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**SKELETAL MUSCLE CALCIUM  
HOMEOSTASIS DURING FATIGUE  
–MODULATION BY KINASES AND  
MITOCHONDRIA**

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**“What is simple is wrong, and what is  
complicated cannot be understood”  
- Paul Valery**



## ABSTRACT

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The use of skeletal muscles in daily activities and even during strenuous exercise resulting in fatigue requires precise regulation of force and the timing of contraction. To achieve such performance characteristics, vertebrate skeletal muscles have developed a unique control mechanism for regulating the free  $\text{Ca}^{2+}$  concentration in their myoplasm ( $[\text{Ca}^{2+}]_i$ ). A basic knowledge of cellular and molecular mechanisms regulating these mechanisms is essential in understanding pathological alterations in  $\text{Ca}^{2+}$  handling. This thesis deals with how these mechanisms are modulated by (i) mitochondria, (ii)  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase II (CaMKII) and (iii) protein kinase A (PKA). (i) Mitochondrial free  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_{\text{mit}}$ ) increased during fatiguing stimulation in most, but not all, slow-twitch soleus fibres and fast-twitch extensor digitorum longus (EDL) fibres and was back to pre-fatiguing levels within 20 min in both fibre types.  $[\text{Ca}^{2+}]_{\text{mit}}$  did not affect tetanic  $[\text{Ca}^{2+}]_i$  and thus, mitochondria do not acutely modulate tetanic  $[\text{Ca}^{2+}]_i$ .  $[\text{Ca}^{2+}]_{\text{mit}}$  was also investigated in mice with skeletal muscle specific disruption of mitochondrial transcription factor A (Tfam KO). Fibres from fast-twitch flexor digitorum brevis (FDB) muscle normally do not accumulate  $\text{Ca}^{2+}$  in their mitochondria during fatigue, but in Tfam KO FDB fibres there was a marked increase in  $[\text{Ca}^{2+}]_{\text{mit}}$ . Tetanic  $[\text{Ca}^{2+}]_i$  was significantly lower in Tfam KO compared to controls, due to downregulation of the sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  buffering protein calsequestrin-1. The increased  $[\text{Ca}^{2+}]_{\text{mit}}$  in Tfam KO could be a means by which ATP-production is boosted. These data suggest that mitochondria do not acutely affect tetanic  $[\text{Ca}^{2+}]_i$ , but that they play a more long-term role in regulation of  $\text{Ca}^{2+}$ -handling by modulating sarcoplasmic reticulum proteins responsible for  $\text{Ca}^{2+}$  buffering. (ii) The modulatory role of CaMKII on  $\text{Ca}^{2+}$  handling was investigated by inhibiting CaMKII in FDB fibres using either KN-93 or an inhibitory peptide. CaMKII inhibition resulted in a significant decrease in tetanic  $[\text{Ca}^{2+}]_i$  when contractions occurred at intervals of 2 s or 300 ms, but not 5 s. Mathematical modelling shows that there is some activation of CaMKII using all protocols but suggests that there is an activity threshold that has to be surpassed to permit sustained SR  $\text{Ca}^{2+}$  release when contractions occur close together in time. (iii) During cold exposure there is an increase in systemic sympathetic activity so the modulatory role of PKA on  $\text{Ca}^{2+}$ -handling was investigated in cold-acclimatized mice. FDB muscles fibres from cold-acclimatized mice display increased resting  $[\text{Ca}^{2+}]_i$ , which was shown to be due to an increased SR  $\text{Ca}^{2+}$  leak. This increased SR  $\text{Ca}^{2+}$  leak was shown to be associated with PKA-mediated phosphorylation of the SR  $\text{Ca}^{2+}$  channel, the ryanodine receptor (RyR), on ser2844 and moderate dissociation of the RyR regulatory protein calstabin-1. An increased leak results in increased SR  $\text{Ca}^{2+}$  cycling and could be a local means of generating heat in the distally and superficially located FDB muscles. Our results show that there are several factors involved in the shaping of skeletal muscle  $[\text{Ca}^{2+}]_i$  handling, some which do so acutely and some are of more importance in the long term.

## LIST OF PUBLICATIONS

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The present thesis is based on the following papers:

- I. Bruton J, Tavi P, **Aydin J**, Westerblad H, Lännergren J  
Mitochondrial and myoplasmic  $[Ca^{2+}]$  in single fibres from mouse limb muscles during repeated tetanic contractions.  
*J Physiol*, 2003; 551:179-90.
- II. **Aydin J**, Andersson DC, Hänninen SL, Wredenberg A, Tavi A, Larsson N-G, Bruton JD, Westerblad H  
Decreased  $Ca^{2+}$  release and force production and increased mitochondrial  $Ca^{2+}$  uptake in mitochondrial myopathy  
Submitted
- III. **Aydin J**, Korhonen T, Tavi P, Allen DG, Westerblad H, Bruton JD  
Activation of  $Ca^{2+}$ -dependent protein kinase II during repeated contractions in single muscle fibres from mouse is dependent on the frequency of sarcoplasmic reticulum  $Ca^{2+}$  release.  
*Acta Physiol*, 2007; 191:131-137
- IV. **Aydin J**, Shabalina I, Reiken S, Zhang S-J, Bellinger AM, Nedergaard J, Cannon B, Marks AR, Bruton JD, Westerblad H  
Skeletal muscle fibres of cold-acclimated wild-type and UCP1 ablated mice display an increased sarcoplasmic reticulum  $Ca^{2+}$  leak.  
Submitted

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## LIST OF ABBREVIATIONS

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|                                 |  |
|---------------------------------|--|
| 8-oxo-dG                        | 8-hydroxy-2'-deoxyguanosine                                |
| $\alpha$ KAP                    | $\alpha$ kinase anchoring protein                          |
| ACh                             | acetylcholine  |
| ADP                             | adenosine diphosphate                                      |
| AMP                             | adenosine monophosphate                                    |
| ATP                             | adenosine triphosphate                                     |
| BAT                             | brown adipose tissue                                       |
| $[\text{Ca}^{2+}]_i$            | free myoplasmic $[\text{Ca}^{2+}]$                         |
| $[\text{Ca}^{2+}]_{\text{mit}}$ | free mitochondrial $[\text{Ca}^{2+}]$                      |
| CaMKII                          | $\text{Ca}^{2+}$ /calmodulin dependent protein kinase II   |
| cAMP                            | cyclic AMP   |
| CGP                             | 7-Chloro-3,5-dihydro-5-phenyl-1H-4,1-benzothiazepine-2-one |
| CK                              | creatine kinase  |
| CM-H <sub>2</sub> DCF           | 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein    |
| CU                              | $\text{Ca}^{2+}$ uniporter                                 |
| CyA                             | cyclosporin A  |
| DHPR                            | dihydropyridine receptor                                   |
| ECC                             | excitation-contraction coupling                            |
| EDL                             | extensor digitorum longus                                  |
| ETC                             | electron transport chain                                   |
| FAD                             | flavin adenine dinucleotide                                |
| FADH <sub>2</sub>               | reduced flavin adenine dinucleotide                        |
| FCCP                            | carbonyl cyanide 4-(trifluoromethoxy)phenyl hydrazone      |
| FDB                             | flexor digitorum brevis                                    |
| H <sub>2</sub> O <sub>2</sub>   | hydrogen peroxide  |
| MHC                             | myosin heavy chain   |
| mPTP                            | mitochondrial permeability transition pore                 |
| mtDNA                           | mitochondrial DNA  |
| NAD <sup>+</sup>                | nicotinamide adenine dinucleotide                          |
| NADH                            | reduced nicotinamide adenine dinucleotide                  |
| NCX                             | $\text{Na}^+/\text{Ca}^{2+}$ exchanger                     |
| NHX                             | $\text{Na}^+/\text{H}^+$ exchanger                         |
| O <sub>2</sub> <sup>•-</sup>    | superoxide anion radical                                   |
| •OH                             | hydroxyl radical   |
| ONOO <sup>-</sup>               | peroxynitrite  |
| P <sub>i</sub>                  | inorganic phosphate  |
| PKA                             | cAMP dependent protein kinase A                            |
| PLB                             | phospholamban  |
| PolgA                           | nuclear encoded catalytic subunit of mtDNA polymerase      |
| ROS                             | reactive oxygen species                                    |
| RyR                             | ryanodine receptor   |
| SERCA                           | sarco/endoplasmic reticulum ATPase                         |
| SLN                             | sarcolipin   |
| SR                              | sarcoplasmic reticulum                                     |
| TCA                             | tricarboxylic acid   |
| Tfam                            | mitochondrial transcription factor A                       |
| UCP                             | uncoupling protein   |



## INTRODUCTION

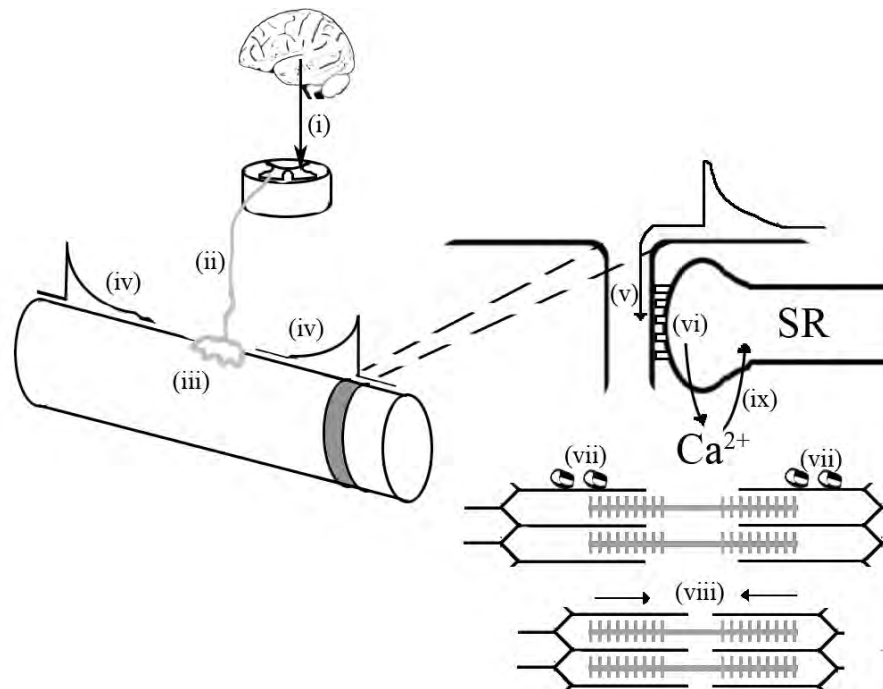
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Movement and communication are everyday processes which in higher vertebrates critically depend on an intact neuronal system and contractions by the highly specialized skeletal muscle cells. The use of skeletal muscles in these daily activities and other physical activities requires precise regulation of force and the timing of contraction. To achieve such performance characteristics, vertebrate skeletal muscles have developed a very unique control mechanism for regulating the free  $\text{Ca}^{2+}$  concentration in their myoplasm ( $[\text{Ca}^{2+}]_i$ ). A basic knowledge of cellular and molecular mechanisms regulating  $[\text{Ca}^{2+}]_i$  is essential in understanding pathological alterations that occur during states such as myositis, myopathies and muscular dystrophies.

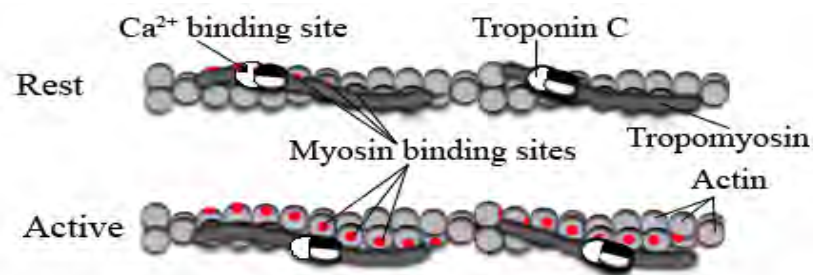
### Skeletal muscle activation

The synchronisation of excitation and contraction is mediated through a series of events shown in Figure 1, commonly referred to as excitation-contraction coupling (ECC), starting with neural impulses from higher motor centres. These travel via descending pathways down to the cell bodies of  $\alpha$ -motoneurons located in the ventral horns of the spinal cord (i). The  $\alpha$ -motoneurons' axons project to a group of muscle fibres (ii). Upon triggering of an action potential, the neurotransmitter acetylcholine (ACh) is released across the neuro-muscular synaptic cleft from the synaptic terminal of the axon (iii). ACh binds to acetylcholine receptors located predominately at the neuromuscular junction of the muscle fibre, resulting in local depolarization of the muscle fibre. This depolarization is sufficient to initiate an action potential (AP) by activating voltage-gated sodium channels in the sarcolemma (iv). The AP propagates in all directions from the neuro-muscular junction and down to the inner parts of the muscle fibre via the transverse tubular system (t-tubules) (v). The t-tubules are narrow invaginations of the sarcolemma forming a branched network of tubules throughout the fibre. APs in the t-tubules activate the voltage-gated sensor protein, the dihydropyridine receptor (DHPR), located in the walls of the t-tubules. The DHPR is in close proximity to the ryanodine receptor (RyR), a  $\text{Ca}^{2+}$ -channel located in the membranes of the sarcoplasmic reticulum (SR).  $[\text{Ca}^{2+}]_i$  is very low in a resting skeletal muscle (50-100 nM) whereas  $[\text{Ca}^{2+}]$  in the SR lumen is high (1-2 mM). The low  $[\text{Ca}^{2+}]_i$  is maintained by an adenosine triphosphate (ATP) driven  $\text{Ca}^{2+}$ -pump, the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA). When activated, the DHPR mechanically interacts with the RyR (Grabner *et al.*, 1999; Takekura *et al.*, 2004), resulting in opening of the RyR. Due to this opening and to the  $\text{Ca}^{2+}$  gradient between the myoplasm and the SR,  $\text{Ca}^{2+}$  is then released into the myoplasm and  $[\text{Ca}^{2+}]_i$  is increased (vi). At rest when  $[\text{Ca}^{2+}]_i$  is low, the regulator protein tropomyosin occupies the binding site for myosin on actin and thereby hinders contraction. When  $[\text{Ca}^{2+}]_i$  increases, it binds to and induces a conformational change of another regulator protein, troponin C (vii). Troponin C is bound to tropomyosin which is moved away from the myosin-binding site on actin allowing the two motor proteins to interact (Figure 2). Cross-bridge cycling is then initiated resulting in contraction of the muscle fibre (viii). When neural impulses cease,  $\text{Ca}^{2+}$ -release stops and  $\text{Ca}^{2+}$  is rapidly pumped back into the SR by SERCA (ix). This leads to dissociation of  $\text{Ca}^{2+}$  from troponin C and

tropomyosin re-occupies and blocks the myosin-binding site on actin, resulting in relaxation of the fibre.



**Figure 1.** Processes involved in excitation contraction coupling. See text for details.

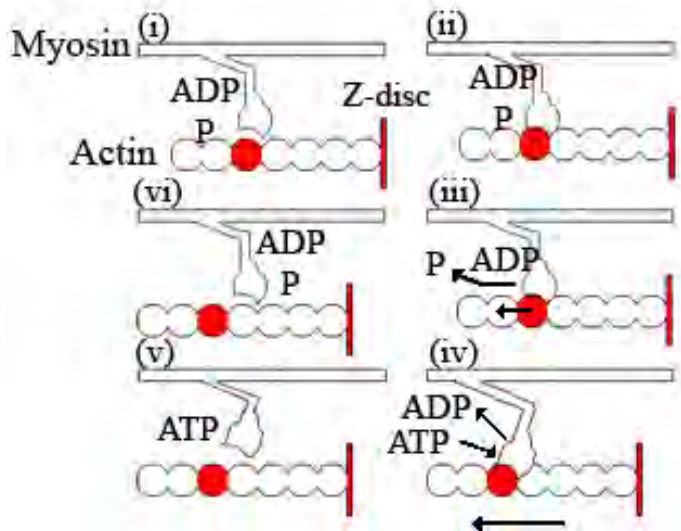


**Figure 2.** In the rested state, access to the myosin binding sites on actin is hindered by tropomyosin. When  $[Ca^{2+}]_i$  increases,  $Ca^{2+}$  will bind to troponin C, inducing a conformational change of tropomyosin which will move away from the myosin binding sites, making them accessible to myosin.

### Cross-bridge cycling

Cross-bridge cycling is driven by hydrolysis of ATP to adenine diphosphate (ADP) and inorganic phosphate ( $P_i$ ). Figure 3 schematically illustrates this process. At rest the myosin heads have ADP and  $P_i$  bound to their ATPase site but do not attach to actin and the two protein filaments can easily slide in relation to each other (i). With SR  $Ca^{2+}$  release and removal of tropomyosin from the myosin binding site, binding between

the myosin head and actin is initiated (ii). Next,  $P_i$  is released from the myosin head and this step is believed to be the force-generating step as actin and myosin go from a weakly bound to a strongly bound state (iii). ADP is then released and the myosin head pulls the actin towards the centre of the sarcomere, resulting in shortening of the sarcomere (iv). ATP from the myoplasm then binds to the myosin head which is detached from myosin (v). The ATP is rapidly hydrolyzed to ADP and  $P_i$  (vi) and the cycle is repeated, assuming that  $[Ca^{2+}]_i$  is still high.



**Figure 3.** The six steps in cross-bridge cycling. Details are given in the text.

### Fatigue

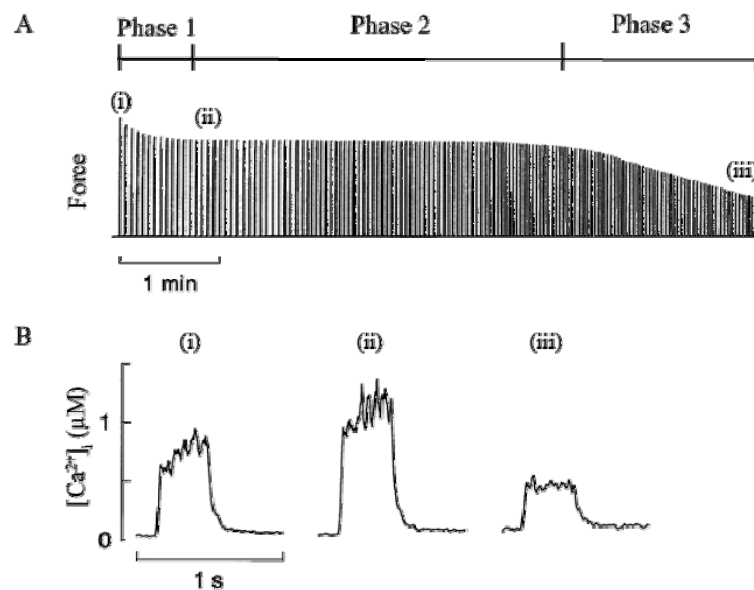
Fatigue is used to describe a phenomenon that occurs after a period of muscular activity. It is subjective and when not clearly defined the term can be used to describe anything between slight tiredness to exhaustion. It is also possible to experience fatigue without prolonged muscle activity as for example after a day that has been mentally challenging but has not involved any major physical effort. We can all relate to these sensations which in themselves are a hint that fatigue involves both a central as well as a peripheral component. A difficult day will affect mainly our central centres, whereas a hard workout in the gym might result in soreness in our muscles for several days. When discussing fatigue it is important to define which particular aspect that is under discussion. In this thesis, only peripheral changes limited to muscle function were investigated and fatigue is defined as an inability to maintain force production during prolonged activation of muscle.

Objective measurements of the changes in muscle function are technically difficult to perform in a field or on a track, so studies are generally laboratory based. It is also difficult to measure muscle function while a subject is moving, so much of the work to date has used isometric contractions both to induce fatigue and to monitor the changes in contractile properties. To study aspects of ECC and SR  $Ca^{2+}$  release is currently not feasible with muscles *in situ*. Much of the current knowledge on the

mechanisms involved in fatigue and ECC has arisen from a mixture of studies performed on humans, isolated whole muscle, intact single fibres and skinned muscle fibres. Skinned muscle fibres are fibres where the sarcolemma is removed either mechanically, using a fine needle, or chemically, using detergents such as saponin or glycerol to make the contractile filaments accessible by means of diffusion. Here we use intact single fibres from mice which enables simultaneous measurements of tetanic  $[Ca^{2+}]_i$  and force. It should be pointed out that each type of preparation has its advantages as well as its drawbacks. Although the intact single fibre method might not precisely reproduce what happens in a muscle *in vivo*, it enables better control of experimental conditions and allows specific intracellular mechanisms to be directly investigated.

### Changes in force and $[Ca^{2+}]_i$ during fatigue

Figure 4 shows typical changes in force and  $[Ca^{2+}]_i$  during fatigue of a single intact fast-twitch mouse muscle fibre induced by repeated short tetani (Westerblad & Allen, 1991; Allen *et al.*, 1995; Allen & Westerblad, 2001). Fatigue occurs in three distinct phases. During phase 1 there is a drop in tetanic force to ~85% of initial force accompanied by an increase in  $[Ca^{2+}]_i$ . Next follows phase 2 with more stable tetanic force and  $[Ca^{2+}]_i$ . The duration of this phase is largely dependent on the fibre types fatigue resistance (see below) and seems to reflect differences in oxidative metabolism as it can be shortened by mitochondrial inhibition with cyanide (Lännergren & Westerblad, 1991; Westerblad & Allen, 1991). In phase 3 both force and  $[Ca^{2+}]_i$  rapidly decrease until stimulation is ceased.



**Figure 4.** Representative changes in force (top) and  $[Ca^{2+}]_i$  (bottom) during fatigue. **A.** Force declines at the onset of fatigue (phase 1). Then follows a longer period where force is stable (phase 2). At the end of fatiguing stimulation, force rapidly declines (phase 3). **B.**  $[Ca^{2+}]_i$  records from representative tetani in A (i-iii). Tetanic  $[Ca^{2+}]_i$  increases during phase 1, remains constant during phase 2, and rapidly decreases in phase 3. Modified from Allen & Westerblad, *J Physiol*, 2001.

### **Skeletal muscle fibre types**

Different skeletal muscles have different contractile and metabolic properties and differ in their fatigue resistance. These differences arise as skeletal muscle is not a homogenous tissue but rather a mixed population of fibres with different properties. The easiest way to spot a difference is to simply look at the colour at the gross anatomical level, where some muscles appear red whereas others are white. This observation was made already in 1873 by Ranvier (cited in Zierath & Hawley, 2004) who also found that dark red muscles contract more slowly, develop tetanus at lower rates of stimulation and are more resistant to fatigue than white muscles. Although these differences in colour are clear in animals such as chicken and mouse, in humans this is not the case.

Defining fibres by their contractile properties divides them into fast-twitch and slow-twitch fibres with some distinct differences (Close, 1972). The twitch of a fast fibre has an earlier peak and faster relaxation time compared to a slow-twitch fibre. The frequency at which a smooth isometric tetanus is reached is different between the two fibre types, with a higher frequency being required in fast fibres (Barclay, 1994). This also means that the force-frequency relationship is different between slow- and fast-twitch fibres. Just as for single twitches, fast fibres have a faster relaxation time from tetanic contractions. Fast-twitch fibres generally also fatigue faster than slow-twitch fibres (Fitts, 1994).

Slow twitch fibres mostly rely on oxidative metabolism and have a high content of mitochondria, myoglobin and oxidative enzymes but are low on glycogen content and glycolytic enzymes compared to fast-twitch fibres (Essen *et al.*, 1975). Therefore they can also be referred to as slow oxidative fibres. Fast twitch fibres differ among themselves with respect to fatigue. Some are more resistant to fatigue as they rely more on oxidative metabolism and are referred to as fast oxidative fibres. Similar to slow fibres, fast oxidative fibres have high myoglobin content but differ in the higher abundance of glycogen. Fast fibres that fatigue more rapidly are limited in their oxidative metabolism capacity and are heavily reliant on energy stored in glycogen and phosphocreatine. These fast glycolytic fibres also have few mitochondria, are low on oxidative enzymes and display low amounts of myoglobin.

The most commonly used fibre type classification system is that based on the isoform of myosin heavy chain (MHC) fibres express. With the MHC system, fibres are divided into four different types: type I, IIa, IIx and IIb with all four types being expressed in rodents whereas humans do not express MHC IIb (Smerdu *et al.*, 1994). The different isoforms of MHC have different ATPase activity, which determines the rate at which ATP is consumed and thereby how fast the fibre is able to contract, with type I being the slowest, type IIa intermediate and IIb/IIx the fastest (Bottinelli & Reggiani, 2000).

The MHC classification system can be used to group fibres according to their functional and metabolic properties which will result in Table 1.

| MHC          | I         | Ila       | Ilb/Ilx    |
|--------------|-----------|-----------|------------|
| Twitch speed | slow      | fast      | fast       |
| Metabolism   | oxidative | oxidative | glycolytic |
| Mitochondria | many      | many      | few        |
| Glycogen     | low       | abundant  | high       |
| Colour       | red       | pink      | white      |

**Table 1.** Grouping of fibre types by myosin heavy chain isoform.

It should, however, be pointed out that the classification of fibre type by MHC is by no means absolute. For example, there are hybrid fibres co-expressing different types of MHC. In human muscle, around 3% of fibres in soleus muscle were found to express both type I and IIa and 28% of fibres from quadriceps were shown to express both IIa and IIx (Larsson & Moss, 1993). No co-expression of type I and IIx was observed. Larsson and Moss (1993) correlated MHC isoform expressions to the maximum shortening velocity ( $V_{max}$ ) and found that fibres exist in a continuum with regards to  $V_{max}$ . These results are interesting and should always be considered when discussing fibre types using MHC isoforms as classification.

Although there is a difference in the speed at which ATP is hydrolysed and consumed in the different MHC isoforms, the MHC type cannot be solely ascribed as the determinant of fatigability. The expression pattern of SERCA also differs within different fibre types where the SERCA1 isoform is expressed in fast type II fibres and the SERCA2a isoform is expressed in slow type I fibres (Lytton *et al.*, 1992). The concentration of SERCA in fast type II fibres is up to eight-fold that found in slow type I fibres (Everts *et al.*, 1989), suggesting that ATP will be consumed at a higher rate in fast type II fibres than in slow type I fibres. Furthermore, the SERCA activity is modulated differently in slow and fast twitch fibres by the endogenous molecules phospholamban (PLB) and sarcolipin (SLN), respectively (Lytton *et al.*, 1992; Odermatt *et al.*, 1998; Periasamy & Kalyanasundaram, 2007). When active, both PLB and SLN lower the  $Ca^{2+}$  affinity of SERCA, but to date the physiological role of PLB has been more investigated than that of SLN (Bhupathy *et al.*, 2007). In addition to these proteins, there are additional proteins with several isoforms, including the SR  $Ca^{2+}$  buffering protein calsequestrin, tropomyosin and troponin C to mention a few, that are expressed differently in fast- and slow-twitch muscle fibres (Bottinelli & Reggiani, 2000). As fast type fibres consume ATP at a higher rate than slow type fibres both at the cross-bridges and at SERCAs, the ability to sustain ATP-production is a major determinant of fatigue resistance. Slow type I fibres not only consume ATP at a lower rate but, as stated above, they also contain more mitochondria and have a higher oxidative capacity than the fast type II fibres and thereby also have better conditions for sustained ATP-production. Hence in general, slow type I fibres are more fatigue resistant than fast type II fibres.

### What causes fatigue

Over the years it has become clear that fatigue involves several complex mechanisms (reviewed by Fitts, 1994; Gandevia, 2001). In principle peripheral fatigue can be caused by disruption at any point between (iii) to (ix) in Figure 1 and previous studies in skeletal muscle have suggested changes in excitability, reactive oxygen species



(ROS) and metabolic changes as underlying mechanisms (Fitts, 1994; Allen *et al.*, 2007).

The hypothesis that reduced excitability causes fatigue is based on that muscle fibres become depolarized with successive or prolonged stimulations. This will eventually lead to APs not being propagated across the sarcolemma and/or down the t-tubules (Allen *et al.*, 2007). Although many studies have showed that repeated skeletal muscle activation leads to increased plasma  $[K^+]$  (Kjeldsen, *et al.*, 1990), increased  $[K^+]$  in the interstitial space (Juel *et al.*, 2000) and a decreased intracellular  $[K^+]$  (Sjøgaard *et al.*, 1985), action potential failure does not seem to occur in humans during exercise (Bigland-Ritchie *et al.*, 1983; West *et al.*, 1996; Sandiford *et al.*, 2005) and cannot be considered as the main causative factor of fatigue.

ROS has been considered to be a mediator of fatigue as treatment with scavengers of ROS such as superoxide dismutase, dimethylsulfoxide and N-acetylcysteine have been shown to delay fatigue development (Shindoh *et al.*, 1990; Reid *et al.*, 1992; Reid *et al.*, 1994). The involvement of ROS seems, however, to be prominent only at low frequencies which was proposed to be due to either reduced SR  $Ca^{2+}$  release or decreased myofibrillar  $Ca^{2+}$  sensitivity (Reid *et al.*, 1994). The exact mechanisms by which ROS exerts its effects are, however, yet to be revealed.

Historically, fatigue has been attributed to metabolic changes and primarily to increased levels of lactic acid formed under anaerobic metabolism. In the cell lactic acid dissociates into lactate and  $H^+$ . Lactate itself does not affect muscle contraction (Posterino *et al.*, 2001) but rather the decreased pH, or acidosis, has classically been considered the cause of fatigue. Evidence in favour of reduced pH causing reduced force in fatigue comes from skinned fibre experiments performed at low temperatures (below 15°C) (Pate *et al.*, 1995). When the same experiments were performed at a more physiological temperature (30°C), the effect of acidosis was very small. These observations are confirmed by work performed in intact single fibres (Westerblad *et al.*, 1997; Bruton *et al.*, 1998), whole muscles (Wiseman *et al.*, 1996) and muscles *in situ* (Baker *et al.*, 1994). These studies suggest that the effects of pH on force are quite small at physiological temperature and that reduced pH is not likely to be the mediator of fatigue, but also that the effects are temperature dependant. Furthermore, humans with McArdle's disease, who have a deficit in myophosphorylase and are unable to utilize glycogen as an energy source because myophosphorylase is needed for the breakdown of glycogen to glucose-6-phosphate, display no decrease in pH during exercise although the onset of fatigue is more rapid than in healthy individuals (Sahlin *et al.*, 1990; Vissing *et al.*, 2001). These observations strongly argue against decreased pH as the dominating mediator of fatigue.

Currently, one popular mechanism behind fatigue is the accumulation of inorganic phosphate ( $P_i$ ) from breakdown of phosphocreatine and possibly ATP during repeated contractions. (Westerblad & Allen, 2002; Steele & Duke, 2003; Myburgh, 2004). Changes in  $P_i$  have been used to explain changes in the different phases of fatigue. The early increase in tetanic  $[Ca^{2+}]_i$  during fatigue has been ascribed to increased levels of  $[P_i]$ . In support of this is the absence of an early increase in  $[Ca^{2+}]_i$  in mice devoid of creatine kinase (CK-/-) which display high resting  $[P_i]$  (Dahlstedt *et al.*, 2000, 2001).  $P_i$

has also been suggested to act on the RyR in phase 1 of fatigue, increasing the channels open probability and thereby causing increased SR  $\text{Ca}^{2+}$  release (Fruen *et al.*, 1994; Balog *et al.*, 2000). Duke & Steele (2001) later showed that the  $\text{P}_i$  effect on RyR is dependent on free  $[\text{Mg}^{2+}]_i$  and that  $\text{P}_i$  inhibits the RyR at concentrations of  $\text{Mg}^{2+}$  observed during the late stage of fatigue (~1.6 mM). Elevated  $[\text{P}_i]$  might also inhibit the SERCA (Dawson *et al.*, 1980; Stienen *et al.*, 1993) or even cause it to run in the reverse mode (Duke & Steele, 2000). Although these findings imply that  $\text{P}_i$  accumulation is necessary for the early increase in tetanic  $[\text{Ca}^{2+}]_i$ , the exact mechanism by which this is achieved is still unclear.

As fatiguing stimulation progresses tetanic  $[\text{Ca}^{2+}]_i$  and myofibrillar  $\text{Ca}^{2+}$  sensitivity both decrease which will result in phase 3 where there is a rapid decline in force (Figure 4, phase 3). These changes are delayed in CK-/- mice (Dahlstedt *et al.*, 2000), suggesting that  $\text{P}_i$  might be responsible for these changes also. The decreased  $[\text{Ca}^{2+}]_i$  release is believed to be due to either inhibition of RyR or formation of  $\text{Ca}^{2+}$ - $\text{P}_i$  precipitates in the SR. While inhibition by  $\text{P}_i$  of RyR has been suggested to occur in a  $\text{Mg}^{2+}$  dependent manner (Duke & Steele, 2001; Steele & Duke, 2003), there is currently no direct evidence to support the hypothesis that  $\text{Ca}^{2+}$ - $\text{P}_i$  precipitation occurs in the SR. There are however a number of studies performed that indirectly support the idea that precipitation occurs in both skinned fibres (Allen & Westerblad, 2001; Steele & Duke, 2003; Dutka *et al.*, 2005) and intact fibres (Westerblad & Allen, 1996c; Kabbara & Allen, 1999). Further studies are required to determine the exact mode of action of  $\text{P}_i$  on late tetanic  $[\text{Ca}^{2+}]_i$  in fatigue.

Ultimately, fatigue is due to a lack of ATP replenishment to drive all the critical steps in contraction and  $\text{Ca}^{2+}$  removal in skeletal muscle.

There are three major systems making ATP available for muscle contraction (Linnane *et al.*, 1998):

- i) the fastest generates ATP from creatine phosphate and ADP.
- ii) the slower anaerobic/glycolytic system uses glucose to generate ATP.
- iii) the slow/oxidative aerobic system is the slowest and generates ATP from glycogen and lipids.

### **The Mitochondria**

Mitochondria are the major providers of energy in the form of ATP during prolonged exercise. Mainly glucose and fatty acids are oxidized in the TCA cycle to yield ATP, carbon dioxide and water. The mitochondria of skeletal muscle are highly adaptive and can adjust their activity to match the ~100 fold increase in energy expenditure when the cell goes from rest to intensive work (Sahlin *et al.*, 1998). The period of time a muscle can sustain contraction (Figure 4, phase 2) is dependent on the ability of its mitochondria to produce ATP by the oxidative system. This is evident not only from the fact that fibre types with more mitochondria are less sensitive to fatigue but also from endurance exercise training studies, where an increase in the size and number of mitochondria (Tarnopolsky *et al.*, 2007) with increased activity of enzymes involved in  $\beta$ -oxidation (Tremblay *et al.*, 1994; Tarnopolsky *et al.*, 2007), enzymes of the TCA cycle (Tremblay *et al.*, 1994) and components in the respiratory chain (Bengtsson *et al.*,

2001) have been observed. These changes increase the capability of the muscle to produce ATP with oxidative metabolism, which results in a later onset of fatigue in endurance-trained individuals (Holloszy & Booth, 1976). It should be noted that mitochondria are not a homogenous population of organelles but differ between cell types in terms of morphology and biochemical properties (Collins & Bootman, 2003). Such differences can also be observed in skeletal muscle where different subpopulations of mitochondria coexist (Krieger *et al.*, 1980; Cogswell *et al.*, 1993; Koves *et al.*, 2005).

Under aerobic conditions, pyruvate is imported into the mitochondria and converted to acetyl coenzyme A which enters the TCA cycle. The eight reactions of the TCA cycle all take place inside the mitochondrial matrix and each reaction is catalyzed by a different enzyme. Four of the reaction steps are oxidation reactions where an electron is transferred to nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) or flavin adenine dinucleotide (FAD), which together with hydrogen become NADH and  $\text{FADH}_2$ . These two molecules then transfer electrons to the electron transport chain (ETC) where oxygen is reduced to water.

The ETC is formed by four different, but closely related, enzyme complexes. The electrons donated by NADH and  $\text{FADH}_2$  are transferred from one complex to another in a series of energy-releasing oxidation reactions. The redox reactions along the ETC pump out hydrogen ions into the mitochondrial intermembrane space, creating a proton gradient. This gradient is ultimately used to drive the generation of ATP from ADP and  $\text{P}_i$  by the mitochondrial  $\text{F}_0\text{F}_1$ -ATPase complex.

### **Mitochondrial $\text{Ca}^{2+}$ uptake**

The ability of mitochondria to sequester  $\text{Ca}^{2+}$  was demonstrated almost 50 years ago in rat kidney cells by DeLuca & Engström (1961). An increase in free mitochondrial  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_{\text{mit}}$ ) has been considered to be an induction signal for apoptosis (Nicotera *et al.*, 1992; Richter, 1993; Hajnoczky *et al.*, 2006). However, more recently it has become more and more evident that the mitochondria also play a role in normal  $\text{Ca}^{2+}$  homeostasis and buffering of the cytosolic  $[\text{Ca}^{2+}]$  has been shown to occur in neurons (David *et al.*, 1998; David, 1999), frog skeletal muscle fibres (Lännergren *et al.*, 2001), cardiac cells (Maack *et al.*, 2006) as well as in non-excitable cells (Montero *et al.*, 2002; Malli *et al.*, 2003).

Sembrowich and co-workers (1985) showed that mitochondria isolated from both fast and slow twitch muscles have the capacity to take up  $\text{Ca}^{2+}$ . Later an increased  $[\text{Ca}^{2+}]_{\text{mit}}$  was reported in exercised muscle from rats (Duan *et al.*, 1990) and humans (Madsen *et al.*, 1996). In rats the increased  $[\text{Ca}^{2+}]_{\text{mit}}$  was suggested to be linked to exercise induced muscle damage (Duan *et al.*, 1990) and in humans there were indications of a reduced ratio of oxidative phosphorylation (Madsen *et al.*, 1996). These observations together suggest that accumulation of mitochondrial  $\text{Ca}^{2+}$  may have detrimental effects on muscle. However, both studies were performed on isolated mitochondria harvested post-exercise and it was not until recently that changes in  $[\text{Ca}^{2+}]_{\text{mit}}$  were measured directly in intact viable muscle fibres (Lännergren *et al.*, 2001). Lännergren *et al.* (2001) showed that muscle fibres from the African clawed frog (*Xenopus laevis*)

take up  $\text{Ca}^{2+}$  during fatiguing stimulation and that this uptake is involved in shaping the tetanic  $[\text{Ca}^{2+}]_i$  transient. The increase in  $[\text{Ca}^{2+}]_{\text{mit}}$  reverted back to its initial value after stopping stimulation, which is in contrast to the reports by Madsen *et al* (1996) and Duan *et al* (1990) where  $[\text{Ca}^{2+}]_{\text{mit}}$  was reported to stay elevated for up to 48 hours. However, when investigating isolated fibres from mouse flexor digitorum brevis (FDB) muscles there was no increase in  $[\text{Ca}^{2+}]_{\text{mit}}$  during fatiguing stimulation (Lännergren *et al.*, 2001). The mouse FDB is not as resistant to fatigue as fibres from *Xenopus laevis* and it is therefore tempting one to speculate that mitochondrial  $\text{Ca}^{2+}$  uptake helps maintaining ATP-production and thereby delays fatigue development.

Usage of  $\text{Ca}^{2+}$  as a signalling molecule is expensive in terms of energy as the  $\text{Ca}^{2+}$  released during each contraction has to be pumped back into the SR. Since  $\text{Ca}^{2+}$  is a second messenger, it would not be unlikely that  $\text{Ca}^{2+}$  also plays a role in driving metabolism. In fact, three dehydrogenases; pyruvate dehydrogenase, isocitrate dehydrogenase, and  $\alpha$ -ketoglutarate dehydrogenase in the TCA-cycle are dependent on  $\text{Ca}^{2+}$  for their full activation (McCormack & Denton, 1993) and  $\text{Ca}^{2+}$  is also required for other mitochondrial functions (Joyal *et al.*, 1995).  $\text{Ca}^{2+}$  has been suggested to directly stimulate oxidative phosphorylation (Kavanagh *et al.*, 2000) and increased  $[\text{Ca}^{2+}]_{\text{mit}}$  increases ATP production in myotubes (Jouaville *et al.*, 1999). These observations stress the importance of  $\text{Ca}^{2+}$  in regulating metabolism and could partly explain the absence of  $[\text{Ca}^{2+}]_{\text{mit}}$  uptake in fast-twitch, easily fatigable mammalian muscles (Lännergren *et al.*, 2001). If this is the case then  $[\text{Ca}^{2+}]_{\text{mit}}$  is more likely to increase in the more fatigue resistant slow-twitch fibres.

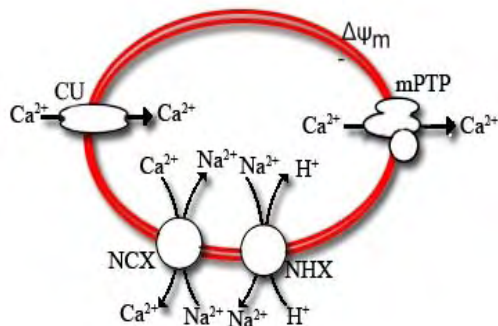
### Mechanisms of mitochondrial $\text{Ca}^{2+}$ uptake and extrusion

Mitochondrial  $\text{Ca}^{2+}$  uptake is dependent on the mitochondrial membrane potential and mediated by means of a  $\text{Ca}^{2+}$  carrier, the  $\text{Ca}^{2+}$  uniporter (CU), located in the mitochondrial inner membrane (Gunter & Gunter, 1994; Bernardi, 1999). It has been shown that the CU is a very specific channel for  $\text{Ca}^{2+}$  (Kirichok *et al.*, 2004). The study by Kirichok *et al.* (2004) also showed that CU was regulated by oscillations in cytosolic  $[\text{Ca}^{2+}]_i$  and that the uptake was inhibited by ru360, a colourless component of ruthenium red. Later, Moreau *et al.* (2006) were able to show that the CU is biphasically regulated by  $\text{Ca}^{2+}$  with the  $[\text{Ca}^{2+}]_i$  required to initiate uptake being very similar (10-20  $\mu\text{M}$ ) to the  $[\text{Ca}^{2+}]_i$  that rapidly deactivates the CU. The authors demonstrated that the CU desensitizes and that it is the time between oscillations in  $[\text{Ca}^{2+}]_i$  and not the concentration itself that determines the uptake. Other factors reported to regulate the CU include  $\text{Mg}^{2+}$  and adenine nucleotides (Litsky & Pfeiffer, 1997). Although the CU has been functionally described, its molecular components and structures have yet to be defined (Saris & Carafoli, 2005). The most recent suggestion is that the two uncoupling proteins (UCPs) UCP-2 and UCP-3 are fundamental contributors to the CU (Trenker *et al.*, 2007).

A second mechanism of mitochondrial  $\text{Ca}^{2+}$  uptake has been reported. The rapid uptake mode (RAM) (Sparagna *et al.*, 1995; Buntinas *et al.*, 2001). As the name implies, mitochondrial  $\text{Ca}^{2+}$  uptake occurs on a millisecond timescale and allows fast changes in  $[\text{Ca}^{2+}]_{\text{mit}}$  to mirror change of  $[\text{Ca}^{2+}]_i$ . However, additional reports on RAM have been

sparse and RAM might instead represent the uptake by CU before desensitization develops as described by Moreau *et al.* (2006).

Efflux of  $\text{Ca}^{2+}$  from the mitochondria is primarily achieved by exchange of  $\text{Na}^+$  by a mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) and  $\text{Na}^+$  is in turn pumped out in exchange for  $\text{H}^+$  by a  $\text{Na}^+/\text{H}^+$  exchanger (NHX) (Brookes *et al.*, 2004) (Figure 5). Another proposed extrusion route is opening of the mitochondrial permeability transition pore (mPTP) (Bernardi & Petronilli, 1996). The mPTP is putatively assembled from cyclophilin D, adenine nucleotide translocator and the voltage dependent anion channel, a group of pre-existing proteins in the mitochondrial inner and outer membranes (Crompton, 1999). mPTP has binding sites for  $\text{Ca}^{2+}$  on the matrix side which are believed to regulate its activity. It has been suggested that flickering of the mPTP between the open and the closed state serves to release  $\text{Ca}^{2+}$  from the matrix (Huser & Blatter, 1999) but prolonged mPTP opening might have pathological consequences (Crompton, 1999).



**Figure 5.** Mitochondrial  $\text{Ca}^{2+}$  uptake is mediated by the  $\text{Ca}^{2+}$  uniporter (CU). Uptake is also dependant on the mitochondrial membrane potential ( $\Delta\psi_m$ ).  $\text{Ca}^{2+}$  is cleared in exchange for  $\text{Na}^{2+}$  by a  $\text{Na}^{2+}/\text{Ca}^{2+}$  exchanger (NCX) and  $\text{Na}^{2+}$  is then removed in exchange for  $\text{H}^+$  by a  $\text{Na}^{2+}/\text{H}^+$  exchanger (NHX). Extrusion of  $\text{Ca}^{2+}$  could also be achieved by induction of the mitochondrial permeability transition pore (mPTP).

### Mitochondrial reactive oxygen species production

The mitochondria are also considered to be the main site of production of reactive oxygen species (ROS) (Wallace, 1999;Orrenius *et al.*, 2007). ROS is produced during oxidative respiration in all aerobic organisms and is generally believed to be beneficial in small amounts, while large quantities of ROS are considered to be detrimental to cells (Wallace, 1999;Tarnopolsky & Raha, 2005;Orrenius *et al.*, 2007).

There is a constant leak of electrons from the ETC, which rapidly form ROS. Of the total oxygen consumption, 1-2% is generally believed to result in formation of ROS (Orrenius *et al.*, 2007) although more recently levels as low as 0.15% have been reported (St Pierre *et al.*, 2002). ROS are by definition small molecules containing unpaired electrons which make them highly reactive. Examples of ROS are the superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) and the hydroxyl radical ( $\cdot\text{OH}$ ) (Orrenius *et al.*, 2007).  $\text{O}_2^{\cdot-}$  is short lived and rapidly converted to  $\text{H}_2\text{O}_2$  by mitochondrial superoxide dismutase.  $\text{H}_2\text{O}_2$  is not a true ROS but is nonetheless highly reactive. It is also more stable than  $\text{O}_2^{\cdot-}$  and can diffuse out of the mitochondria.  $\text{H}_2\text{O}_2$  can also form the highly reactive hydroxyl radical ( $\cdot\text{OH}$ ) by reacting with transition metals such as  $\text{Fe}^{2+}$ . If  $\text{O}_2^{\cdot-}$  is

produced simultaneously with nitric oxide (NO) the two could react to form the highly reactive nitrogen species peroxynitrite, ONOO<sup>-</sup> (Orrenius *et al.*, 2007).

### **Mitochondria, ROS and ageing**

Ageing of skeletal muscle involves a decrease in muscle mass caused by a decrease in fibre size and number, primarily of type IIb fibres (Larsson *et al.*, 1978). Skeletal muscle can undergo both complete cell death as well as partial cell death where only individual myonuclear are fragmented and age-related muscle wasting is thought to be related to the loss of myonuclei through an apoptosis-like mechanism (Marzetti & Leeuwenburgh, 2006).

The decline of basal metabolic rate is characteristic of ageing which, has placed mitochondria under attention in the biology of ageing due to their central role in ATP production. The involvement of ROS was suggested already in 1956 by Harman who outlined the free radical theory of ageing. This theory states that organisms age because cells accumulate free radical damage with the passage of time. Targets of ROS affected in ageing include proteins, lipids and DNA (Navarro & Boveris, 2007; Orrenius *et al.*, 2007).

Protein oxidation has a major importance in the molecular mechanisms involved in cell turnover and cell cycle and in triggering apoptosis (Navarro & Boveris, 2007). The proteolytic enzymes that degrade modified proteins decline with ageing and less effective removal results in accumulation of oxidized proteins (Navarro & Boveris, 2007). Furthermore, ROS can also lead to formation of 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) in mtDNA which could cause deleterious effects by preventing or impairing replication of DNA (Chomyn & Attardi, 2003). 8-oxo-dG is routinely used as an indicator of DNA damage and has been shown to be 16 times higher in mtDNA than in nuclear DNA (Richter *et al.*, 1988). This has led to the suggestion of a vicious cycle in which mitochondrial DNA (mtDNA) mutagenesis caused by ROS leads to production of defective mtDNA-encoded proteins of the ETC chain (Ozawa, 1995). The insertion of defective proteins into the complexes of the ETC would in turn lead to less effective oxidative phosphorylation with increased ROS production, causing more mtDNA mutations and the whole process is repeated. This detrimental, autocatalytic event will eventually result in apoptosis (Marzetti & Leeuwenburgh, 2006).

It is currently unknown whether an increased ROS production is preceded by mtDNA damage or vice versa. The involvement of mtDNA mutagenesis in ageing is supported by the findings of Trifunovic *et al.* (2004) who generated knock-in mice expressing a defective proof-reading version of PolgA, the nuclear encoded catalytic subunit of mtDNA polymerase which resulted in a prematurely ageing phenotype with accelerated accumulation of mtDNA mutations. However, in a subsequent report the same group demonstrated that these mice do not have increased formation of ROS (Trifunovic *et al.*, 2005). Similar findings were reported by another group who also generated mice with defective PolgA, which also displayed an accumulation of mtDNA mutations without an increase in H<sub>2</sub>O<sub>2</sub> production or increased oxidative damage to proteins and lipids (Kujoth *et al.*, 2005). Together, these studies show that mtDNA

mutations, but not ROS, are a determinant factor in ageing, and hence challenge the mitochondrial theory of ageing outlined by Harman (1956).

### **Mitochondrial myopathy – the Tfam model**

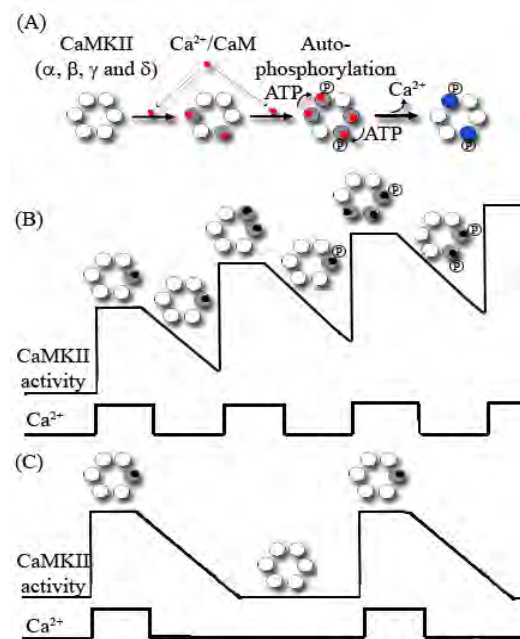
Mitochondrial transcription factor A (Tfam) is a nuclear-encoded transcription factor for mtDNA. Tfam is essential for maintenance of mtDNA and plays significant roles in transcription, replication, damage sensing and DNA repair (Larsson *et al.*, 1998; Ekstrand *et al.*, 2004). Tissue specific disruption of Tfam in skeletal muscle causes a myopathy characterized by ragged-red fibres with increased numbers of morphologically abnormal mitochondria and progressively deteriorating respiratory function (Wredenberg *et al.*, 2002). It was proposed that the increase in mitochondrial mass was a compensatory mechanism to counteract the respiratory deficiency. Muscles from Tfam knock-out mice (Tfam KO) do not fatigue more rapidly than their wildtype littermates but display lower absolute forces and especially at lower frequencies. The force reduction was believed to be due to a deficit in the force generating cross bridges and/or a changed  $\text{Ca}^{2+}$  homeostasis. To further clarify the force deficit, experiments using single fibres are necessary and these experiments were undertaken in this thesis.

### **Modulation of $\text{Ca}^{2+}$ transients by kinases**

In recent years, the involvement of kinases in modulating ECC has been increasingly studied and has focused mainly on two specific kinases,  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase II (CaMKII) and cyclic AMP (cAMP) dependent protein kinase A (PKA). The second part of this thesis will deal with modulation of tetanic  $[\text{Ca}^{2+}]_i$  by CaMKII and PKA.

### **CaMKII**

CaMKII belongs to a group of enzymes termed  $\text{Ca}^{2+}$  decoders that can respond to variations in frequency, duration and amplitude of the  $\text{Ca}^{2+}$  signalling (De Koninck & Schulman, 1998). CaMKII is composed of 8-12 subunits (Bennett *et al.*, 1983; Kuret & Schulman, 1984) and there are four different subunit isoforms:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  (Hudmon & Schulman, 2002). Skeletal muscle CaMKII is composed of  $\gamma$ -,  $\delta$ - and  $\beta_m$ -subunits with the last one being a splice variant specific for skeletal muscle (Bayer *et al.*, 1998; Sacchetto *et al.*, 2000). Upon activation, the subunits are autophosphorylated on thr286 ( $\alpha$ ) or thr287 ( $\beta$ ,  $\gamma$  and  $\delta$ ), prolonging the enzymes' period of activity (Yamagata & Obata, 1998). An activated subunit can activate neighbouring subunits as well as other molecules. With successive  $\text{Ca}^{2+}$  transients, CaMKII accumulates in an active state and may persist in this state even after removal of the  $\text{Ca}^{2+}$  transient (De Koninck & Schulman, 1998; Hudmon & Schulman, 2002) (Figure 6).



**Figure 6.** CaMKII is activated upon binding of  $\text{Ca}^{2+}/\text{CaM}$  and activated subunits can phosphorylate neighbouring subunits, prolonging the activity of the enzyme. If the enzyme is phosphorylated it can remain active after dissociation of  $\text{Ca}^{2+}$  (A). When  $\text{Ca}^{2+}$  transients are delivered close together in time the activity of CaMKII accumulates and the activity can be sustained even after removal of  $\text{Ca}^{2+}$  (B). If  $\text{Ca}^{2+}$  transients occur more separated in time, there will not be any accumulation of CaMKII activity which will return to basal levels between stimulations (C).

### CaMKII and ECC

The majority of the current knowledge on CaMKII and ECC originates from studies performed on cardiac cells where CaMKII is involved in fine-tuning of the cytosolic  $\text{Ca}^{2+}$  transients by modulating SR  $\text{Ca}^{2+}$  release (Maier *et al.*, 2003; Kohlhaas *et al.*, 2006; Guo *et al.*, 2006) and enhancing SR  $\text{Ca}^{2+}$  reuptake (Bassani *et al.*, 1995; Li *et al.*, 1998; DeSantiago *et al.*, 2002). Experiments on isolated cardiac RyR (RyR2) incorporated into lipid bilayers suggested that CaMKII mediated phosphorylation of RyR2 could either increase (Witcher *et al.*, 1991; Hain *et al.*, 1995) or decrease (Lokuta *et al.*, 1995; Dzhura *et al.*, 2000) its open probability. Although these findings have given contradictory results, more recent studies performed on intact cardiomyocytes have shown that CaMKII is closely associated with RyR2 (Zhang *et al.*, 2003; Currie *et al.*, 2004; Wehrens *et al.*, 2004) and that overexpressing a cardiac specific subunit of CaMKII, CaMKII $\delta_c$ , results in increased SR  $\text{Ca}^{2+}$  release (Maier *et al.*, 2003; Kohlhaas *et al.*, 2006; Guo *et al.*, 2006). Furthermore, blocking CaMKII using the pharmacological inhibitor KN-93 resulted in a marked decrease of spontaneous SR  $\text{Ca}^{2+}$  release (Maier *et al.*, 2003). Thus, the results of whole cell experiments are in favour of CaMKII increasing RyR2 open probability. The site of CaMKII phosphorylation on RyR2 was initially reported to be ser2809 (Witcher *et al.*, 1991), however Wehrens *et al.* (2004) later suggested that CaMKII phosphorylates ser2815, a site distinct from ser2809 (which is also the site for PKA phosphorylation, see below). To add further to the controversy, additional sites have been reported (Rodriguez *et al.*, 2003). Cardiac cells overexpressing CaMKII $\delta_c$  have an increased relaxation rate which, has been suggested to be due to phosphorylation of either SERCA2a (cardiac and slow-twitch skeletal muscle isoform) at ser38 (Toyofuku *et al.*, 1994; Xu *et al.*, 1993; Hawkins *et al.*, 1994) or its regulatory protein PLN at thr17 (Hagemann *et al.*, 2000).



In comparison to cardiac muscles, very little work has been done on skeletal muscle and the question whether and how CaMKII is involved in fine tuning of tetanic  $[Ca^{2+}]_i$  transients remains open. Identified targets involved in ECC for CaMKII in skeletal muscle are PLN (Damiani *et al.*, 2000; Sacchetto *et al.*, 2000), triadin (Colpo *et al.*, 2001; Rose *et al.*, 2006) and SERCA2a (Damiani *et al.*, 2000). In skeletal muscle a specific anchoring protein found in the SR,  $\alpha$  kinase anchoring protein ( $\alpha$ KAP), binds and localizes CaMKII in close proximity to the RyR1 (Bayer *et al.*, 1998). Phosphorylation of RyR1 by CaMKII has been demonstrated to occur by Dulhunty *et al.* (2001), resulting in an increased open probability of the channel. However, although the authors demonstrate a functional effect of RyR1 phosphorylation, no attempt was made to determine the site of phosphorylation. A general belief seems to be that the site targeted by CaMKII in skeletal muscle and RyR1 is ser2844. However these assumptions are based on findings in cardiac muscle and, likewise, discussions regarding functional effects on RyR1 are often based on a mixture of findings from RyR1 and RyR2 (see for example Sacchetto, 2005) in the absence of data from skeletal muscle.

CaMKII activity was shown to increase in skeletal muscle after exercise in humans (Rose & Hargreaves, 2003) and later Rose *et al.* (2007) also demonstrated that the same occurs in stimulated muscles from rat. Furthermore, Tavi *et al.* (2003) demonstrated that inhibiting CaMKII in intact muscle fibres during a bout of contractions resulted in reduced SR  $Ca^{2+}$  release, suggesting a role for CaMKII in  $Ca^{2+}$  handling. One goal of this thesis will be to further elucidate the effect on SR  $Ca^{2+}$  release and to see whether the involvement of CaMKII differs during work of different intensities.

### **PKA and ECC**

The activation pattern of PKA is different from that of CaMKII and is not linked to  $Ca^{2+}$  but rather to the second messenger cAMP. Stimulation of membrane bound  $G_s$ -protein coupled  $\beta$ -adrenergic receptors by the catecholamines adrenaline and noradrenaline causes an elevation of the second messenger cyclic AMP by activation of adenylyl cyclase (Wallukat, 2002). Skeletal muscle express both the  $\beta_1$  and  $\beta_2$  isoforms of the  $G_s$ -coupled adrenergic receptors with  $\beta_2$  being the dominating isoform in both fast- and slow-twitch muscles (Kim *et al.*, 1991; Jensen *et al.*, 1995). Acute  $\beta$ -adrenergic stimulation has long been known to increase force production in skeletal muscle (Brown *et al.*, 1948; Williams & Barnes, 1989; Cairns *et al.*, 1993) and this was suggested to be mediated by PKA-induced phosphorylation of RyR1 (Cairns *et al.*, 1993). One situation during which adrenergic activation occurs is during cold exposure (LeBlanc *et al.*, 1967).

Recently there has been some controversy regarding the effects of PKA-mediated phosphorylation on RyR. Marks and co-workers (Ward *et al.*, 2003; Reiken *et al.*, 2003b) have shown that an elevated PKA-mediated phosphorylation of RyR1 on ser2844 results in dissociation of the RyR1 inhibiting protein calstabin-1 (also known as FKBP12) resulting in more leaky channels with a decrease in associated action-potential activated SR  $Ca^{2+}$  release and reduced force production. This has however been more intensely studied in cardiac muscle where phosphorylation of ser2809 on

RyR2 in the same fashion leads to dissociation of calstabin-2 (also known as FKBP12.6) (Marx *et al.*, 2000, Lehnart *et al.*, 2006). These findings have been replicated by some (reviewed by Danila & Hamilton, 2004), but other groups have reported that there is no dissociation of calstabin-1 after phosphorylation by PKA and suggest that other signals beside phosphorylation are necessary to induce dissociation (Li *et al.*, 2002; Stange *et al.*, 2003). Indeed it has been demonstrated that other ECC proteins in skeletal muscle can be phosphorylated by PKA, including the DHPR (on ser687) (Lu *et al.*, 1995) and phospholamban (on ser16) (Liu *et al.*, 1997).

### **Skeletal muscle in cold-acclimatization**

One by-product of breakdown of ATP is heat (de Meis, 2005). During exercise this is very evident but not vital. However, during exposure to cold it is necessary to increase heat production. Shivering is one mechanism by which mammals generate heat in response to cold (Hemingway, 1963). Shivering is when muscles are asynchronously activated to maintain core body temperature. During prolonged cold exposure rodents can increase their heat production by increasing metabolism in their brown adipose tissue (BAT) by activation of uncoupling protein 1 (UCP-1) (Harper & Himms-Hagen, 2001; Cannon & Nedergaard, 2004). UCP-1 sits on the inner membrane of mitochondria and is a proton channel that allows protons to leak out of the matrix and reduce the proton gradient. The activation of UCP-1 will result in an increased ATP consumption as the ATPase will run in the reverse mode in order to restore the proton gradient. The increased consumption rate of ATP will result in more heat being emitted and the blood passing through BAT will carry the heat to the nearby visceral organs.

Cold exposure activates the sympathetic nervous system and increased plasma levels of noradrenaline in response to cold have been reported (LeBlanc *et al.*, 1967; Roth *et al.*, 1988; Dulloo *et al.*, 1988). To further elucidate the role of prolonged adrenergic stimulation on skeletal muscle and especially  $\text{Ca}^{2+}$  homeostasis we used intact muscle fibres from animals acclimatized to cold.

## AIMS

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The overall aim of this thesis is to investigate modulation of intracellular  $\text{Ca}^{2+}$  handling by mitochondria, CaMKII and PKA in skeletal muscle. The specific aims are:

- To investigate the mechanisms for mitochondrial  $\text{Ca}^{2+}$  uptake and how this is related to fatigue in skeletal muscle.
- To study changes in tetanic  $[\text{Ca}^{2+}]_i$  and  $[\text{Ca}^{2+}]_{\text{mit}}$  and ROS in mice with mitochondrial myopathy in order to determine the cause of the reduced force production in these animals.
- To study the involvement of CaMKII in SR  $\text{Ca}^{2+}$  release during exercise of different intensities
- To study the effects of chronic  $\beta$ -adrenergic stimulation on skeletal muscle in cold-acclimatized mice.

## MATERIAL AND METHODS

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### Animals

Animals were housed at room temperature with a 12 h:12 h light-dark cycle. Food and water were provided *ad libitum*. The following rodents were used: NMRI (Naval Marine Research Institute) mice (study I and III), skeletal muscle specific Tfam knock-out mice (Tfam KO) (Wredenberg *et al.*, 2002) with matching wildtype littermates (study II) and UCP1 knock-out mice (UCP -/-) (Enerbäck *et al.*, 1997) with matching wildtype littermates (study IV). Mice were euthanized by cervical dislocation and muscles were excised. All procedures were approved by the Stockholm North local ethics committee.

### Cold acclimatization

In study IV adult female UCP1 -/- mice and their matching wildtype littermates were divided into age- (7-8 weeks) and body weight (17-18 g)-matched groups and kept at 24°C or were cold acclimatized by first placing them at 18°C for 4 weeks and then at 4°C for 4-5 weeks before sacrifice. The intermediate step at 18°C is required to allow for survival of the UCP1 -/- mice at 4°C (Golozubova *et al.*, 2001).

### Single fibre dissection and mounting

Single fibres were isolated under a dark-field illumination microscope using a pair of jeweller's forceps and micro-iris scissors. The extensor digitorum longus (EDL) muscles were split into four bundles, whilst the soleus muscles were split longitudinally. In each segment a small group of fibres lying on the outer margin of the bundle were selected and all other fibres dissected away by cutting them midway along their length. A single fibre in the remaining small bundle was selected and then isolated by removing the other fibres and connective material that holds the fibres together. The flexor digitorum brevis (FDB) muscles were split into three bundles after which each bundle was trimmed down to two or three fibres. One fibre was selected and the rest were cut through the sarcolemma, leaving only the selected fibre viable. The fibres ability to contract was tested by focal electrical stimulation. After isolation of a single fibre, the tendons were gripped by platinum-foil micro-clips and the preparation was transferred to the perfusion channel of an experimental chamber, placed on the stage of an inverted microscope. The bottom of the chamber consisted of a thin glass cover slip and fibres were mounted as close as possible to this. The fibre was flanked by platinum electrodes which were used for stimulation.

### Force measurements

Force from mechanically dissected fibres was measured in study I-IV using an Akers AE801 force transducer vertically mounted in the experimental chamber. The force transducer was provided with a glass tube extension with a fine platinum hook at the end. On the other end, there was a hook on an adjustable holder. The tendon clips were attached to the hooks, suspending the fibres horizontally. The fibre was stretched to the optimal length, i.e. the length giving the maximal tetanic force, and

fibre diameters were measured in order to obtain the developed force per cross-sectional area.

### Myoplasmic $[Ca^{2+}]_i$ measurements

The fluorescent  $Ca^{2+}$  indicator indo-1 (Invitrogen/Molecular Probes) was used to measure free myoplasmic  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) in *study I-IV*. Fibres were loaded with indo-1 by backfilling it into a glass microelectrode and penetrating the sarcolemma with the tip of the electrode. The indo-1 was pressure-injected into the fibres. The fluorescence was measured in a system consisting of a Xenon lamp, a monochromator and two photomultiplier tubes. Excitation was set to  $360 \pm 5$  nm and light emitted was collected at  $405 \pm 5$  nm and  $495 \pm 5$  nm. After correction of background, the ratio of the light emitted at 405 nm and 495 nm ( $R$ ) was translated to  $[Ca^{2+}]_i$  by using the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_D \beta (R - R_{min}) (R_{max} - R)^{-1} \quad (\text{Eqn 1})$$

where  $K_D$  is the dissociation constant of indo-1,  $\beta$  is the ratio of the 495 nm signals at very low and saturating  $[Ca^{2+}]_i$ ,  $R_{min}$  is the ratio of 405/495 nm signals at very low  $[Ca^{2+}]_i$  and  $R_{max}$  is the ratio of 405/495 nm signals at saturating  $[Ca^{2+}]_i$ . Values for  $K_D$  and  $\beta$  were assumed to be similar to those established previously in mouse toe fibres, whilst  $R_{min}$  and  $R_{max}$  were established intracellularly as described previously (Andrade *et al.*, 1998).

### Force- $[Ca^{2+}]_i$

Force- $[Ca^{2+}]_i$  curves were produced in *study I, II and IV* by plotting the force measured during the last 100 ms of 10-100 Hz stimulations against the  $[Ca^{2+}]_i$  measured in the same contraction. These data were then fitted with the following Hill equation:

$$P = P_{max} [Ca^{2+}]_i^N / (Ca_{50}^N + [Ca^{2+}]_i^N)^{-1} \quad (\text{Eqn 2})$$

where  $P$  is the tetanic force,  $P_{max}$  the peak tetanic force,  $Ca_{50}$  is the  $[Ca^{2+}]_i$  that gives 50% of  $P_{max}$  and  $N$  is a measure of the slope of the relationship.

### Enzymatic dissociation

Enzymatic dissociation was used in *study II* as described by Liu and co-workers (1997) with slight modifications. Briefly, the whole FDB was isolated and placed into a petri dish containing Dulbecco's modified eagle medium (DMEM) (Invitrogen/Gibco) with 10% foetal bovine serum (Invitrogen/Gibco) and 0.3% collagenase type I (Sigma-Aldrich). Muscles were then placed in a water-saturated incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ -95% air for 2-3h. For full dissociation muscles were transferred to a dish with fresh DMEM and gently pipetted up and down about ten times. Dissociated fibres were then transferred to laminin coated petri dishes and allowed to attach for 15 min before DMEM supplemented with 0.55  $\mu\text{g/ml}$  transferrine (Sigma), 0.5 ng/ml selenium (Sigma), 10 mU/ml insulin (Novo Nordisk A/S) and 1  $\mu\text{l/ml}$  antibiotic/antimycotic solution (Sigma) was added. Fibres were then kept at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ -95% air up to 24 hours before experiments.

### Mitochondrial dyes and laser confocal microscopy

Laser confocal microscopy images were obtained in *study I and II* using a BioRad MRC 1024 confocal unit with a krypton-argon mixed gas laser run at 15 mW and attached to a Nikon Diaphot 200 inverted microscope. In *study I* mitochondrial  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_{\text{mit}}$ ) was monitored using Rhod 2-AM. The dye was excited with 568 nm light and the light emitted was collected through a 585 nm long pass filter. Rhodamine 123 (R123), a indicator for the mitochondrial membrane potential, was also used in *study I*. R123 was excited with 488 nm light and emission was collected through a 522 nm band-pass filter. In *study II* MitoSox Red was used to monitor the production of mitochondrial superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) and 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein (CM-H<sub>2</sub>DCF; 5  $\mu\text{M}$ ) was used as a general detector of reactive oxygen species (ROS). MitoSox Red was excited with 488 nm light and emission collected through a 585 nm long-pass filter. CM-H<sub>2</sub>DCF was excited using 488 nm light and emission collected through a 515 nm long-pass filter. Changes in fluorescence at any time point (F) were expressed relative to fluorescence at rest ( $F_0$ ) as  $F/F_0$ . During repeated tetanic stimulations, 10 s pauses were included at regular intervals for obtaining confocal images. All dyes were from Invitrogen/Molecular Probes.

### Stimulation protocols

A variety of different stimulation protocols were used to induce fatigue. Fatigue was defined as the point where force dropped to 40% of initial. Mechanically dissected fibres were allowed to rest for at least 30 min after injection of indo-1. Then force was recorded using 10-100 Hz pulse trains administered every minute in order to establish the force-frequency relationship. After this, fibres were allowed to rest another 10 min before fatiguing stimulation was applied. The following different protocols were used:

*Study I.* EDL fibres were stimulated with up to 500 tetani using 70 Hz pulse trains of 200 ms duration given every 2 s. The protocol for the soleus differed in number of tetani and train duration in that up to 1000 tetani were used with the pulse train duration set to 500 ms. When the mechanisms for mitochondrial  $\text{Ca}^{2+}$  uptake and extrusion were assessed, fibres were subjected to two series of 25 tetani using 70 Hz pulse trains of 200 ms duration at 2 s intervals. Fibres were first stimulated in the absence of drug and 45-60 min later in the presence of drug. Images were obtained at rest, after 10 and 25 tetani and during recovery.

*Study II.* Fatigue was induced in mechanically dissected FDB fibres injected with indo-1 by evoking up to 50 tetani using 70 Hz pulse trains, 300 ms duration, given every 2 s. The same stimulation was used when monitoring  $[\text{Ca}^{2+}]_{\text{mit}}$ , with the inclusion of 10 s pauses every tenth tetani in order to obtain confocal images. When studying changes in ROS with MitoSox Red or CM-H<sub>2</sub>DCF in chemically dissociated fibres, 1 Hz stimulation was used.

*Study III.* To look at the frequency dependency of CaMKII activation three different stimulation protocols were adopted, all of which used 70 Hz pulse trains but differed in train duration, interval and number. Protocols were: long interval (350 ms trains, at 5 s intervals, 25 times), fatigue (350 ms trains, at 2 s intervals, 50 times) and high intensity (200 ms trains, at 300 ms intervals, 20 times).

#### **Drugs used for assessing $[Ca^{2+}]_{mit}$ uptake and release**

The mechanisms for skeletal muscle mitochondrial  $Ca^{2+}$  uptake and extrusion were assessed using drugs targeting the different complexes involved. The  $Na^+/Ca^{2+}$  exchanger (NCX) was inhibited using 10  $\mu$ M of 7-Chloro-3,5-dihydro-5-phenyl-1H-4,1-benzothiazepine-2-one (CGP), the mPTP was inhibited using 4  $\mu$ M cyclosporine A (CyA) and the mitochondrial membrane potential was disrupted using 0.5  $\mu$ M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

#### **CaMKII inhibitory agents**

The CaMKII inhibitory peptide AC3-I (KKALHRQEAVDCL) was assembled on an ABI 433A Peptide Synthesizer using Fmoc chemistry, according to routine procedures. AC3-I is a modified CaMKII substrate with the amino acid sequence HRQEAVDCL, corresponding to the autophosphorylation site (thr286/287) on CaMKII, except for alanine replacing threonine to prevent phosphorylation. The peptide was dissolved in 150 mM KCl, 10 mM Hepes, pH 7.1 to a final concentration of 1–10 mM and then mixed with the indo-1 solution and injected into isolated muscle fibres. Fibres were allowed to rest for 60 min after the injection. The amount of injected peptide was estimated from the change in indo-1 fluorescence, as described elsewhere (Tavi *et al.*, 2003). The cell permeable pharmacological inhibitor of CaMKII, KN-93, was purchased from Calbiochem. Fibres were superfused by 3  $\mu$ M KN-93 for 30 min before being tested.

#### **Mathematical modelling of CaMKII activation**

The  $Ca^{2+}$  dependency of CaMKII activation was simulated in *study III* as previously described (Tavi *et al.*, 2003) by utilizing a previously published reaction scheme (Bhalla & Iyengar, 1999). Briefly, the model takes the experimentally obtained  $[Ca^{2+}]_i$  signal as an input and simulates the corresponding  $Ca^{2+}$  binding dynamics of calmodulin, calcineurin/protein phosphatase 2B, protein phosphatase 1 and CaMKII based on the reaction pathway equations. The only difference from the model published by Bhalla & Iyengar (1999) was that the total amount of CaMKII was set to 1  $\mu$ M instead of 7  $\mu$ M. The model was incorporated into Matlab 6.5 (Mathworks, USA) as time-dependent differential equations and simulated using the Matlab solver function 'ode15s', based on numerical differentiation formulas.

#### **$Ca^{2+}$ tail analysis**

In *study IV* SR  $Ca^{2+}$  pumping and SR  $Ca^{2+}$  leak were assessed using a model originally described by Klein and co-workers (1991) by measuring the tails of elevated  $[Ca^{2+}]_i$  after 100 Hz tetani. Tails were averaged and fitted to a double exponential function and the exponential fits were then used to obtain  $d[Ca^{2+}]_i/dt$  vs.  $[Ca^{2+}]_i$  at regular

intervals. The results were plotted and the following equation was fitted to the data-points:

$$d[Ca^{2+}]_i / dt = A [Ca^{2+}]_i^n - L \quad (\text{Eqn 3})$$

where A reflects the rate of SR  $Ca^{2+}$  uptake, L is the SR  $Ca^{2+}$  leak, and  $n$  is a power function (Westerblad & Allen, 1996b; Klein *et al.*, 1991). To enable comparisons of A and L between groups,  $n$  was set to 4 (Klein *et al.*, 1991).

### Immunoprecipitation and western immunoblotting of RyR1

*Study IV.* FDB muscles were isolated and immediately frozen in liquid nitrogen. Muscle homogenate was prepared by isotonic lysis and RyR1 was immunoprecipitated from 100 µg of homogenate with 2 µl anti-RyR antibody in 0.5 ml of a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM  $Na_3VO_4$ , 0.5% Triton-X100, and protease inhibitor cocktail (Roche) for 1 hr at 4 °C. Protein content was determined using the Bradford assay (Bio-Rad). The samples were incubated with protein A Sepharose beads (Amersham Pharmacia) at 4 °C for 1 h, and the beads were washed three times with RIPA buffer. The product was size-fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (4-20% gradient) and transferred onto nitrocellulose membranes for 2 hours at 200 mA (SemiDry transfer blot, Bio-Rad). After incubation with blocking solution (LICOR Biosciences) to prevent non-specific antibody binding and a wash in Tris-buffered saline with 0.1% Tween-20, membranes were incubated for 1–2 h at room temperature with primary antibodies diluted in blocking buffer: anti-calstabin (1:2500), anti-RyR (1:5,000), or anti-phospho-RyR2-pSer2809 (1:5000), which detects PKA-phosphorylated mouse RyR1- pSer2844. After three washes, membranes were incubated with infrared-labelled secondary antibodies (1:10 000 dilution, LICOR Biosystems). Band densities were quantified using the Odyssey Infrared Imaging System (LICOR Biosciences). All antibodies were manufactured in the lab of Professor A. M. Marks, Columbia University, New York, USA.

### RNA isolation and quantitative PCR

In study II mRNA levels of several  $Ca^{2+}$ -handling proteins were assessed as follows: Total RNA from wild-type and knockout Tfam EDL muscle tissue samples was isolated using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the First Strand cDNA Synthesis Kit (MBI Fermentas), and quantitative PCR reactions were performed with the ABI 7700 Sequence Detection System (Applied Biosystems) using TaqMan chemistry. The results were normalized to 18S rRNA quantified from the same samples. The primers and fluorogenic probes are listed in Table 2.

### Statistics

Values are presented as mean ± SEM. Statistically significant differences were determined using Student's t-test, unpaired or paired as necessary. The significance level was set at  $P < 0.05$ .



|                                | Sense primer<br>(forward)     | Antisense primer<br>(reverse) | Fluorogenic probe                 |
|--------------------------------|-------------------------------|-------------------------------|-----------------------------------|
| DHPR<br>(XM_358335)            | CGAGGAGGTTGGC<br>CAGG         | GCATAGAGAAGCCAA<br>AGTTGTCG   | CCCAACCACGGCATCACC<br>CACT        |
| RyR1<br>(NM_009109)            | ACAGGACACTCTTG<br>TATGGCCAC   | AGGCAACTCAGGTAC<br>ATACGACTG  | CCATCCTGCTCCGGCAGC<br>C           |
| Triadin                        | GTCTGTCACCGAAG<br>ACATTGTGA   | TGTGATAATCAGAGC<br>GATGACAAG  | CAGCTCCCCTGCAGCCTG<br>GC          |
| Junctin<br>(AF289490)          | GAGAAAGAACTGA<br>AGGCCTGTCA   | AGATTTCTCTGCCCT<br>TTAGCA     | AATGGCTCACCTTCCTGA<br>CTCCAGTTCA  |
| Calsequestrin-1<br>(NM_009813) | TGCGGCTGGCACTG<br>CT          | ATCTTCCCCTGGACC<br>CC         | TTTGTAAGTGGTCTAGG<br>GACGCCCA     |
| SERCA1<br>(NM_007504)          | CAGGAAGGGAGCA<br>CAATGGA      | CCCCGAAATAGGACA<br>AACATTC    | CCGCGCACTCCAAGTCCA<br>CAGA        |
| NCX<br>(AF004666)              | TTGTTTTCCCATGTT<br>GACCATATAA | GAGCCAGTACATTCA<br>GTGGTTTCA  | TGCAGATACAGAGGCAG<br>AAACAGGAGGAA |
| 18S                            | TGGTTGCAAAGCTG<br>AAACTTAAAG  | AGTCAAATTAAGCCG<br>CAGGC      | CCTGGTGGTGCCCTCCG<br>TCA          |

**Table 2.** Oligonucleotide sequences for primers and probes used in quantitative PCR.

## RESULTS AND DISCUSSION

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### Mitochondrial $\text{Ca}^{2+}$ uptake during fatigue (Paper I)

In this study we wanted to establish whether mitochondrial  $\text{Ca}^{2+}$  uptake occurs in intact slow-twitch muscle fibres, if this is related to induction of fatigue and also by which mechanisms the  $\text{Ca}^{2+}$  uptake is mediated. A secondary objective was to confirm that  $\text{Ca}^{2+}$  uptake does not occur in fast-twitch fibres as reported previously (Lännergren *et al.*, 2001).

#### *Tetanic $[\text{Ca}^{2+}]_i$ and force in single soleus and EDL fibres*

This study is the first to describe the changes both in tetanic  $[\text{Ca}^{2+}]_i$  and force in single soleus fibres during repeated contractions. Our results show that tetanic  $[\text{Ca}^{2+}]_i$  is maintained during a bout of 1000 contractions and does not significantly differ from the initial value at the end of stimulation ( $1.34 \pm 0.16 \mu\text{M}$  vs.  $1.61 \pm 0.21 \mu\text{M}$ ) (Figure 2, *paper I*). Force on the other hand declined over the same period and was significantly reduced at the end of stimulation ( $238 \pm 25 \text{ kN m}^{-2}$  vs.  $324 \pm 26 \text{ kN m}^{-2}$ ,  $P < 0.05$ ). The mean force- $[\text{Ca}^{2+}]_i$  relationship shows that the force decrease might be due to a decreased  $\text{Ca}^{2+}$  sensitivity as the curve is shifted to the right in the last tetanus when compared to the first (Figure 3, *paper I*). An additional feature which could be ascribed to changed  $\text{Ca}^{2+}$  sensitivity was noted in all soleus fibres, namely a slowing in the rate of force production (Figure 4, *paper I*). Slowed SR  $\text{Ca}^{2+}$  release is not likely to be the cause as no changes in the time course of  $[\text{Ca}^{2+}]_i$  transient were observed. Furthermore, no change was seen in rate of force relaxation, suggesting that the reuptake of  $\text{Ca}^{2+}$  by SERCA and the cross-bridge cycling remained unchanged during the series of stimulation.

#### *Mitochondrial $\text{Ca}^{2+}$ uptake in soleus and EDL*

Of the 14 soleus fibres used in the study, ten took up  $\text{Ca}^{2+}$  into their mitochondria during fatiguing stimulation. This uptake was not uniform throughout the fibres but rather occurred only in the mitochondria just below the sarcolemma (Figure 5, *paper I*).  $[\text{Ca}^{2+}]_{\text{mit}}$  initially increased, reaching a maximum value after about 50 tetani (Figure 6a, *paper I*) and thereafter it slightly declined throughout the rest of the stimulation series. At the end of the stimulation period,  $[\text{Ca}^{2+}]_{\text{mit}}$  was ~60% of its maximal value. Rhod-2 fluorescence was also monitored after cessation of stimulation and at 5 min of recovery,  $[\text{Ca}^{2+}]_{\text{mit}}$  had fallen to 50% of its final value and was completely restored to its resting value within 20 min (Figure 6b, *paper I*).

To assure that the recorded signal actually originated from the mitochondria, we used dual-labelling with Rhod-2 and R123. The data showed that Rhod-2 is loaded into the mitochondria (Figure 1a, *paper I*). However, as only the mitochondria closest to the membrane showed accumulated  $\text{Ca}^{2+}$ , we performed additional control experiments to assure that Rhod-2 was taken up uniformly in mitochondria throughout the fibres. By inducing localized damage to muscle fibres using maximum power of the laser of the confocal microscope, we were able to record a gradual increase in the Rhod-2

fluorescence as  $\text{Ca}^{2+}$  accumulated in all mitochondria in the damaged area, indicating that the dye was loaded into all mitochondria (Figure 1c, *paper I*). Furthermore, this also showed that all mitochondria have the ability to accumulate  $\text{Ca}^{2+}$ .

Contrary to what was expected for a fast-twitch fibre type, eight of the nine EDL fibres used in the study also took up  $\text{Ca}^{2+}$  in their mitochondria. Similarly to what was observed for the *soleus*, uptake of  $\text{Ca}^{2+}$  was restricted to mitochondria located just below the sarcolemma (Fig 8, *paper I*). The peak in  $[\text{Ca}^{2+}]_{\text{mit}}$  in EDL occurred after 50 tetani (Fig 6a, *paper I*) and declined through the remainder of the stimulation series, reaching ~70% of its peak value at the end of stimulation. The recovery was also very similar to that observed for *soleus* with the signal reverting to 50% of its final value within 5 min after stopping stimulation and back to its resting value within 20 min (Figure 6b, *paper I*).

#### *Effects of mitochondrial $\text{Ca}^{2+}$ accumulation on force development and fatigue*

As data from earlier studies (Duan *et al.*, 1990; Madsen *et al.*, 1996) have suggested that accumulation of  $\text{Ca}^{2+}$  in the mitochondria has a negative effect on force production, we examined the relationship between peak mitochondrial  $\text{Ca}^{2+}$  and final force (Figure 9, *paper I*). Our results show that  $[\text{Ca}^{2+}]_{\text{mit}}$  does not have any negative effects on force and, if anything, there is a positive relationship between mitochondrial  $\text{Ca}^{2+}$  uptake and force development in *soleus* ( $P = 0.029$ ) but not in EDL ( $P = 0.866$ ) (Figure 9, *paper I*). When induction of fatigue was compared between *soleus* fibres that accumulated  $\text{Ca}^{2+}$  in their mitochondria with *soleus* fibres that did not take up  $\text{Ca}^{2+}$ , there was no significant difference regarding how many tetanic contractions were produced until force fell to 40% ( $719 \pm 116$  vs.  $553 \pm 116$ ). Thus it seems that there is no significant difference in terms of fatigue development between fibres that accumulate mitochondrial  $\text{Ca}^{2+}$  and fibres that do not.

#### *Mechanisms of mitochondrial $\text{Ca}^{2+}$ extrusion*

Uptake of  $\text{Ca}^{2+}$  into mitochondria in other cell types has been shown to depend on the mitochondrial membrane potential with  $\text{Ca}^{2+}$  passively entering through the  $\text{Ca}^{2+}$ -uniporter and being extruded either through a NCX or by opening of the mitochondrial permeability transition pore (mPTP) (Brookes *et al.*, 2004; Bernardi & Petronilli, 1996). By dissipating the mitochondrial membrane potential using FCCP, we were able to show that the entry of  $\text{Ca}^{2+}$  into the mitochondria of skeletal muscle is dependent on normal polarization of the mitochondria (Figure 10, *paper I*). Inhibiting the NCX using CGP resulted in accumulation of  $\text{Ca}^{2+}$  in the mitochondria whereas blocking the mPTP using CyA had no such effect suggesting that  $\text{Ca}^{2+}$  extrusion in skeletal muscle mitochondria is limited to the NCX.

#### *Does mitochondrial $\text{Ca}^{2+}$ uptake modulate tetanic $[\text{Ca}^{2+}]_i$ during fatigue?*

Although administration of FCCP to *soleus* fibres ablated the increase in  $[\text{Ca}^{2+}]_{\text{mit}}$  it had no effect on tetanic  $[\text{Ca}^{2+}]_i$  in *soleus* fibres. Furthermore, there was no change in tetanic  $[\text{Ca}^{2+}]_i$  at times when  $[\text{Ca}^{2+}]_{\text{mit}}$  was at its maximal value. These two observations suggest that  $[\text{Ca}^{2+}]_{\text{mit}}$  buffering does not occur in mouse skeletal muscle unlike frog

skeletal muscle fibres (Lännergren *et al.*, 2001), neurons (David *et al.*, 1998,1999) and cardiac muscle (Maack *et al.*, 2006).

To conclude, this study showed that an increase in  $[Ca^{2+}]_{mit}$  can be seen in most but not all fibres from both *soleus* and EDL and that the  $Ca^{2+}$  uptake is limited to the mitochondria closest to the sarcolemma although all mitochondria are capable of accumulating  $Ca^{2+}$ . Accumulation of mitochondrial  $Ca^{2+}$  is not associated with impaired contractile performance and does not buffer the tetanic  $[Ca^{2+}]_i$ . Finally, the rise in  $[Ca^{2+}]_{mit}$  is dependent on normal mitochondrial polarization and extrusion occurs mainly via NCX.

### **Force and $Ca^{2+}$ in myopathy of mitochondrial origin (Paper II)**

In this study isolated fibres from mice with a skeletal muscle specific disruption of the nuclear encoded mitochondrial transcription factor A (Tfam KO) were used in order to investigate the reduction in force previously demonstrated (Wredenberg *et al.*, 2002).

#### *Reduced force in Tfam KO is due to decreased releasable SR $[Ca^{2+}]$*

We investigated the underlying cause of reduced force production. This was done by studying force and  $[Ca^{2+}]_i$  in isolated muscle fibres of FDB muscles. Similar to EDL muscle, isolated fibres from Tfam KO mice displayed a lower force production which was significantly different from their wildtype littermates at 40 to 100 Hz ( $P < 0.05$ ) (Figure 2, *paper II*), whereas tetanic  $[Ca^{2+}]_i$  was lower at all frequencies ( $P < 0.05$ ) (Figure 2, *paper II*). SR  $[Ca^{2+}]$  storage capacity was assessed by 100 Hz stimulation in the presence of 5 mM caffeine (Allen & Westerblad, 1995) and the results show a marked decrease in the SR  $Ca^{2+}$  stores in fibres from Tfam KO mice compared to fibres from wildtype mice (Figure 2, *paper II*). Plotting the force- $[Ca^{2+}]_i$  relationship (Eqn 2, Material and Methods) showed no significant differences in either the  $[Ca^{2+}]_i$  required to obtain 50% of maximal force ( $Ca_{50}$ ) or in the steepness of the relationship (N), i.e. no difference in myofibrillar  $Ca^{2+}$  sensitivity. Thus the lower force production observed in Tfam KO is predominately due to decreased SR  $Ca^{2+}$  release. We investigated if this reduction in  $Ca^{2+}$  release was due to decreases in the SR  $Ca^{2+}$  handling or buffering proteins. PCR was used to quantify the gene expression and it showed a significant decrease in mRNA expression only for the SR luminal  $Ca^{2+}$  binding protein calsequestrin-1 (CASQ1) (Figure 3, *paper II*). The RNA expression was decreased by ~60% compared to wildtype controls, indicating the cause of the decreased SR  $Ca^{2+}$  loading capacity which is in accordance with our measurements of tetanic  $[Ca^{2+}]_i$ .

#### *Tfam KO accumulate $Ca^{2+}$ in their mitochondria*

Wredenberg *et al.* (2000) demonstrated that Tfam KO mice do not have altered levels of ATP-production and they suggested that the increase in mitochondrial number was a compensation for the respiratory chain dysfunction. As mitochondrial  $Ca^{2+}$  uptake has been shown to stimulate the respiratory chain in skeletal muscle (Kavanagh *et al.*, 2000), and also several key enzymes in the tricarboxylic acid cycle (McCormack & Denton, 1993), we argued that an increased  $[Ca^{2+}]_{mit}$  could boost ATP-production in Tfam KO muscle fibres. In accordance with previous observations (Lännergren *et al.*, 2001) we could not detect any increase in  $[Ca^{2+}]_{mit}$  with Rhod-2 during fatigue in FDB

fibres from wildtype mice (Figure 5, *paper II*). Mitochondria of Tfam KO also showed no increased  $[Ca^{2+}]_{mit}$  at rest. However, during fatiguing stimulation there was a four-fold increase in Rhod-2 signal with the maximum being reached after 30 tetani in fibres from two-month old animals.  $[Ca^{2+}]_{mit}$  started decreasing at the end of stimulation and had reverted back to its original value after ten minutes. The increase in  $[Ca^{2+}]_{mit}$  stimulates mitochondrial ATP-production and could be a beneficial adaptive response early in the myopathic disease process (McCormack & Denton, 1993; Jouaville *et al.*, 1999). However, in four-month old animals (the age for animals used in all other experiments),  $[Ca^{2+}]_{mit}$  was still significantly increased above the pre-fatigue value by ~60% 10 minutes after the end of stimulation. It can be speculated that this prolonged increase in  $[Ca^{2+}]_{mit}$  might have other detrimental effects on cell function (Duchen, 2000; Brookes *et al.*, 2004).

#### *Is mitochondrial buffering responsible for the reduced tetanic $[Ca^{2+}]_i$ ?*

Mitochondrial  $Ca^{2+}$  uptake has been shown to buffer  $[Ca^{2+}]_i$  transients in frog skeletal muscle fibres (Lännergren *et al.*, 2001), in neurons (David *et al.*, 1998; David, 1999) and in other non-excitable cell types (Montero *et al.*, 2002; Malli *et al.*, 2003). These findings might imply that the increased mitochondrial  $Ca^{2+}$  uptake in Tfam KO cells acts as a buffer and causes the observed decrease in tetanic  $[Ca^{2+}]_i$ . However, increased  $Ca^{2+}$  buffering results in slowed  $[Ca^{2+}]_i$  transients in muscle fibres (Lännergren *et al.*, 2001; Baylor & Hollingworth, 1988), and no such slowing was observed in Tfam KO fibres (see Figure 1, *paper II*). Furthermore, while  $[Ca^{2+}]_{mit}$  increased during the series of repeated contractions in Tfam KO fibres, tetanic  $[Ca^{2+}]_i$  was actually better maintained in Tfam KO than in control muscle cells (Figure 4, *paper II*) which would not be the case if buffering occurred. These observations further strengthen the idea that decreased calsequestrin-1 leads to reduced SR  $Ca^{2+}$  storage capacity, which in turn leads to reduced SR  $Ca^{2+}$  release.

#### *No increased ROS production in Tfam KO*

Mitochondrial dysfunction is often associated with increased production of ROS (Esposito *et al.*, 1999; Tarnopolsky & Raha, 2005) so we monitored mitochondrial super oxide ( $O_2^{\cdot -}$ ) production. During repeated stimulation, we observed no difference between Tfam KO and wildtype muscle fibres in production of  $O_2^{\cdot -}$  (Figure 6a, *paper II*). However, assessment of basal  $O_2^{\cdot -}$  production using 100  $\mu$ M  $H_2O_2$  to product inhibit mitochondrial superoxide dismutase, thereby preventing the conversion of  $O_2^{\cdot -}$  to  $H_2O_2$ , revealed that Tfam KO have a significantly lower production of ROS (Figure 6b, *paper II*). To show that the observed signal was due to impaired respiratory function, we did the same experiment using toe fibres from control mice where complex I was inhibited using 2.4  $\mu$ M rotenone. This resulted in a reduced production of  $O_2^{\cdot -}$ , similar to what was found in Tfam KO (Figure 6c, *paper II*).

Tfam KO skeletal muscle fibres display lower force production and altered properties in their  $Ca^{2+}$  handling. Our results show that the reduction in force is due to a decreased SR pool of releasable  $Ca^{2+}$  probably caused by a downregulation of calsequestrin-1. Also, the mitochondria in skeletal muscle of Tfam KO but not wildtype mice accumulate  $Ca^{2+}$ . The recovery of  $[Ca^{2+}]_{mit}$  to the baseline was delayed in four-months vs. two-month old Tfam KO animals.  $[Ca^{2+}]_{mit}$  is not involved in the decreased

force production as no buffering of the tetanic  $[Ca^{2+}]_i$  occurred. Lastly, Tfam KO fibres do not have an increased production of ROS.

### **Modulation of tetanic $[Ca^{2+}]_i$ by CaMKII (Paper III)**

Another aspect of this thesis was to clarify the role of CaMKII in SR  $Ca^{2+}$  release. Our idea was to determine whether  $Ca^{2+}$  itself can promote further  $Ca^{2+}$  release as it has been reported to act on several proteins involved in ECC (Damiani *et al.*, 2000; Sacchetto *et al.*, 2000; Colpo *et al.*, 2001; Rose *et al.*, 2006). Furthermore we wanted to investigate if CaMKII in intact muscle fibres is activated in a frequency dependent manner as demonstrated *in vitro* by De Koninck & Schulman (1998).

#### *Inhibition of CaMKII markedly reduces $Ca^{2+}$ release and force*

To determine the contribution of CaMKII to SR  $Ca^{2+}$  release, a pharmacological inhibitor KN-93 and an inhibitory peptide AC3-I were used with three different stimulation protocols all using 70 Hz trains but differing in train duration, intervals and number of invoked contractions.

When contractions were separated by 5 s, there was no effect of inhibiting CaMKII on either tetanic  $[Ca^{2+}]_i$  or force (Figures 4 and 5, *paper III*). However, when using contractions separated with 2 s intervals (Figure 1, *paper III*), controls displayed the expected pattern of fatigue with a rapid rise in  $[Ca^{2+}]_i$  during the first five tetani, reaching a maximum after about 15 tetani and returning back to the initial value ( $100 \pm 11\%$ ) at the end of stimulation. When CaMKII was inhibited using AC3-I, the early rise in tetanic  $[Ca^{2+}]_i$  was markedly smaller compared to controls and maximal  $[Ca^{2+}]_i$  was reached after 10 tetani and then started to decline reaching  $97 \pm 11\%$  of its initial value when stimulation stopped. The effect of CaMKII inhibition on both tetanic  $[Ca^{2+}]_i$  and force was more marked when using KN-93, where there was only a slight increase in tetanic  $[Ca^{2+}]_i$  in the first three stimulations. Then  $[Ca^{2+}]_i$  rapidly dropped reaching  $67 \pm 11\%$  of its initial value at the end of stimulation. The impaired  $[Ca^{2+}]_i$  was reflected in force production where controls were able to produce  $47 \pm 3$  contractions before force dropped to 40%. With AC3-I the number of tetani was  $33 \pm 3$  and with KN-93 force dropped to 40% after  $15 \pm 3$  contractions.

Decreasing the interval between contractions to 100 ms gave results similar to those obtained when using 2 s intervals (Figure 3, *paper III*). In control fibres bathed in standard Tyrode, tetanic  $[Ca^{2+}]_i$  increased steadily reaching a maximum after 10 tetani after which it started to decline and was not significantly different from the initial value ( $110 \pm 20\%$ ) after 20 repeated stimulations. Force was maintained for 10 contractions and then steadily decreased reaching  $61 \pm 7\%$  in the last contraction. Inhibiting CaMKII with KN-93 resulted in  $[Ca^{2+}]_i$  reaching its maximum level after only four contractions and thereafter it dropped to  $46 \pm 13\%$  of its initial value. Force started to decline after three tetani and was almost completely abolished after 20 repeated stimulations ( $8 \pm 2\%$ ).

Taken together, these results show that activation of CaMKII is essential for sustaining SR  $[Ca^{2+}]_i$  release when stimulations are close together in time.

### *Mathematical modelling of CaMKII-activation*

In order to further elucidate the involvement of CaMKII in SR  $\text{Ca}^{2+}$  release, we used mathematical modelling based on the previously published model of Bhalla & Iyengar (1999) to look at the activation patterns of CaMKII (Figure 6, *paper III*). Modelling provided us with the information on how the activity of CaMKII changes over time with different stimulation protocols.

The modelling data suggest that there is a CaMKII activity threshold that has to be surpassed in order to affect SR  $\text{Ca}^{2+}$  release. This conclusion is based on a number of findings. Firstly, there was no effect on SR  $\text{Ca}^{2+}$  release with CaMKII inhibition when contractions were separated by 5 s (Figures 4 and 5, *paper III*) although CaMKII was moderately activated. Secondly, the total time in high  $[\text{Ca}^{2+}]_i$  at which an effect of CaMKII inhibition could be observed was 1400 ms both with stimulations occurring every 2 s and 300 ms. Thirdly, the maximum level of activity produced by the 5 s protocol was surpassed at the time point of 1400 ms in total time in high  $[\text{Ca}^{2+}]_i$  when stimulations were given at shorter intervals.

### *Site of action?*

Targets for CaMKII in fast-twitch skeletal muscle include RyR1 (Dulhunty *et al.*, 2001) and triadin (Colpo *et al.*, 2001; Rose *et al.*, 2006). Furthermore, it has been shown that CaMKII is anchored in close vicinity to the RyR1 in skeletal muscle (Bayer *et al.*, 1998), which is more or less the pivotal point in ECC. The direct effect observed on SR  $\text{Ca}^{2+}$  release in our single fibre experiments and our modelling, together with the above observations, strongly support the notion that proteins involved in ECC are targeted.

### **Ca<sup>2+</sup> changes in cold-acclimatization (Paper IV)**

The final paper in this thesis deals with the effect on SR  $\text{Ca}^{2+}$  release of chronic adrenergic exposure. UCP-1 KO mice were used because they cannot use the non-shivering metabolic thermogenesis in brown fat and in this respect they represent a model of human thermoregulation in the cold. Intact fibres from the FDB were studied as these muscles are not involved in shivering and any effects seen would be due to systemic effects of cold acclimatization, such as increased adrenergic stimulation.

### *No differences between genotypes*

When we looked at  $\text{Ca}^{2+}$  release in fibres from the FDB, there was no significant difference between fibres taken from UCP-1 and wildtype animals kept at the same temperature. Interestingly, when genotype was disregarded and mice instead grouped according to the holding temperature, there was a ~40% increase in tetanic  $[\text{Ca}^{2+}]_i$  levels at 40-100 Hz in cold acclimated animals (4°C) compared to animals kept at room temperature (24°C) (Figure 2a, *paper IV*). As there was no effect of genotype on  $\text{Ca}^{2+}$  release, data were pooled for each temperature (Figure 2b, *paper IV*).

### *Cold acclimatized mice display increased tetanic and basal $[\text{Ca}^{2+}]_i$*

Cold acclimated mice displayed an increased level of tetanic  $[\text{Ca}^{2+}]_i$  compared to mice kept at room temperature and submaximal force increased accordingly. However,

there was no difference between maximal force production between the two groups and plotting force vs.  $[Ca^{2+}]_i$  (Figure 2c, *paper IV*) revealed that there was no difference in either the  $[Ca^{2+}]_i$  needed to obtain half-maximum tetanic force ( $Ca_{50}$ ;  $0.61 \pm 0.05$  vs.  $0.62 \pm 0.05$   $\mu M$ ) or the steepness of the relationship ( $N$ ;  $3.9 \pm 0.4$  vs.  $4.7 \pm 0.7$ ), i.e. there was no difference in myofibrillar  $Ca^{2+}$  sensitivity.

Resting  $[Ca^{2+}]_i$  did not differ between genotypes ( $< 5\%$ ) but was significantly higher ( $P < 0.01$ ) in pooled data for cold acclimated mice ( $86 \pm 8$  nM) compared to mice kept at room temperature ( $57 \pm 7$  nM). This difference could either be due to an increased SR  $Ca^{2+}$  leakage or a diminished SR  $Ca^{2+}$  pumping. To distinguish between these two possibilities, the tails of increased  $[Ca^{2+}]_i$  after tetanic contraction were analysed using a previously published model (Klein *et al.*, 1991; Westerblad & Allen, 1996a). The  $[Ca^{2+}]_i$  tail analysis showed a four-fold higher SR  $Ca^{2+}$  leakage in cold acclimated mice ( $29$  nM  $s^{-1}$ ) compared to controls ( $7$  nM  $s^{-1}$ ) (Figure 3, *paper IV*). The pumping rate was halved in cold acclimated mice ( $1.6 \times 10^{-7}$  vs.  $3.2 \times 10^{-7}$  nM $^{-3}$   $s^{-1}$ ). By entering the obtained values back for A and L into Equation 3 (Eqn 3, Material and Methods) and calculating  $[Ca^{2+}]_i$  at rest (i.e. when  $d[Ca^{2+}]_i/dt=0$  and there is no net movement of  $Ca^{2+}$  across the SR membrane) we estimated that  $\sim 70\%$  of the increased resting  $[Ca^{2+}]_i$  in cold acclimated mice is due to increased SR  $Ca^{2+}$  leak and  $\sim 30\%$  to slowed SR  $Ca^{2+}$  pumping.

#### *Does cold exposure induce PKA-mediated RyR phosphorylation?*

Since it has been known for over 50 years that adrenergic stimulation increases skeletal muscle force production (Brown *et al.*, 1948) and that there is an associated increase in sympathetic activity during cold acclimation (Dulloo *et al.*, 1988), we speculated that this could be the cause of the observed changes in force and tetanic  $[Ca^{2+}]_i$  handling in muscle fibres from cold acclimated mice. This seemed reasonable since  $\beta$ -adrenergic stimulation in skeletal muscle increased tetanic  $[Ca^{2+}]_i$  and force (Cairns *et al.*, 1993) and had effects on RyR1 and SR  $Ca^{2+}$  release (Reiken *et al.*, 2003b; Ward *et al.*, 2003).

We therefore assessed PKA-mediated phosphorylation of RyR1 at ser2844 in cold acclimated and control mice. An increased adrenergic stimulation could impair contractile function in the long run as prolonged stimulation can induce hyperphosphorylation of the RyR1, causing almost complete dissociation of calstabin-1 which results in leakier RyR channels and decreased SR  $Ca^{2+}$  release (Ward *et al.*, 2003; Reiken *et al.*, 2003a). Our results show a marked increase in RyR1 phosphorylation ( $\sim 80\%$ ,  $P < 0.001$ ) and minor calstabin-1 depletion ( $\sim 20\%$ ,  $P < 0.01$ ) in data from cold acclimated mice (Figure 4, *paper IV*). Thus, the FDB muscles of our cold acclimated mice seem to be in a state where the positive effects of  $\beta$ -adrenergic stimulation predominate. This is obvious from the increased force generation discussed above but was also seen during fatiguing stimulation where force production in fibres from cold-acclimated mice was better maintained (data not shown).



#### *Cycling of $\text{Ca}^{2+}$ as a mean of generating heat*

Cycling of SR  $\text{Ca}^{2+}$  has been estimated to account for ~50% of a resting muscles energy expenditure (Marie & Silva, 1998) and heat generation could therefore be augmented by increasing the rate of SR  $\text{Ca}^{2+}$  release at rest. The contribution of increased SR  $\text{Ca}^{2+}$  release to heat generation is very clear in malignant hyperthermia (Mickelson & Louis, 1996; Melzer & Dietze, 2001). Moreover, several types of fishes warm the brain and eyes by prolonged SR  $\text{Ca}^{2+}$  cycling by a thermogenic organ consisting of specialized non-contracting skeletal muscle cells (Block *et al.*, 1994; Morrissette *et al.*, 2003). The FDB muscles used in our studies are not involved in the shivering response and their distal localization prevents them from playing any significant role in maintaining core body temperature. But nonetheless they can generate heat by recycling of  $\text{Ca}^{2+}$  by SERCA (Block *et al.*, 1994; de Meis *et al.*, 2005) which would be important in maintaining force production in superficial muscles during cold exposure.

## CONCLUSION

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In this thesis we looked at changes in  $\text{Ca}^{2+}$  handling focusing on modulation by mitochondria and the two kinases CaMKII and PKA. It is clear from these results that the mechanisms that are involved during fatigue are diverse and that peripheral fatigue cannot be attributed to a single factor.

### *Mitochondrial $\text{Ca}^{2+}$ uptake and fatigue*

We have shown that mitochondrial  $\text{Ca}^{2+}$  uptake in skeletal muscle occurs in both *soleus* and EDL so the notion that mitochondrial  $\text{Ca}^{2+}$  uptake is a fibre type specific event is not supported. Furthermore, we also show that fatigability is not correlated to mitochondrial  $\text{Ca}^{2+}$  uptake even though there is a statistical significant relationship between increased  $[\text{Ca}^{2+}]_{\text{mit}}$  and force production in *soleus* fibres. For the same reason, involvement of  $\text{Ca}^{2+}$  in regulation of energy metabolism in skeletal muscle is not directly evident either, as  $\text{Ca}^{2+}$  uptake was absent in 30% of *soleus* fibres without any noticeable effects on fatigue. This might be an indication that other factors also are important in regulating metabolism. In the mitochondria, such factors are presumed to include ATP,  $\text{P}_i$  and phosphocreatine (Walsh *et al.*, 2001).  $\text{Ca}^{2+}$  could also be involved in mitochondrial protein turnover (Joyal *et al.*, 1995), which would not be reflected as an acute effect on fatigability.

In contrast to control mice, FDB fibres from Tfam KO displayed an increase in  $[\text{Ca}^{2+}]_{\text{mit}}$ . This differed markedly from what was observed in *soleus* and EDL in that the uptake was seen in mitochondria throughout the fibre and not restricted to the mitochondria closest to the sarcolemma. The same observation has been made earlier in mice devoid of creatine kinase (Bruton *et al.*, 2003), which implies that the increased  $[\text{Ca}^{2+}]_{\text{mit}}$  could be an adaptive response to increase ATP production.

Another adaptive response in Tfam KO mice is the reduced SR  $\text{Ca}^{2+}$  release which might be due to down-regulation of calsequestrin-1. The decrease of releasable SR  $\text{Ca}^{2+}$  could be a mean by which Tfam KO reduce ATP-consumption by the cross-bridges and SERCA but *in vivo* this would have severe consequences as myopathic subjects would have to work at much higher frequencies compared to healthy individuals in order to perform the same physiological task.

It would be of great interest to further study the mitochondrial  $\text{Ca}^{2+}$ -uptake in muscle from myopathic mice. If moderate accumulation of  $[\text{Ca}^{2+}]_{\text{mit}}$  is an adaptive response which becomes lethal for cells when excessive, blocking of the mitochondrial  $\text{Ca}^{2+}$ -uptake in the late stage could possibly prolong the life span.

### *CaMKII is essential during fatiguing stimulation*

Our paper (*paper III*) is the first to look at the frequency effect of CaMKII in intact skeletal muscle single fibres and shows that inhibiting CaMKII during repeated contractions results in lower tetanic  $[\text{Ca}^{2+}]_i$  and force when stimulations occur at intervals of 2 s or less but not when the interval is 5 s. It is likely that the duration and interval between tetanic  $[\text{Ca}^{2+}]_i$  govern the extent of CaMKII activation and the

amplitude is of lesser importance as the autonomous activity is fully saturated at lower frequencies (1-10 Hz) (Eshete & Fields, 2001).

We also propose, based on mathematical modelling in conjunction with the findings in single muscle fibre experiments, that there is a CaMKII activity threshold that has to be surpassed in order to affect repeated SR  $\text{Ca}^{2+}$  release when contractions are close together in time.

Although no affected target proteins were identified in our study, the direct effect observed on SR  $\text{Ca}^{2+}$  release together with our mathematical modelling leads us to believe that CaMKII target proteins directly involved in ECC (Colpo *et al.*, 2001; Dulhunty *et al.*, 2001; Rose *et al.*, 2006) are affected. Future studies should aim at resolving if additional proteins involved in ECC are phosphorylated by CaMKII and also how the observed effect on  $\text{Ca}^{2+}$  release is mediated.

#### *Increased PKA-mediated $\text{Ca}^{2+}$ leak in cold acclimatized mice*

When investigating the adrenergic effects on  $\text{Ca}^{2+}$  handling during cold exposure, we found an increase in tetanic  $[\text{Ca}^{2+}]_i$  and force. Fibres from cold-acclimatized mice also displayed an increased resting  $[\text{Ca}^{2+}]_i$  which was due to an increased leak, induced by PKA-mediated phosphorylation of RyR1 on ser2844. Our results indicate that the cold acclimatized animals are in a state where beneficial effects of PKA-mediated RyR1 phosphorylation are at advantage (Cairns *et al.*, 1993). These results may differ from the observations by Marks and co-workers (Reiken *et al.*, 2003b; Ward *et al.*, 2003) where ser2844 phosphorylation was shown to decrease SR  $\text{Ca}^{2+}$  release. However, the magnitude of RyR ser2844 phosphorylation observed by Marks and colleagues was very high (six-fold, compared to controls) and calstabin-1 was completely dissociated from phosphorylated RyRs. This could explain the impaired SR  $\text{Ca}^{2+}$  release as the RyR gating control mediated by calstabin-1 would be abolished, resulting in a very much leakier SR than observed in our cold acclimatized mice where calstabin-1 is moderately dissociated (~20%). The proposal that cycling of  $\text{Ca}^{2+}$  between SR and myoplasm could be a mean of generating heat is a phenomenon well known in malignant hyperthermia (Mickelson & Louis, 1996; Melzer & Dietze, 2001) as well as in specialized heater cells in several types of fishes (Block *et al.*, 1994; Morrisette *et al.*, 2003). In FDB muscles this heat-generation could be important to preserve muscle function, due to the distal and superficial localization of this muscle.

Although we demonstrate an increased leak, this was done using mathematical models. Future studies should include incorporation of RyR from cold-acclimatized mice into lipid bilayers and measurements of its open probability in order to verify these results.

## Concluding Remarks

In this thesis the modulation of tetanic  $[Ca^{2+}]_i$  by mitochondria, CaMKII and PKA was investigated. The findings that  $[Ca^{2+}]_{mit}$  does not acutely modulate  $[Ca^{2+}]_i$  in normal muscle fibre might instinctively be interpreted as that mitochondria are not involved in regulating  $[Ca^{2+}]_i$ . However, results from Tfam KO presented herein suggest otherwise with altered free SR  $Ca^{2+}$  content in what we believe to be an attempt to lower ATP-consumption. This is an indication of that mitochondria truly are players in modulation of tetanic  $[Ca^{2+}]_i$  and implicates a mitochondrial-SR interplay although the mechanisms, for how this is achieved are unknown and need further attention. Furthermore, this observation together with previous findings where  $[Ca^{2+}]_{mit}$  is involved in regulating several aspects of mitochondrial function suggests that  $[Ca^{2+}]_{mit}$  uptake might be more important in the long-term, involving modulation of nuclear protein expression.

The versatility of  $Ca^{2+}$  is truly remarkable as it not only can regulate its further release by activating CaMKII, but when circulating between SR and myoplasm, it can also be used to generate heat. Although  $Ca^{2+}$  might be simple in its molecular form, nature's utilization of the ion is far from simple and we can only hope to one day be able to accurately decode the information carried by the  $Ca^{2+}$ .

To summarize, the major findings of this thesis are:

- Mitochondrial  $Ca^{2+}$  uptake does not modulate tetanic  $[Ca^{2+}]_i$
- Decreased force production in Tfam KO muscle is due to a reduced SR  $Ca^{2+}$  content
- CaMKII activation is necessary for sustained SR  $Ca^{2+}$  release
- Muscle fibres from cold acclimatized mice display an increased SR  $Ca^{2+}$  leak

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