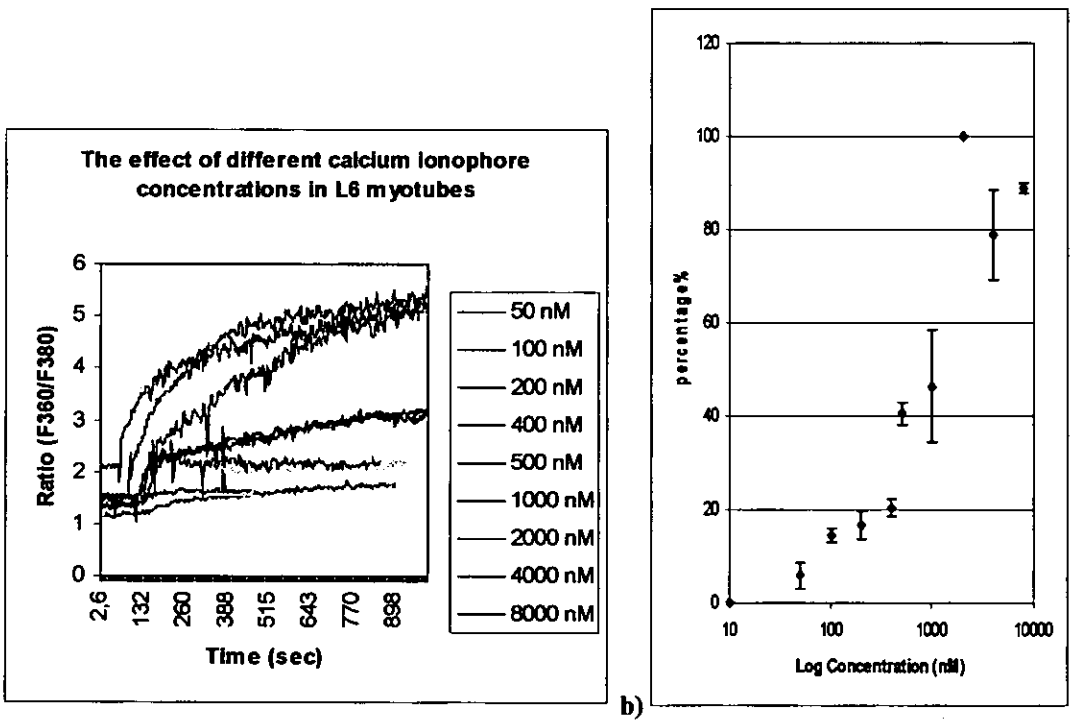


Figure 3.25. **a)** The cells were loaded for 50 min at room temperature in the dark with 8 μM fura-2. After 10 min equilibration time, $[Ca^{2+}]_{bulk}$ was monitored in a fluorometer and expressed as a ratio of fuorescence intensities at 340 and 380 nm (F_{340}/F_{380}) as described in Methods. The traces represent the effect of 50 nM to 8μM 4-bromo-calcium ionophore concentrations on intracellular calcium changes. **b)** Changes in intracellular Calcium as an effect of 4-bromo calcium ionophore; presented as a dose response curve (n=3, error bars ± SD).

These results demonstrate that increasing the concentration of the ionophore increased the intracellular free Ca concentration in a dose-dependent manner.

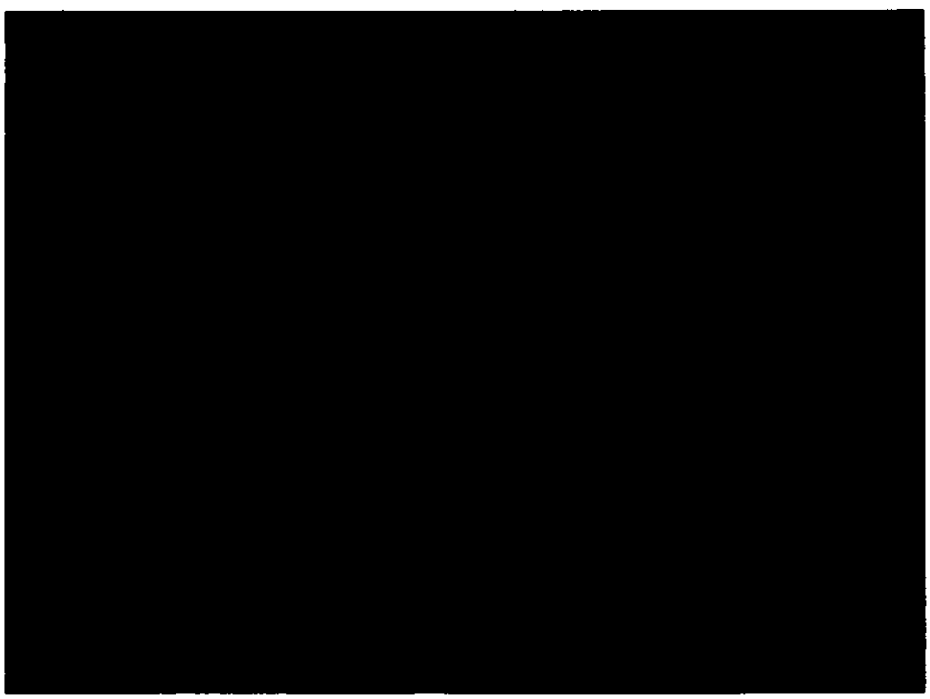
3.2.2. The effect of increased intracellular calcium in NFAT localization and translocation

The cell line was in differentiation medium (α-MEM) when incubated for 24 hours with 1 μM ionophore. This ionophore concentration was chosen after a series of experiments, which revealed that this is the concentration, produced the optimal intracellular calcium increase. This study revealed that higher ionophore concentrations cause problems to cell function since cell death was observed in the cell cultures. However, these observations were in contrast to the work of Meiβner and co-workers (2001) who observed an NFAT translocation to the nucleus in response to a 4 x 10^{-7} M ionophore concentration. These were pelimenary data and were not included. When the Ca^{2+} ionophore (Br-A23187) (1 μM) was added to the medium, a partial fast-to-slow transformation occurred, since NFAT transolocated to the nucleus (Figure 3.27).

Initially, the localization of NFAT was investigated before ionophore treatment took place. Immunohistochemical studies for NFAT, as described in the methods, were performed using a secondary antibody with a Cy3 (fluorescent red) conjugate. The study revealed that NFAT is almost entirely located in the cytosol in a resting state (Figure 3.26: 1-3).

1.

2.



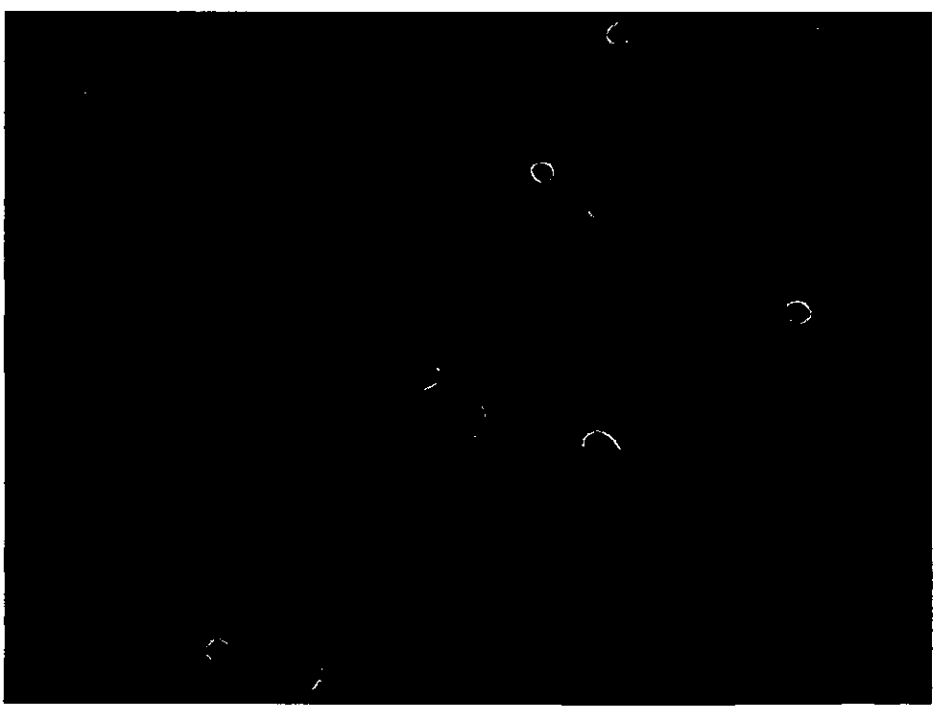
3.

Figure 3.26. Immunohistochemical photographs of L_6 myotubes in x40 magnification. *1-3* Cell cultures were grown (as described in the methods section) for about 9 days on petridishes and then for 5 more days on glass coverslips. They were then fixed and stained for NFAT. Photograph 1 illustrates control samples. *2.* DAPI stain of the section shown in picture 1. *3.* Overlay picture of the section shown in pictures 1 and 2, which illustrates NFAT and DAPI stain respectively. NFAT is localised almost entirely in the cytosol. (n=7)

The second part of the study involved the investigation of a possible NFAT transolocation after incubation for 24 h with 1 μM 4-bromo-calcium ionophore. A variety of ionophore concentrations (Figure 3.25), were tested in a pelimenary study indicating this concentration (1 μM) to be the optimal.

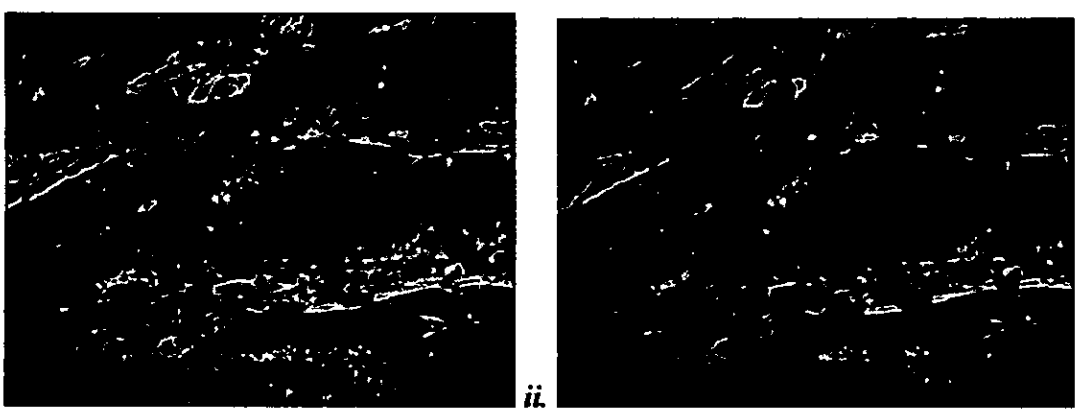
A.

B.



C.

D.



i. ***ii.***

Figure 3.27. Immunohistochemical photographs of L_6 myotubes in a x40 magnification. The cell cultures were grown for about 9 days on petridishes and then for 5 more days on glass coverslips. They were then cultured for one more day (24 h) in the presence of Ca^{2+} ionophore Br-A23187 (1 μM) on glass coverslips, fixed and stained for NFAT. Photographs ***A*** and ***B*** illustrate NFAT and DAPI stain respectively for the same section, while ***C*** is an overlay picture of the two. NFAT translocation is demonstrated (n=3) ***D.i.*** Control culture that did not receive any treatment. ***D.ii.*** Overlay picture of the control section shown in Figure *D.i.*, which illustrates NFAT and DAPI stain respectively. NFAT is localised almost entirely in the cytoplasm. (n=3)

Figures 3.27 $_{A, B, C}$ illustrate that NFAT is localised almost entirely in the nucleus after the Ionophore treatment. An overly of Figures 3.27 $_{A, B}$ shows (presented as Figure 3.27 $_{C}$) that NFAT is located were the DNA lays hence in the nucleus.

In conclusion, the experiments performed in myotubes as shown before, have established the effect of various ionophore concentrations in the intracellular calcium stores. These changes cause a rise in the cytosolic calcium concentration, activating the calcineurin pathway (Figure 1.20 in Chapter 1-Introduction) by binding to calmodulin (Chin et al., 1998). This pathway is thought to switch on slow genes and cause the production of type I proteins (Olson and Williams, 2000 **a,b**). In addition, this hypothesis can be confirmed by investigating the NFAT kinetics within the myotubes of the L_6 cell line. As mentioned before NFAT in phosphorylated by Calcineurin A (CnA), which exposes its "nuclear localization signal" causing it to translocate to the nucleus with CnA bound to it, and in turn causing slow gene activation and hypertrophy in skeletal muscles (in combination with other transcription factors like MEF2, GATA2) (Chin et al., 1998; Olson and Williams, 2000; Dunn, Chin, and Michel., 2000). Finally, all the results presented in this study will be further explained and discussed in the next chapter.

DISCUSSION

4.1. Induction of a fast- oxidative phenotype by chronic muscle stimulation and investigation of the effect of cyclosporin A : histochemical and metabolic studies

4.1.1 Effect of chronic stimulation on muscle fiber differentiation

4.1.2 Effect of chronic stimulation in Calcineurin content of different muscle fiber types

4.1.3 The effect of chronic stimulation on enzyme activities of four metabolic pathways

4.2. Investigation of the effect of increased intracellular Ca^{2+} in NFAT translocation on L_6 Muscle cell cultures

DISCUSSION

4.1. Induction of a fast- oxidative phenotype by chronic muscle stimulation and investigation of the effect of cyclosporin A : histochemical and metabolic studies

4.1.1 Skeletal muscle Plasticity: Effect of chronic stimulation on muscle fiber differentiation

Skeletal muscle has the ability to adapt specifically to chronic exercise. Olson and Williams (2000) have suggested that "skeletal myofibers exhibit highly specialized characteristics with respect to size, metabolism, and contractile function" (Olson and Williams, 2000). These changes, when it comes to protein isoforms and quantities, have been established in the past (Saltin and Gollnick, 1983).

Skeletal muscle makes up 71% of the total hindlimb mass of the rat. Of this, 76% was occupied by fast-twitch glycolytic (type IIA) fibers, 19% by fast-twitch oxidative glycolytic (type IIb) fibers, and 5% by slow-twitch oxidative (type I) fibers (Armstrong and Phelps, 1984). Thus, the fast-twitch glycolytic (type IIA) fiber type is clearly the predominant fiber type in the rat hindlimb in terms of muscle mass. The results of this study taken from the TA muscle samples agree with the Armstrong and Phelps study outcomes, since type IIa fibers cover the 70% of the muscle, while type I and type IIb cover about 15% and 15% respectively (Figures 3.1-3.4). The following table illustrates the percentage of the three different fiber types in some specific muscles of the rat (Armstrong and Phelps, 1984). Figure 100 (which is located in

Appendix 1) illustrates the anatomy of the muscles located in the lateral surface of the lower leg and the extensor surface of the rat foot (which were used for experimentation) (Greene, 1959).

Table 4.1 The percentage of the three different fiber types in some specific muscles of the rat (as cited in Armstrong and Phelps, 1984).

	Fast-twitch oxidative glycolytic (type IIb)	*Fast-twitch glycolytic (type IIa)*	*Slow-twitch oxidative (type I)*
	Population %	Population %	Population %
Soleus	13±4	0	87±4
TA red	57±8	38±9	5±1
TA white	27±6	72±6	1±1
EDL	42±7	56±8	2±1

The majority of mammalian muscles contain muscle fibers with a mixture of myosin (myosin heavy chain MHC and light chain MLC) isoforms (Mayne et al., 1996; Jarvis et al., 1996). Some of the clearly identifiable fiber types though, demonstrate intermediate properties, so they sometimes might appear to be of the fast or the slow type but they share qualities. These fibers have been shown to be the type IIa fibers, which have both a fast contractile speed and a well-developed oxidative metabolism. Every muscle fiber type has its own type of innervation. Type I fibers are innervated by the type I motor neurons with postural activity, while fast type IIb fibers are innervated by the high frequency burst motorneurons. On the other hand, fast IIa fibers have a different type of innervation than the two since they have mixed properties (Jarvis et al., 1996; Olson and Williams, 2000). When stimulating a muscle is very important to know that it is not just the frequency but also the duration and the aggregate number of impulses delivered to the muscle that can determine that level of differentiation (Jarvis et al., 1996).

This study, among other things, is investigating the role of the Calcineurin signalling pathway in the adaptive capacity of fast rat skeletal muscle when stimulated

chronically with 10 Hz for a period of 3 weeks. Histochemical evidence illustrate that the small population of type I fibers normally found in the control muscles showed no marked increase after 3 weeks of stimulation, instead they decreased about 20% in average. On the other hand, the fast fiber population had the histochemical appearance of type IIa fibers, which increased about 30%, with a marked decline in fast type IIb (10%)and IIx fibers (Figures 3.1 and 3.2). Mitochondrial stain revealed a marked increase in mitochondrial content (Figures 3.5 and 3.6). The fibers which were chronically stimulated with 10 Hz could be classified as slow type I fibers. The other histochemical results obtained from the ATPase stain argue with the previous observation since they show no increase of slow fibers, even though they illustrate (Figures 3.1 and 3.2) a lack of fast type IIb fibers. This indicates that the first aspect influenced by chronically stimulating muscle fibers (10 Hz) is the mitochondrial content. Stimulating the rat skeletal muscle for 3 weeks seems to be inadequate to cause a marked increase in the slow fiber population. Even though there was not a big increase in type I fiber population, based on these histochemical data cyclosporin A (CsA) was not able to prevent neither the decrease of the fast fibers (type IIb) (Figures 3.3 and 3.4) nor the increase in the mitochondrial content of all the fibers in the stimulated muscles (Figures 3.7 and 3.8). Another important observation is reduction of the muscle fiber size as well as the changes in the shape, which are thought to be caused by endurance exercise (Figures 3.2, 3.4, 3.6 and 3.8). It has also been observed that there is a difference in the muscle fiber composition, especially in the Tibialis Anterior muscle samples, between the central and peripheral parts of the rat muscle. For this reason, the results presented are from the same part of the muscle (Figures 3.1-3.8). Purely histochemical data can be misleading (Mayne et al., 1996) and for

this reason further immunohistochemical as well as metabolic studies were performed.

Furthermore, similar (mostly in rabbit muscle) studies have illustrated that after continuous stimulation at a frequency of 10 Hz for a period more than 10 weeks, the properties acquired by the fast-twitch fibers of these muscles are more closely related to those of slow-twitch, or type I, fibers (typical slow muscle isoform composition). In addition, they have slow contractile speed; they show remarkable resistance to fatigue, have well-developed blood supply and predominately oxidative metabolism (Mayne et al., 1996; Pette and Vrbová, 1992; Salmons and Henriksson, 1981).

4.1.2 Effect of chronic stimulation in the Calcineurin content of different muscle fiber types

The first part of the study involved the investigation of muscle adaptation. This type of study though does not provide any data that help explain the signaling processes in molecular level, which link contraction patterns to specific gene regulation. This second part investigates the calcineurin pathway involved in muscle fiber differentiation (Chin et al., 1998) and more specifically the differences in calcineurin concentration between slow and fast fibers.

Calcineurin is a serin-threonine protein phosphatase that has been shown to be activated by increased intracellular free calcium levels (Chin et al., 1998; Olson and Williams, 2000). When calcineurin is activated dephosphorylates the nuclear factor of activated T cells (NFAT). This dephosphorylation results in the exposure of NFATs nuclear localization sequence and its nuclear translocation (Meiβner *et al.*, 2001). Nuclear NFAT binds DNA at the specific NFAT enhancer sequences located in the regulatory regions of the "slow" skeletal muscle genes (Chin *et al.*, 1998).

The combination of immunohistochemical staining and western blotting for Calcineurin A, has revealed a marked difference in concentration between slow and fast fibers (Figures 3.9- 3.15). For the immunohistochemical technique, a specific primary antibody for CnA and a specific secondary antibody for the primary mentioned before were used. Differences in the fluorescence among fibers (as a result of the fluorescent secondary antibody) were interpreted as differences in the Calcineurin content among the different fiber types. In turn Western blot technique was used in order to identify whether type I-slow fibers or type II-fast fibers contain a higher amount of the Calcineurin protein. Western blotting was proven to be very useful and consistent technique, since substantial difficulties were experienced when muscle fiber were to be identified through a combination of immunohistochemical and ATPase staining in order to justify which fiber type contains more of the protein. As illustrated in Figure $3.15_{A,B}$, there is up to 8 times more calcineurin (relative concentrations) in the control muscles than in the stimulated. This indicates that fast fiber types contain a higher concentration of the protein. Calcineurin pathway seems to be down regulated in the muscle samples that received 10 Hz chronic stimulation for 3 weeks. The maintenance of a high intracellular calcium concentration, which we would expect as a result of chronic stimulation (chronic stimulation imitates the slow-glycolytic motor neuron firing) (Olson and Williams, 2000), in turn results in a constantly activated calcineurin pathway. It seems that a homeostatic mechanism causes the down-regulation of the calcineurin pathway, which is interpreted as a reduction of the calcineurin concentration.

A very important study by Chin et al. (1998) identified that fiber type specific gene expression in skeletal muscles is controlled by the calcineurin signaling pathway. This

pathway was shown to selectively up-regulate slow fiber specific gene promoters. The transcription factors identified, that mediate the transcriptional activation involving the slow genes, were of the NFAT and MEF2 families. Finally, this study investigated the inhibitory effect of Cyclosporin A on the calcineurin pathway, and revealed that the drug prevents the slow-to-fast transformation (Chin et al., 1998). Swoap and coworkers (2000), in contradiction to Chin et al.'s hypothesis, have presented data showing that apart from the slow gene activation the calcineurin pathway could increase the expression of some fast genes (Swoap et al., 2000). Additional evidence illustrates that the calcineurin pathway is involved in IGF-1 induced skeletal muscle hypertrophy (Semsarian et al., 1999), which are in contradiction to the Swoap et al. (2000) study. All these studies show that the actual role of Calcineurin in skeletal muscle plasticity is yet to be clarified. In addition, investigating the activation of other pathways, also caused by a potential calcium increase, is essential and has to be taken under consideration. There has been clear evidence for connections between signal transduction pathways, known as "cross-talk," which have been shown to effect muscle fiber differentiation. Some of these pathways "switch on" at the same with the calcineurin pathway since they are also calcium activated and obviously can either contribute or resist the fast-to-slow conversion of a chronically stimulated fast skeletal muscle. An important category of pathways, which are also thought to be calcium activated and cause slow gene transcriptional activation, is the MAPK/ERK kinase (MEK1) pathways (Perry et al., 2001; Cox, Quinn and McDermott, 2000; Cruzalegui and Bading, 2000; Treisman, 1996). There are three distinct classes of MAPKs : the extracellular signal regulated kinases (ERKs), the c-Jun NH_2- terminal Kinases/stress activated protein kinases (JNK SAPKs), and the p38 MAPKs (Cox, Quinn and McDermott, 2000). These different MAPK pathways have been characterized, based

on their initial receptors, to be responsive to mitogein (ERKs) or to cellular stress, such as p38 and JNK MAPKs, which are all calcium related responses (Cox, Quinn and McDermott, 2000; Cruzalegui and Bading, 2000; Treisman, 1996). In addition, there is MyoD family (Parry, 2001; Cox, Quinn and McDermott, 2000), which consists of four members: MyoD, Myf-5, myogenin, and MRF4 and are all expressed exclusively in skeletal muscle cells. There has been evidence that an up-regulated expression of MyoD causes a slow-to-fast differentiation, since it translocates to the secondary myonuclei and induces transcription of MHC IIb (Parry, 2001). Moreover, there is also evidence for cross-talk between the insulin and p38 signaling pathways (Blair et al., 1999). All these and more signaling pathways have been shown to induce gene expression and interact with each other (Cross-talk) in heart muscle, which unfortunately is not comparable to skeletal muscle (Molkentin and Dorn II, 2001). Finally, it is essential to identify the stimulating patterns that cause different calcium responses in the skeletal muscle cells, which in turn trigger different signal transduction pathways. It is therefore clear that muscle fiber differentiation is not just controlled by calcineurin pathway but instead is mediated by an interlinked network of several contraction-responsive signal transduction pathways.

4.1.3 The effect of chronic stimulation on enzyme activities of four metabolic pathways

Glycolysis is the chain of chemical reactions involved in the breaking down of the sugars. In the first stage of cellular respiration, occurring with or without the presence of oxygen, glucose is converted to two molecules of pyruvic acid. The final energy gain from the glycolysis occurs in the hydrolysis of phosphoenolpyruvate to pyruvate and the concomitant phosphorylation of ADP to ATP. For every molecule of hexose

phosphate consumed, two molecules of ATP are produced to finally bring the net yield of ATP to two molecules for each molecule of glucose (Zubay et al., 1995).

In order for glycolysis is to continue the pyruvate produced must be further metabolised. A molecule of NAD^+ is reduced for every molecule of phosphoglyceraldehyde that is oxidized and this procedure is essential for its regeneration. Because equimolar amounts of NADH and pyruvate are produced in glycolysis, a simple way to reoxidize the NADH is by transfer of electrons to pyruvate, forming lactate. Skeletal muscle tissue uses this strategy in times of exertion when the oxygen supply is not enough in order to keep up with the energy requirements of the tissue. The reduction of pyruvate is catalysed by the enzyme *lactate dehydrogenase* (LDH) (Zubay et al., 1995). Moreover, carbohydrate metabolism is controlled in part by *creatine kinase (CK)* one of the high energy phosphate transfer enzymes. On the other hand, other enzymes investigated were *malate dehydrogenase* and *hexokinase*, which are involved in the final oxidation step of the TCA cycle and glycogen metabolism respectively.

The observations made in this study involved the continuous stimulation of rat skeletal muscle for a period of 3 weeks. The investigation of the metabolic changes, were interpreted as changes in four enzyme activities belonging to different metabolic pathways (as mentioned before). In all the enzymes investigated in this study, there are no significant changes in enzyme activities after stimulating for 3 weeks at 10 Hz, even though the changes occur towards the 'expected' direction, based on the similar published experiments (Henriksson et al., 1986; Mayne et al., 1996; Chi et al., 1986; Meißner et al., 2001). Henriksson and co-workers (1986) have suggested that " a transformation takes place within individual muscle fibers and not through a process

of degeneration and regeneration that would affect the relative proportions of fiber types but not their terminal state of differentiation" (Henriksson et al., 1986).

Both oxidative and glycolytic enzymes have been shown to be affected in other studies of endurance training, where the muscle were chronically stimulated at low (2.5 Hz) and high (10 Hz) frequencies for up to 10 weeks (Henriksson et al., 1986; Mayne et al., 1996; Chi et al., 1986; Meiβner et al., 2001). This study illustrates a progressive but insignificant decline both in *Lactate Dehydrogenase* and *Creatine kinase* levels between control and stimulated muscles. Even though the reduction in activity can be concluded from figures 3.17 and 3.19, is not though statistically significant within the first 3 weeks. On the other hand, *Malate Dehydrogenase (MDH)* and *Hexokinase (HEX)* activities, varied in an increasing but still insignificant fashion towards the stimulated muscles compared to controls (Figures 3.21 and 3.23). In addition, no marked changes, apart from lactate dehydrogenase, were detected when cyclosporin was used in combination to stimulation.

The study by Henriksson and co-workers (1986) illustrated the effect of 10 Hz chronic stimulation in many enzyme activities, among which are the ones investigated in this study. The study presented marked changes in all the above enzymes within the first 3 weeks. The reason for this difference might be explained by the fact that rabbit, which Henriksson and co-workers used, is very different animal model compared to rat skeletal muscle. In this last animal model (Rat) it is very difficult to get any changes in enzyme activities within 3 weeks, because it requires a longer period of stimulation (Jarvis et al., 1996). Any attempt to compare the results obtained in this study with similar studies performed in rabbit would be impossible, since for the same period of chronic stimulation the changes would be much higher and definitely significant (Henriksson et al., 1986; Mayne et al., 1996; Chi et al., 1986; Meiβner et

al., 2001). Finally, the lack of significance in the recordings of the enzyme activities for this study makes conclusions impossible. Even though a rise in the mitochondrial content has been established (Figures 3.5- 3.8) the only conclusion is that the 3 weeks stimulation period was insufficient, in order to cause a marked change in the metabolism of the muscles investigated although the techniques demonstrated here have shown to be the valid approach to the study of transformations.

4.2. Investigation of the effect of increased intracellular Ca^{2+} in NFAT translocation on L_6 Muscle cell cultures

Motorneurons and their activity play an essential role in muscle fiber determination and differentiation. It has been shown that muscle fiber differentiation and fast-to-slow transformation can be triggered by cross-innervation or chronic stimulation (Salmons and Sreter, 1976; Pette, 1984; Henriksson et al., 1986; Mayne et al., 1996; Chi et al., 1986). In addition, data demonstrating the importance of $[Ca^{2+}]_i$ changes, were presented in studies using muscle cell cultures as a model of investigation (Seigneurin et al., 1996). Calcium ionophore treatment has been shown to lead to a reversible fast-to-slow transformation in cultured muscle cell lines (Meiβner et al., 2001; Friday, Horsley, and Pavlath, 2000; Kubis et al., 1997). It has also been shown the ionophore treatment induces fiber transformation and not a hypertrophic response (Meiβner et al., 2001). This ionophore-induced transformation is comparable to chronic low frequency stimulation (Salmons and Sreter, 1976). Friday, Horsley, and Pavlath (2000), in their study present data suggesting that at least three distinct steps require calcium, based on their differing requirements for extracellular calcium: commitment to differentiation, phenotypic differentiation, and cell fusion (Friday, Horsley, and Pavlath, 2000). A different study by Dolmetsch and co-workers (1997)

suggests that changes in intracellular calcium differing in amplitude and duration result in differential activation of transcription factors in lymphocytes (Dolmetsch et al., 1997). In addition, it has been shown that a low sustained plateau in the level of $[Ca^{2+}]_i$ leads to the activation of NFAT, while large a large transient rise lead to the activation of a different transcription factor NFkB (Meiβner et al., 2001). Finally, Chin and co-workers has shown that similar mechanisms are involved in the regulation of fiber type specific gene expression in skeletal muscle (Chin et al., 1998). The molecular mechanisms though, which initiate differentiation of myoblasts are not well understood.

There is evidence illustrating that calcium -calmodulin-regulated serine-threonine phosphatase calcineurin plays a key role in calcium dependent signalling in rabbit skeletal muscle cell culture (Meiβner et al., 2001). A calcium ionophore addition of $1x\ 10^{-6}$ M has been shown to produce a steady but not unphysiologically high intracellular calcium plateau increase (Figure 3.25). This study presents data showing the effect of this intracellular calcium plateau increase on the calcineurin pathway and muscle fiber differentiation. Immunohistochemical results presented on Figures 3.26 1-3. and 3.27: Di,ii. , illustrate that in a resting state of the L_6 myotubes NFAT is localised almost entirely in the cytosol. The increase in intracellular calcium caused by the ionophore addition (1×10^{-6} M) in the cell line, resulted in the activation of the calcineurin pathway. The elevated calcium bound to the calmodulin domain of calcineurin causing its activation. Calcineurin in turn phosphorylated NFAT exposing its nuclear localization sequence and resulting in its translocation to the nucleus. The NFAT translocation was immunohistochemically detected using a fluorescent secondary antibody (Figures 3.27: A, B and C). Moreover, Abbott et al. (1998) have suggested that in muscle cells (in contrast to lymphocytes) each NFAT isoform (as

described in the introduction) undergoes nuclear translocation only at specific stages of myogenesis, which suggests that they may regulate distinct subsets of genes necessary for muscle cell physiology (Abbott et al., 1998).

Furthermore, in L_6 cell cultures like in skeletal muscle research, all the possible interactions of the calcineurin pathway with other signalling pathways have to be taken under consideration. This means that other transcription factors mentioned before like MEF2, or GATA2 (during IGF-induced skeletal muscle hypertrophy) are involved in the calcineurin pathway and muscle fiber differentiation in muscle cell cultures (Abbott et al., 1998; Meiβner et al., 2001; Musaro, et al., 1999), but their exact relationship is yet to be determined.

CONCLUSION

In summary, this study has defined a calcium-dependent pathway that regulates the commitment of both skeletal muscle and myoblasts to the differentiation pathway. Calcineurin activity is both necessary and sufficient to induce differentiation in the presence of adequate extracellular calcium environment. In rat skeletal muscle, 10 Hz of chronic electrical stimulation for 3 weeks resulted in a marked reduction of the fast fiber type family. In addition, the same stimulation patter caused a marked increase in the mitochondrial content of the fibers but still was not able to elicit a significant metabolic change. The calcineurin pathway was down regulated as a result of chronic stimulation indicating adaptation. Moreover, there is strong evidence concerning the calcineurin content of different fiber types, since it appears to be significantly higher in fast fibers. Even though the time period for this stimulations pattern was insufficient to cause marked changes in the metabolism of the skeletal muscles of the

rat, histological studies revealed that cyclosporin A was unable to prevent the fast-to-slow transformation. In L_6 myocytes evidence support the relation between the increased intracellular calcium and NFATc1 translocation and possibly DNA binding through the calcineurin pathway. Finally, further studies will identify and clarify the upstream signals that regulated calcium changes and trigger the activation of different signalling pathways as well as the downstream targets of calcineurin in skeletal muscle.

Scope for further studies

1. Histochemical and metabolic studies in rat skeletal muscle after the effect of 10 Hz chronic stimulation for 10 weeks. Investigation of the effect of cyclosporin A under these conditions.
2. Investigate the involvement of calcineurin-dependent signalling pathways in controlling the expression of MHCI, MHCIIa and MHCIIb under the effect of chronic stimulation in rat skeletal muscle. Investigate the effect of cyclosporin A under this treatment.
3. Investigate the NFAT kinetics under the effect of various intracellular calcium changes in L_6 skeletal muscle cells.
4. The effect of different intracellular calcium changes on a range of transcription factors such as NFkB, ERKs, c-jun, p38 kinetics and Jak/STAT in L_6 skeletal muscle cells. In this study, western blots can be used in order to identify the protein in the skeletal muscle and secondly investigate if there are differences in relative concentration between fast and slow muscle. In addition, by using cytosolic and nuclear extractions of fast and slow muscle is

possible to investigate the kinetics of the mentioned proteins if they indeed translocate to the nucleus as a results of calcium changes. Different patterns of calcium increases (plateau or spiking patterns) and a variety of intracellular concentrations can be investigated using the same model. Additional and more accurate information could be obtained by using Northern blotting techniques for RNA.

5. Finally, with the given technology it possible to investigate the activity of every single gene in a given sample by using Microarrays. This technique enables us to investigate whether genes involved in skeletal muscle hypertrophy or fiber differentiation are activated after a given treatment. It is also possible to block gene activity ("knocking out genes") and investigate the importance of a certain gene in muscle fiber differentiation.

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APPENDIX 1

All figures (Fig.100; Fig.187; Fig.188; Fig.320) in this appendix have been taken from *Greene, 1959.*

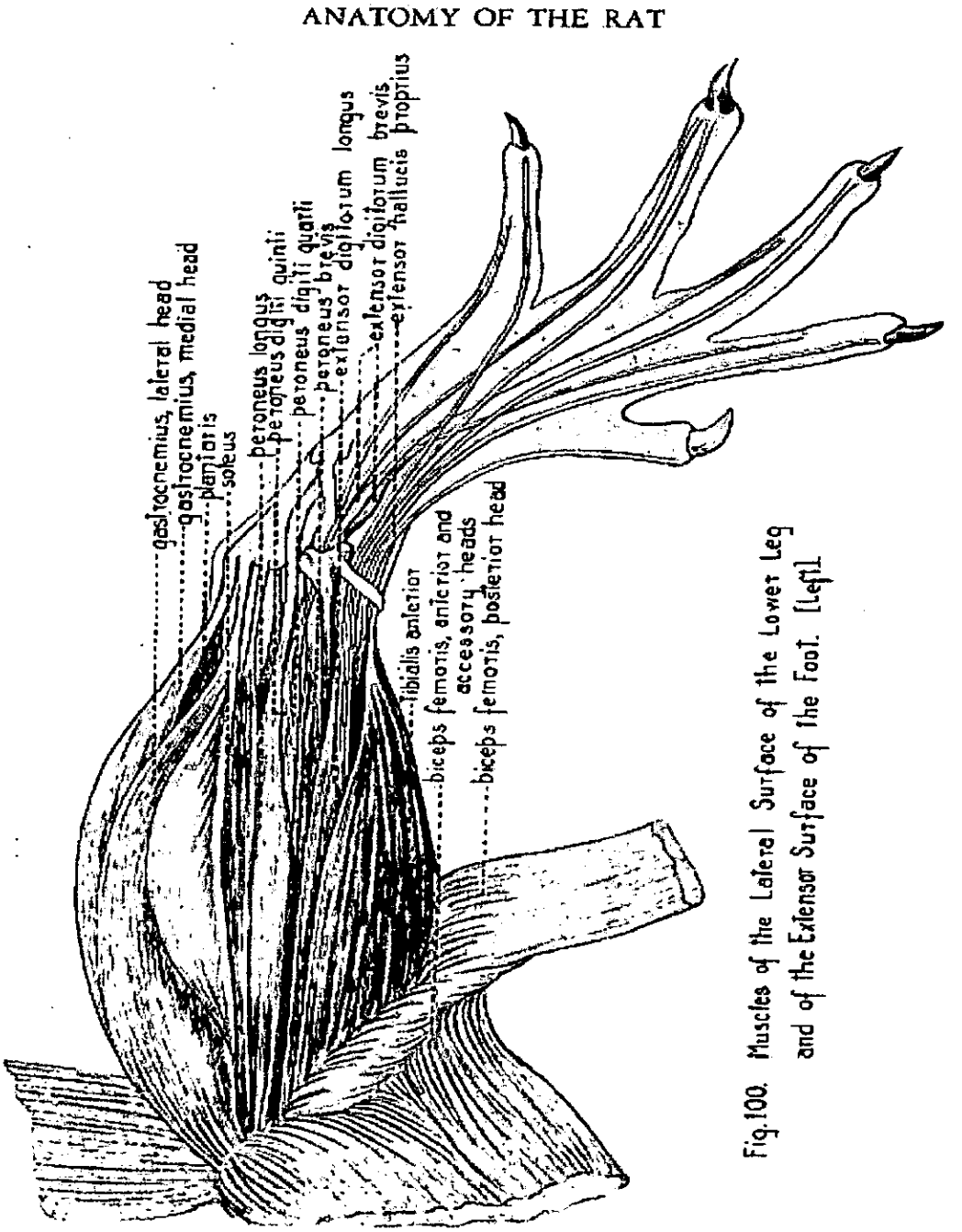

Fig.100. Muscles of the Lateral Surface of the Lower Leg and of the Extensor Surface of the Foot. (Left)

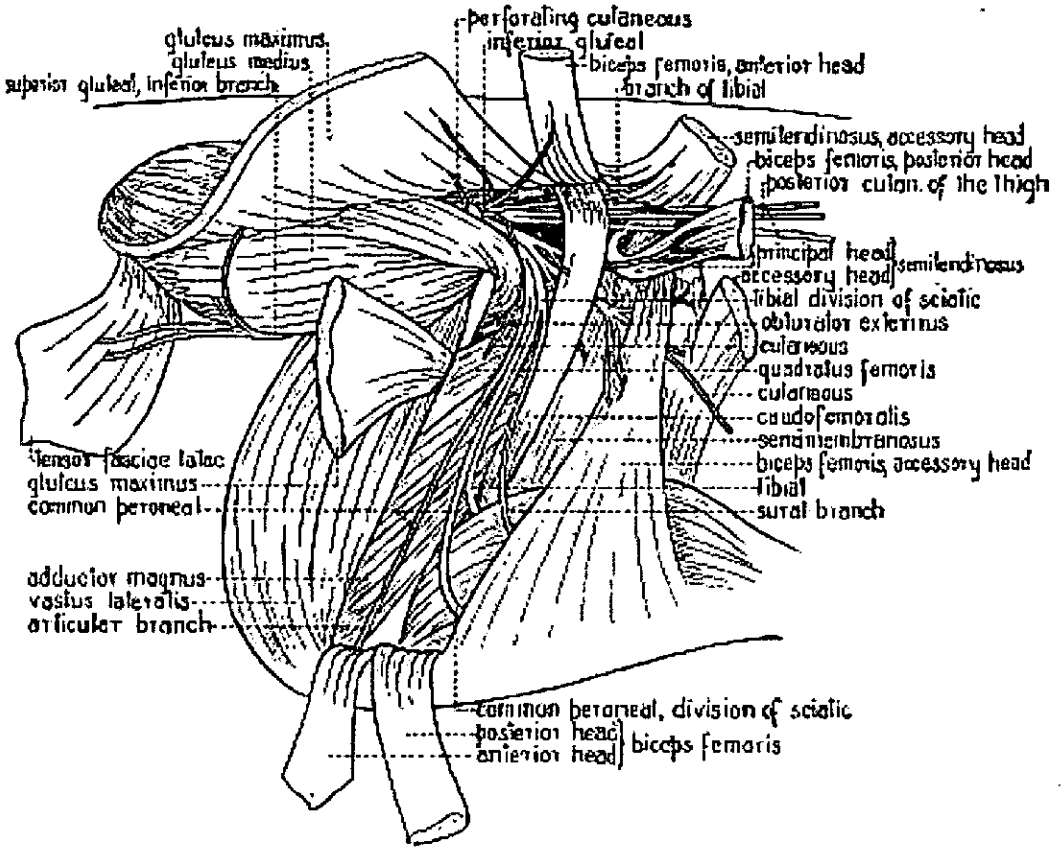

Fig. 187. Lateral aspect of the left thigh dissected to show divisions of the sciatic nerve.

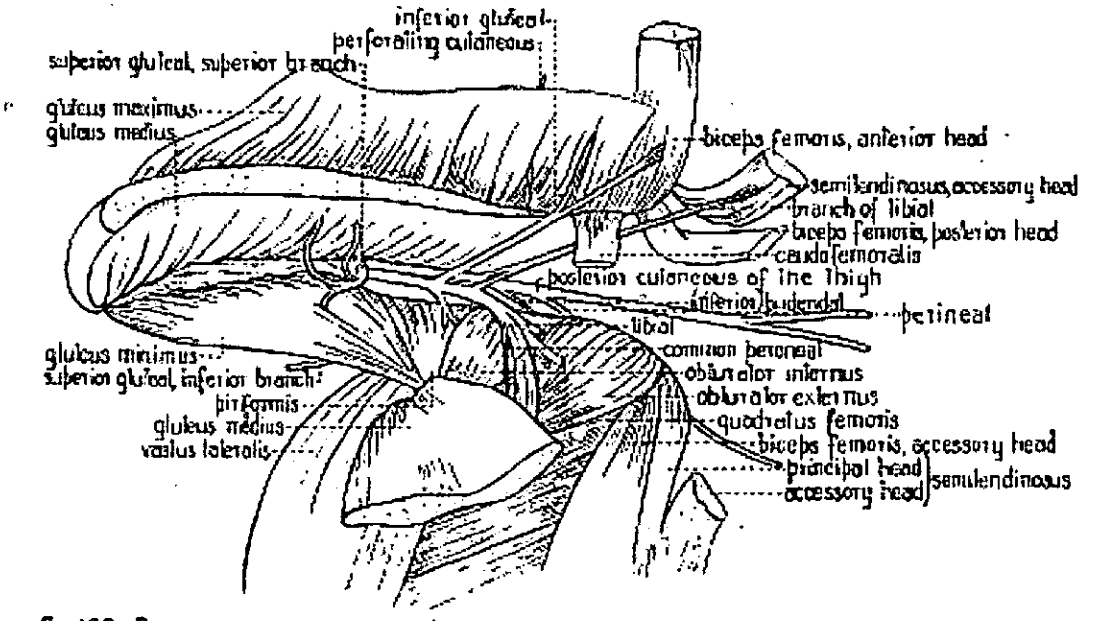

Fig. 188. Deep dissection of left lateral hip region, showing nerves from the sacral plexus

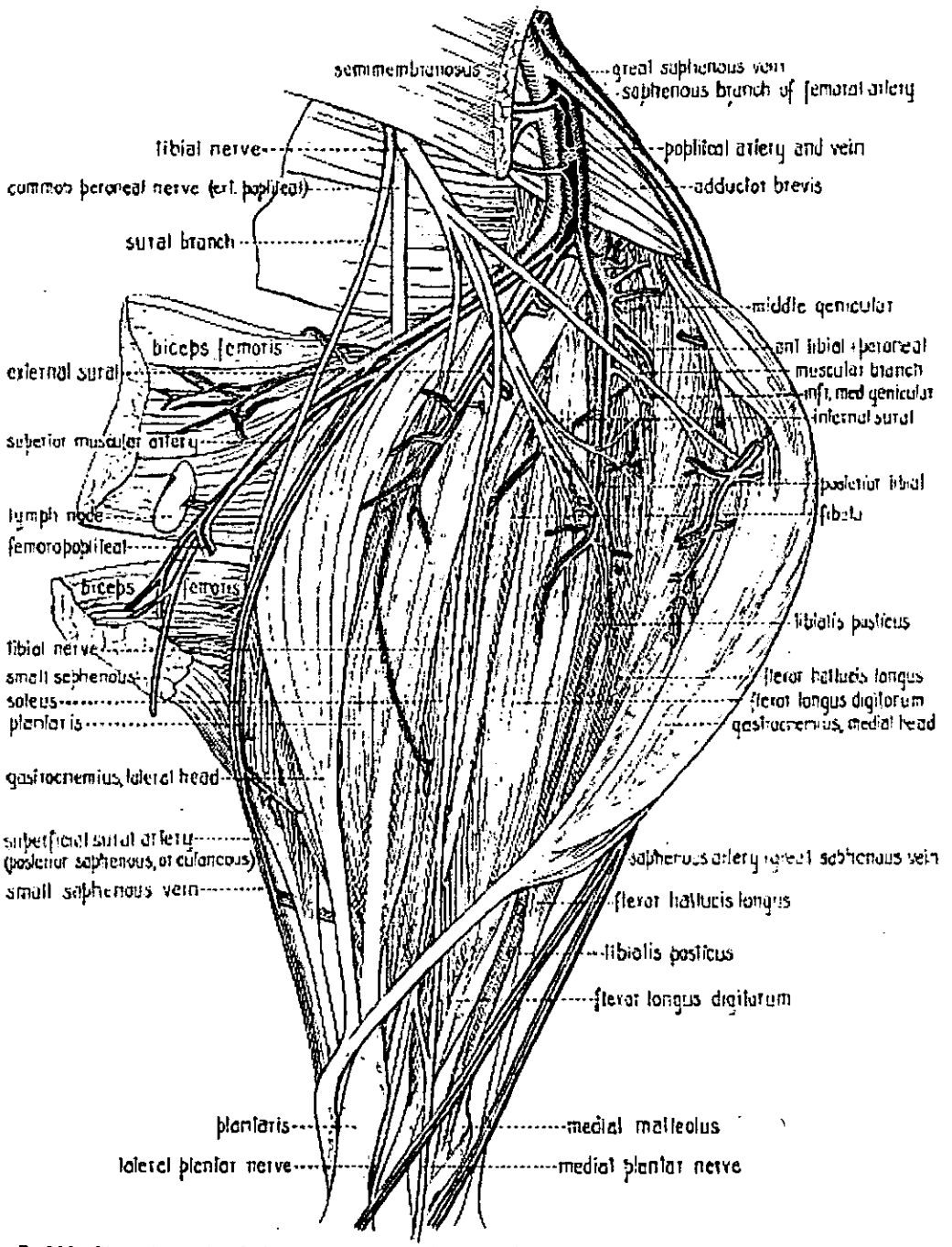

Fig320 Blood Vessels of the Lower Leg. Axillary View